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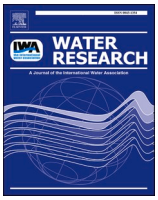

Biotransformation of starch-based wastewater into bioplastics: Optimization of poly(3-hydroxybutyrate) production by *Cupriavidus necator* DSM 545 using potato wastewater hydrolysate

S. González-Rojo *, A.I. Paniagua-García, R. Díez-Antolínez

Centro de Biocombustibles y Bioproductos, Instituto Tecnológico Agrario de Castilla y León (ITACyL), Polígono Agroindustrial del Órbigo p. 2-6, Villarejo de Órbigo 24358, León, Spain

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ABSTRACT

Biodegradable biopolymers, such as polyhydroxyalkanoates (PHAs), have emerged as an alternative to petrochemical-based plastics. The present work explores the production of PHAs based on the biotransformation of potato processing wastewater and addresses two different strategies for PHA recovery. To this end, culture conditions for PHA synthesis by *Cupriavidus necator* DSM 545 were optimized on a laboratory scale using a response surface methodology-based experimental design. Optimal conditions rendered a PHB, poly(3-hydroxybutyrate), accumulation of 83.74 ± 2.37 % (5.1 ± 0.2 gL^{-1}), a 1.4-fold increase compared to the initial conditions. Moreover, polymer extraction with non-halogenated agent improved PHB recovery compared to chloroform method (PHB yield up to 78.78 ± 0.57 %), while maintaining PHB purity. (99.83 ± 4.95 %). Overall, the present work demonstrated the potential valorization of starch-based wastewater by biotransformation into PHBs, a high value-added product, and showed that recovery approaches more eco-friendly than the traditional treatments could be applied to PHB recovery to some extent.

1. Introduction

Owing to the dramatic increase in the production of plastics, our society has become increasingly concerned about the pollution resulting from the use of these polymers. Plastic waste, and microplastics in particular, play an unequivocal role in environmental pollution (Ebrahimi et al., 2022), and their ubiquitous presence in the environment leads to chronic exposure in organisms, posing a risk to ecosystems and human health (Campanale et al., 2020). To overcome these difficulties associated with traditional plastics, biodegradable biopolymers have emerged as an alternative to petrochemical-based polymers, and in this regard, polyhydroxyalkanoates (PHAs) are well-known as promising candidates (Ertan et al., 2021).

PHAs are intracellularly produced polyesters consisting of hydroxyalkanoate monomers, classified as fully biodegradable polymers, synthesized by microorganisms using various substrates from carbohydrates to alcohols or gases (Alvarez-Santullano et al., 2021; Alves et al., 2017; Ertan et al., 2021). They are produced as granules and represent a carbon energy reserve that allows cells to survive under conditions of nutrient deficiency or stress (Kalia et al., 2021). Specifically, PHAs are synthesized when there is an excess of carbon and a limitation of nitrogen and phosphorus sources in the media (Taguchi and Matsumoto, 2020). In bacteria, poly(3-hydroxybutyrate) (PHB) is the most studied and characterized microbial PHA to date. PHB, a short chain-length PHA, exhibits interesting thermoplastic properties such as resistance to a wide temperature range (30 °C-120 °C), non-toxicity, insolubility in water, rapid biodegradation under both aerobic and anaerobic conditions, and biocompatibility (Alves et al., 2017; Kalia et al., 2021; Asrar and Hill, 2001). The polymer composition, and thus the mechanical and thermal properties, define the future processability of the polymer, which is related to the bacterial strain producer and the fermentation conditions (Zhila et al., 2021). *Cupriavidus necator*, formerly known as *Ralstonia eutropha* or *Wautersia eutropha*, is considered to be one of the natural PHB-producing bacteria in large quantities, reaching up to 80 % of its dry cell weight when renewable feedstocks are used (Arikawa and Matsumoto, 2016).

Due to the intracellular accumulation of PHAs, it is necessary to extract the biopolymer out of the cells, which involves breaking and removing the cell membrane. Traditional methods to accomplish this extraction use halogenated solvents, with chloroform being the most

* Corresponding author.
E-mail address: gonrojsi@itacyl.es (S. González-Rojo).

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commonly used (Mannina et al., 2019; Samrot et al., 2021). Despite the known environmental and human health hazards of this solvent, the process provides high PHA purity in a simple, fast and efficient manner (Mohammadi et al., 2012). However, recent trends focus on the development of new technologies that use environmentally friendly and recyclable solvents for PHA extraction, such as dimethyl carbonate, ethyl acetate, 1,2-propylene carbonate, all of which dissolve the polymer, implying an additional recovery step (Bartels et al., 2020; Kosseva and Rusbandi, 2018; Marudkla et al., 2018; Samrot et al., 2021) In contrast, other approaches aim to solubilize the non-PHA cellular material, such as acids or alkalis. For example, NaOH promotes saponification of the membrane lipids, thereby increasing membrane permeability and facilitating the release of PHA granules that are not solubilized by the alkaline agent (Mohammadi et al., 2012). This approach requires an additional step of centrifugation or filtration to recover the released PHA granules (Pagliano et al., 2021).

Even now, the industrial production of PHAs presents the inconvenience of a great economic demand, not only for the recovery process itself, but also for the cost of substrates. This makes the entry of PHAs into the plastics market, traditionally represented by petroleum-based plastics, very challenging. To reduce these high production costs, new alternatives point to the use of renewable feedstocks as cost effective carbon sources. In this regard, organic wastewaters from the agri-food industry could be used as a low-cost substrate and as a high-value bioproducts producer (de Oliveira et al., 2019; Dutt Tripathi et al., 2021; Gottardo et al., 2022; Neelamegam et al., 2018). These approaches, consistent with circular economy strategies, demonstrate the feasibility of using wastewater as a valuable substrate for the sustainable production of high-value bioproducts such as PHAs. Therefore, in this manuscript, a simple and efficient treatment of potato processing wastewater has been developed for the recovery of a high-value product, specifically PHB. To achieve this goal, the best conditions for enzymatic hydrolysis of potato wastewater were first optimized to render sugars accessible to the microorganisms. Subsequently, the optimal culture conditions for *C. necator* DSM 545 were established to produce the highest PHB concentration, and finally, a non-halogenated PHB recovery method based on alkali treatment was tested.

2. Material and methods

2.1. Potato wastewater characterization and hydrolysis

The process water was a by-product of potato chip production, kindly donated by Aperitivos Gus S.L. (Apex Group, Riego de la Vega, León, Spain) in June 2021. Initial characterization was performed to determine starch content by polarimetry (RD 2257/1994), chemical oxygen demand (LCK514, Hach), total solids (APHA, 2017) and nitrogen and ammonium content (LCK338 and LCK304, Hach). The hydrolysis conditions of potato wastewater were optimized based on the method described by Abanoz et al., 2012, using the technique of response surface methodology (RSM). For this purpose, a Box–Behnken design was applied, following the guidelines given in Section 2.5 (Table 1). The wastewater was completely homogenized by magnetic stirring, and then 25 mL aliquots were introduced into an Erlenmeyer flask. The pH was then adjusted to 6.5 and an autohydrolysis pretreatment was performed in an autoclave (Sanyo, Tottori, Japan) at 120 °C for 20 min. After cooling, α-amylase (Sigma Aldrich, Spain) was added for liquefaction and the mixture was heated at 85 °C for 30 min with a magnetic stirrer. After cooling, the pH was adjusted to 5.0 with 2 M HCl and then amyloglucosidase (Sigma Aldrich, Spain) was added. Afterwards, samples were incubated in an orbital shaker (Infors HT Ecotron, Switzerland) at 55 °C and 200 rpm. The suspension was then centrifuged at 4000 rpm for 20 min at 4 °C, and the supernatant was filtered through a paper filter and stored at 4 °C until used.

2.2. Bacterial strains and initial culture conditions for PHB production

C. necator DSM 428, *C. necator* DSM 531, *C. necator* DSM 545 and *C. necator* DSM 13513 were acquired from the Leibniz Institute DSMZ-German collection of microorganisms and cell culture (Braunschweig, Germany). A minimal salt medium (MSM) supplemented with analytical grade glucose (Sigma Aldrich, France) or glucose from potato wastewater hydrolysate was used for preliminary screening of the candidate microorganism for PHA production. This PHA production medium comprised of (per liter of culture): KH_2PO_4 (Sigma Aldrich, Japan), 2.4 g; Na_2HPO_4 (Panreac, Spain), 2.5 g; $MgSO_4$ (VWR Int., Belgium), 0.5 g; ammonium iron (III) citrate (Acros Organics, Belgium), 0.05 g; $CaCl_2$ (Sigma Aldrich, Germany), 0.02 g; $NaNO_3$ (Acros Organics, Belgium), 2.82 g; and glucose, 20 g. The pH of the medium was adjusted to 7.0 with 2 M NaOH and the broth was autoclaved at 121 °C for 20 min before use.

Seed inocula were prepared by growing two loopful of each strain glycerinate in 100 mL flasks containing 50 mL nutrient broth (Oxoid, UK). The flasks were incubated in an orbital shaker (Infors HT Ecotron, Switzerland) at 30 °C and 150 rpm for 18–20 h (mid-log phase). Seed inocula were then inoculated into 100 mL of PHA production medium in 250 mL flasks at approximately 10 % (v/v) (initial OD_{600nm}: 0.1). The flasks were maintained at 30 °C and 150 rpm for a maximum of 72 h. The experiments were performed in triplicate. Cell growth was monitored by optical density measurement at 600 nm (Ultrospec 10, Biochrom, USA) and bacterial density was determined using a Bürker counting chamber

Table 1
Glucose concentration and percentage of starch hydrolysis values recorded during the Box–Behnken RSM design for the optimization enzymatic pretreatment. Note that the percentage of hydrolysis is shown respect to the initial starch concentration in starchy wastewater. Run numbers highlighted in bold correspond to central points. U: enzyme units.

	Independent variables (factors)			Response	
Run	α-amylase (U)	Amyloglucosidase (U)	Saccharification time (min)	[glucose] (g/L)	Starch hydrolysis (%)
1	1980	9.95	75	30.11	35.16
2	1089	54.73	75	66.76	77.96
3	1980	54.73	30	48.55	56.70
4	198	9.95	75	29.24	34.15
5	1089	9.95	30	22.65	26.45
6	1089	99.5	120	84.50	98.68
7	198	54.73	30	54.33	63.44
8	1089	9.95	120	33.19	38.76
9	198	99.5	75	85.22	99.52
10	1980	54.73	120	75,79	88.51
11	1089	54.73	75	76.89	89.79
12	198	54.73	120	74.48	86.98
13	1980	99.5	75	83.73	97.78
14	1089	54.73	75	69.76	81.46
15	1089	99.5	30	77.23	90.19

(Paul Marienfeld GmbH&Co. KG, Germany). Biomass was measured as dry cell weight (DCW) and used to quantify PHB content (see Section 2.4). In addition, cells were freeze-dried (at −82 °C and 0.2 bar - Lyoquest −55, Telstar, Spain) for PHA extraction. To calculate kinetic parameters, cell count data were used to fit the growth curve to the Gompertz model:

$$Y = ae^{-e^{(b-ct)}} \quad (1)$$

where Y is the log(N/N_0) (N is the bacterial density at given time and N_0 is the initial bacterial density), t is the time, and a, b, and c are constant parameters (Deseure et al., 2021). The constant parameters were estimated using Statistica 7 software (StatSoft Inc., USA), and the specific growth rate (μ_{max}), lag time (λ), and doubling time (G) were calculated according to the following equations:

$$\mu_{max} = a{\cdot}c \ \left(h^{-1}\right) \quad (2)$$

$$\lambda = \frac{b-1}{c} \ (h) \quad (3)$$

$$G = \frac{ln2}{\mu_{max}}(h). \quad (4)$$

Moreover, culture parameters such as volumetric productivity (r_p), growth yield ($Y_{X/S}$), and polymer yield ($Y_{P/S}$) were calculated according to Pereira et al., 2019 and described in the following equations:

$$r_p = \frac{\Delta P}{\Delta t} \quad (5)$$

$$Y_{X/S} = \frac{\Delta X}{\Delta S} \quad (6)$$

$$Y_{P/S} = \frac{\Delta P}{\Delta S} \quad (7)$$

where ΔX (g/L) and ΔP (g/L) are the active biomass (X as DCW) and the polymer produced (in terms of PHB), respectively, Δt (h) is the corresponding time of cultivation, and ΔS (g/L) is the total hexose consumed (including glucose and fructose) during culture. Data represent averages of three independent cultures inoculated with independent inocula (mean±SD, $n = 3$, biological triplicates). The bacterial strain with the highest ability to produce PHA and with an appropriate growth rate was selected for further fermentations (Table 2).

2.3. Optimization of culture conditions for PHB production

Once *C. necator* DSM 545 was selected for PHB production, reference conditions were established for future comparisons. Thus, *C. necator* DSM 545 was grown in triplicate in PHA production medium (see MSM in Section 2.2) supplemented with potato wastewater hydrolysate (containing 20 g/L glucose). Next, two successive RSM-based approaches were carried out to improve PHB production using hexoses (glucose and fructose) as the carbon source from potato wastewater hydrolysate. A Box-Behnken design with RSM was executed as is explained in Section 2.5. The experiments were performed in 100 mL PHA production medium as described in Section 2.2 using *C. necator* DSM 545 as seed culture. The culture was maintained for 72 h and cell count (data not shown), biomass, hexose consumption and PHB production were measured, the latter being used as input for the RSM model.

2.4. Analytical methods

2.4.1. Dry cell weight (DCW)

Biomass was quantified as DCW (g/L). Aliquots of 2 mL broth were taken and cell pellets were washed once with distilled water. Cell pellets were dried to constant weight in an air oven (PSelecta, Spain) at 50 °C. The biomass was also used to quantify PHB (in the form of 3-hydroxybutyryc acid) as described below.

2.4.2. Hexose quantification

Glucose and fructose (coming from tautomerization of glucose during autoclaving) contents were quantified by HPLC-RID according to Hijosa-Valsero et al. (2017) and expressed as g/L. The percentage of starch hydrolysis was calculated as follows:

$$\%\ hydrolysis = \frac{\left[glucose, \frac{g}{L}\right] \times 0.9}{\left[starch, \frac{g}{L}\right]} \times 100 \quad (8)$$

2.4.3. PHA determination

Polymer quantification was performed using two different strategies. On the one hand, a colorimetric kit (KHDBA kit, Megazyme Ltd. Ireland) was used for a rapid screening of the R-3-hydroxybutyric acid, according to the manufacturer's instructions. Briefly, approximately 10–15 mg of dried cells were resuspended in 400 μL of 0.5 M NaOH and incubated for 1 h at 85 °C in a shaking thermomixer (Eppendorf, Germany). After cooling, the samples were neutralized by adding 100 μL of 1 M HCl. PHB content was analyzed colorimetrically from the supernatant by measuring the absorbance at 492 nm in a microplate reader (Spectrostar Nano, BMG Labtech, Germany). Data were expressed as the amount (g) of PHB contained in 100 g of lyophilized cells (% of PHB content). In addition, the PHA content was analyzed by gas chromatography (GC). Briefly, approximately 10–15 mg of lyophilized cells were placed in glass vials. Then, 50 μL of benzoic acid (PanReac, Spain) (20 g/L in 1-propanol) (Acros Organics, Germany) was added as internal standard, followed by 1.5 mL digestion solution (1-propanol:HCl 37 % (4:1; v:v)) and 1.5 mL 1,2-dichloromethane (Fisher Scientific, Germany). The samples were then digested at 100 °C for 3 h. After cooling the solution,

Table 2
Kinetics, culture parameters, and DSC properties of biopolymer during the initial strain selection obtained from MSM supplemented with potato wastewater hydrolysate. The microorganisms are different strains of *C. necator*. Data are shown as mean±SD ($n = 3$). Asterisks indicate differences between variants for the same culture parameter ($p < 0.001$). $\mu_{máx}$: maximum growth rate; λ: lag phase duration; G: duplication time; r_p: volumetric productivity; $Y_{X/S}$: growth yield; $Y_{P/S}$: polymer yield; T_m: melting temperature; ΔH_f: enthalpy of fusion; T_d: decomposition temperature; X_c: degree of crystallinity.

C. necator	$\mu_{máx}$ (h^{-1})	λ (h)	G (h)	r_p ($gL^{-1}h^{-1}$)	$Y_{X/S}$	$Y_{P/S}$	T_m (°C)	ΔH_f (J/g)	T_d (°C)	X_c
DSM 428	0.248 ± 0.035	3.96 ± 0.92	2.86 ± 0.44	0.003 ± 0.001	0.299 ± 0.022	0.036 ± 0.009	174.66 ± 0.59	−81.71 ± 3.73	294.52 ± 0.92	0.56 ± 0.03
DSM 531	0.231 ± 0.031	26.99 ± 1.09	3.07 ± 0.45	0.007 ± 0.001	0.351 ± 0.007	0.076 ± 0.010	173.91 ± 0.48	−70.84 ± 3.64	294.37 ± 0.53	0.49 ± 0.02
DSM 545	0.273 ± 0.005	20.51 ± 0.92	2.54 ± 0.05	0.030 ± 0.004*	0.449 ± 0.004*	0.161 ± 0.021*	176.74 ± 0.23	−82.85 ± 0.68	296.01 ± 0.06	0.57 ± 0.00
DSM 13513	0.202 ± 0.022	4.76 ± 1.32	3.46 ± 0.35	0.003 ± 0.001	0.363 ± 0.011	0.031 ± 0.005	170.91 ± 1.96	−40.88 ± 7.21	294.65 ± 2.18	0.28 ± 0.05
PHB/PHV standard	–	–	–	–	–	–	154.35 ±0.09	−54.26 ± 0.33	282.65 ± 1.79	0.37 ± 0.00

3 mL of Milli Q water was added and the tubes were mixed by vortexing for 2 min. To recover the organic phase, samples were centrifuged at 2000 rpm for 5 min, and the resulting methyl esters were filtered through PTFE filters (Clarify, China) and analyzed by GC coupled to a flame ionization detector (FID). Poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P(3HB-*co*-8 mol% 3HV)) (Sigma Aldrich) was used as a reference standard. PHA content was analyzed using a gas chromatograph (7890A GC, Agilent Technologies) equipped with FID and a 30 m polyethylene glycol (PEG) column (30 m × 0.25 mm, i.d: 0.25 μm film thickness) (HP-INNOWax, Agilent Technologies). Helium (purity 99.999 %) at 1 mL/min was used as the carrier gas. The column temperature was started at 60 °C, then increased at 5 °C/min to 160 °C, and finally ramped up to 250 °C at 15 °C/min. The injector temperature was maintained at 250 °C, the injector volume was 1 μL, and the split ratio was set to 16:1. The detector temperature was set at 260 °C. GC results were expressed as the percentage of PHB containing on the lyophilized biomass.

2.4.4. PHA extraction and thermal analysis

Biopolymer was extracted from lyophilized cells by magnetic stirring in chloroform (Fisher Scientific, Germany) (at a biomass:solvent ratio of 1:200, w:v) at 50 °C for 3 days. Cell debris and unsolved material were removed by vacuum filtration using 0.45 μm GxF filters (Filterlab). To obtain a biopolymer film, the solution was filtered directly onto a glass Petri dish and allowed to evaporate. The protocol described by Rodrigues et al. (2022) for PHA extraction using NaOH was followed with slight modifications. Briefly, 100 mg of lyophilized cells were mixed with 10 mL of 0.3 M NaOH at approximately 10 g/L of biomass. The mixture was placed on a glass vial and incubated in an orbital shaker at 30 °C for 4 h. The contents were centrifuged at 4000 rpm for 20 min and washed once with 20 mL of distilled water. After removal of water, the pellet was dried at 50 °C for 24 h to constant weight. The resulting residue was homogenized in a mill to obtain a uniform powder for subsequent analysis. The PHA yield parameters were described by Mannina et al. (2019) as equations given below:

$$PHA\ yield = \frac{Extracted\ polymer\ weight}{Biomass\ weight} \quad (gravimetrically\ determined) \tag{9}$$

$$PHA\ purity = \frac{PHA\ mass}{Extracted\ polymer\ mass} \quad (determined\ by\ GC) \tag{10}$$

$$PHA\ recovery = \frac{PHA\ yield \times PHA\ purity}{PHA\ in\ microbial\ cells} \tag{11}$$

The polymers, both films and powders, were subjected to differential scanning calorimetry (DSC) measurements (Mettler Toledo, Switzerland) following the indications of López-Abelairas et al., 2015. Approximately, 1–2 mg of biopolymer was loaded into a covered aluminum crucible and heated from 25 °C to 400 °C at a heating rate of 10 °C min^{-1} in a N_2 atmosphere (at a flow rate of 50 mL/min). The baseline was calibrated by scanning the temperature range used with two empty pans. Calibration was performed using high purity indium. The melting temperature and enthalpy of fusion (ΔH_f) were calculated from the maximum and the area of the first endothermic peak, respectively, whereas the decomposition temperature (T_d) was calculated from the second endothermic peak. Crystallinity fraction (X_c) was calculated as follows:

$$X_c = \frac{\Delta H_f}{\Delta H_f^o} \tag{12}$$

where ΔH_f^o is the melting enthalpy of the 100 % crystalline polymer PHB, estimated to be 146 J g^{-1} (López-Abelairas et al., 2015).

2.5. Statistical design, modeling and analysis

2.5.1. Box–Behnken design

The optimization of the enzymatic hydrolysis and the culture conditions were carried out using a Box-Behnken design linked to RSM. The experimental design comprised 15 experimental runs, including 3 central points, and had 3 independent variables, 1 replicate, and 1 block. The range of each independent variables is shown in Tables 1 and 3 (see supplementary material). Glucose release for optimization of enzymatic hydrolysis and PHB accumulation (% of DCW) for optimization of culture conditions were considered as response variables for the model. Experimental data generated for the RSM were fitted to the model equation of Box–Behnken design using Minitab 17 software (Minitab, Stage Collage, Pennsylvania, USA). Statistical parameters used for model evaluation were *p*-value, the R-squared (R^2), and the test for lack-of-it. Statistical significance was set as p-value < 0.05. Contour and surface plots were plotted to determine the interaction effects of all independent response variables. The combination of predicted values that optimized the fitted response was used to validate the model by performing experiments in triplicate.

2.5.2. Statistical analysis

For comparative purposes, statistical analysis was performed using Graphpad Prism v.8.0.2 (GraphPad Software, Inc., San Diego, CA, USA), except as noted above. The normality of the data was tested using the Shapiro-Wilk's test. Parametric analysis of variance (ANOVA test) was performed, and significant differences were detected using the Bonferroni *post hoc* test ($p < 0.05$). All data are expressed as mean±SD.

3. Results and discussion

3.1. Potato wastewater hydrolysis

As expected, potato wastewater was characterized by a high starch content, up to about 80 g/L depending on the batch, which was higher than the values reported by other authors (Abdel-Raheam et al., 2022; Chen et al., 2019; Huang et al., 2005), who used potato production water containing less starch, ranged from 1.5 to 4 times lower than those used in this study. Moreover, the residue used in this work had a scarce nitrogen source. This scenario could be very useful for PHA production, since this polymer is produced during unbalanced growth, mainly under nitrogen starvation (Cavalheiro et al., 2012) and when the carbon-to-nitrogen (C/N) ratio is increased (Ahn et al., 2015; Chin et al., 2022).

Cupriaviadus necator DSM 545 is unable to directly utilize starch as a carbon source; however, this strain is a recombinant mutant of the *C. necator* H16 strain with the ability to utilize glucose and fructose for growth and PHA production (Ertan et al., 2021; Poomipuk et al., 2014). Since the selected feedstock was deficient in free glucose (data not shown), hydrolysis of potato wastewater was essential to provide glucose to the microorganism. Autohydrolysis alone is not sufficient to release glucose monomers, as this treatment yielded only about 0.17 g/L of glucose. However, this thermal pretreatment helped to destabilize the starch architecture and facilitate subsequent enzymatic action. The initial conditions of the enzymatic hydrolysis were set according to the previous data of Abanoz et al. (2012). In order to reduce the amount of enzymes used and the time of hydrolysis by amyloglucosidase, without compromising the glucose concentration obtained, a design by RSM was performed to find the best conditions for this purpose. The fifteen hydrolysis conditions produced a fluctuating glucose concentration, ranging from 22.7 g/L to 85.2 g/L (Table 1). Statistical analysis revealed that the two-way interaction was not statistically significant ($p > 0.05$). Therefore, the model was re-evaluated after removing the statistically insignificant terms. The new model explained 97.41 % of the variation, and its predicted R-squared value of 90.97 % indicated that the model had the power to predict responses for new observations (see

Table 3
Growth and culture parameters recorded for *C. necator* DSM 545 incubation during the first RSM design for the optimization of culture conditions. For each condition tested, the percentage of hexose consumed, biomass (as DCW), percentage of PHB content, volumetric productivity (r_p), growth yield ($Y_{X/S}$), and polymer yield ($Y_{P/S}$) after 72 h of incubation are shown. Run numbers highlighted in bold correspond to central points.

Run	[hexoses] (g/L)	[$NaNO_3$] (g/L)	pH	C/N ratio	Consumed hexoses (%)	DCW (g/L)	PHB (%)	r_p ($gL^{-1}h^{-1}$)	$Y_{X/S}$	$Y_{P/S}$
1	25.00	0.50	6.00	35.8	44.04	5.97	82.13	0.0670	0.5630	0.4624
2	35.00	0.50	6.50	50.1	25.29	5.16	86.12	0.0594	0.6633	0.5712
3	15.00	0.50	6.50	21.5	71.00	4.48	76.05	0.0459	0.4586	0.3487
4	35.00	1.25	7.00	36.3	7.02	2.05	36.13	0.0085	0.8031	0.2901
5	25.00	1.25	6.50	26.0	64.32	7.09	80.05	0.0766	0.4861	0.3891
6	35.00	1.25	6.00	36.3	38.86	6.66	71.09	0.0620	0.4818	0.3425
7	25.00	0.50	7.00	35.8	33.86	3.99	79.14	0.0407	0.5041	0.3989
8	25.00	1.25	6.50	26.0	67.20	7.50	77.91	0.07862	0.4974	0.3875
9	25.00	2.00	6.00	20.4	63.49	7.19	70.82	0.0670	0.4476	0.3169
10	25.00	2.00	7.00	20.4	54.32	6.04	73.62	0.0588	0.5155	0.3795
11	25.00	1.25	6.50	26.0	65.12	7.07	77.08	0.0730	0.4821	0.3716
12	15.00	1.25	6.00	15.6	97.78	6.08	75.40	0.0603	0.4228	0.3187
13	15.00	1.25	7.00	15.6	88.75	5.13	75.77	0.0525	0.4453	0.3374
14	15.00	2.00	6.50	12.2	96.44	5.81	66.36	0.0519	0.4287	0.2844
15	35.00	2.00	6.50	28.5	37.87	5.88	71.57	0.0546	0.4745	0.3396

supplementary material). The regression model equation for optimizing potato wastewater hydrolysis was as described in the following equation:

$$[glucose]\left(\frac{g}{L}\right) = -10.22 + 0.00637 \times [alpha\ amylase] + 1.228 \times [amyloglucosidase] + 0.571 \times Time - 0.000003 \times [alpha\ amylase] \times [alpha\ amylase] - 0.00572 \times [amyloglucosidase] \times [amyloglucosidase] - 0.00260 \times Time \times Time \quad (13)$$

The optimized model showed that the best conditions for enzymatic hydrolysis of potato wastewater would be a liquefaction step with 972 U α-amylase and a saccharification treatment with 99.50 U amyloglucosidase for 60 min, resulting in an estimated glucose concentration of 83.33 g/L. This model was validated experimentally in triplicate, yielding a hydrolysate with a glucose content of 81.8 ± 0.6 g/L and a hydrolysis yield of 95.5 ± 0.7 %, which was considered very successful. The incubation time during saccharification was reduced by almost fifty times compared to the original methodology, in which the authors took this time up to 48 h (Abanoz et al., 2012).

Potato starchy wastewater is a rich medium containing high concentrations of starch, reducing sugars, organic matter, etc. making it a promising residue for the production of multiple value-added products (Chauhan et al., 2023). However, its utilization in microbial culture processes for PHA production is still limited. Some studies focus on the different methods used for starch hydrolysis. Enzymatic hydrolysis is considered one of the most commonly used methods and the α-amylase and amyloglucosidase enzymes are the most suitable for this purpose (Chen et al., 2019). Haas and coworkers hydrolyzed potato starch with industrial liquozyme supra® (α-amylase) by boiling for 3 h and AMG300 BrewQ® (glucoamylase) by incubation at 60 °C for 18 h, rendering up to 550 g/L of glucose and approximately 82.5 % of hydrolysis, the latter being somewhat lower than the present results, even though the time required for hydrolysis was longer (Haas et al., 2008). Furthermore, Rusendi and Sheppard hydrolyzed potato processing waste using 9 times higher α-amylase (with twice the incubation time of the present study at 70 °C) and added 27 times higher glucoamylase (with three times the incubation time at 55 °C). They achieved approximately 98 % of hydrolysis after 60 min of α-amylase incubation, a value similar to that obtained in the present work (Rusendi and Sheppard, 1995). Other starch-based substrates, such as cassava or wheat and rice bran, have been hydrolyzed using the same enzymes at a higher concentration, up to 50 times higher, with starch hydrolysis ranging from 47.7 % to 85 %, respectively (Poomipuk et al., 2014; Shamala et al., 2012). In light of these results, the optimized conditions described in the present work not only reduced the amount of enzymes, but also reduced the time of hydrolysis without reducing the percentage of starchy wastewater hydrolysis, making the process more energy and economically efficient.

3.2. Culture in potato wastewater hydrolysate and strain selection

Once the microbial cells capacity to utilize the media for PHA production was assessed (data not shown), an initial strain selection based on the metabolism of the potato wastewater hydrolysate was performed. It is known that the four strains are natural PHA producers from a very different types of residues such as waste oils, paper, molasses, spent coffee grounds, etc. (Al Battashi et al., 2021; Cruz et al., 2014; Dalsasso et al., 2019; Loan et al., 2022; Obruca et al., 2015). However, *C. necator* DSM 428 and closely related strains such as DSM 531 have a limited ability to grow on glucose as the sole carbon source (Orita et al., 2012). In the present study, all strains were able to grow and to produce PHB to some extent; however, the strain with the highest volumetric productivity (0.030 ± 0.004 $gL^{-1}h^{-1}$), growth yield (0.449 ± 0.004) and polymer yield (0.161 ± 0.021) after 72 h of culture was strain DSM 545 (Table 2). Although DSM 545 did not show significant differences with respect to the other strains, it developed the maximum growth rate of 0.273 ± 0.005 h^{-1}, with a lag phase of 20.51 ± 0.92 h and a generation time of 2.54 ± 0.05 h (Table 2). Moreover, DSM 545 produced significantly ($p < 0.01$) the higher biomass (6.25 ± 0.05 g/L) and the higher polymer accumulation and concentration (35.79 ± 4.51 %, 2.24 ± 0.30 g/L – note that these values correspond to PHB quantification by the colorimetric method) at 72 h (Fig. 1).

C. necator DSM 428 (also known as H16) is one of the most important strains used for PHAs production, being able to accumulate up to 80 % of its dry cell weight (Arikawa and Sato, 2022). However, in the current study, DSM 428 was one of the less productive strains (2.22 ± 0.16 g/L biomass; 11.97 ± 2.13 % PHB, 0.27 ± 0.06 g/L PHB), along with DSM 13513 (2.82 ± 0.09 g/L biomass; 8.39 ± 1.14 % PHB, 0.24 ± 0.03 g/L PHB) and DSM 531 (2.53 ± 0.06 g/L biomass; 21.62 ± 3.20 % PHB, 0.54 ± 0.07 g/L PHB). These results were far away from the data reported in the bibliography for these strains, which could utilize different substrates and produce PHB when using volatile fatty acids from waste paper, waste cooking oil, or dairy wastewater (Al Battashi et al., 2021; Loan et al., 2022; Pagliano et al., 2020). Moreover, Haas and coworkers reported that DSM 428 reached 11.3 g/L of dried biomass and 66 % PHB; DSM 531 yielded 3.5 g/L biomass and 46 % PHB and DSM 545 achieved 14.0 g/L of dry biomass, containing 78 % PHB, when chicory root hydrolysate was used (Haas et al., 2015). These values are higher than those showed in the present work, presumably due to the high concentration of fructose contained in the production media and the preference

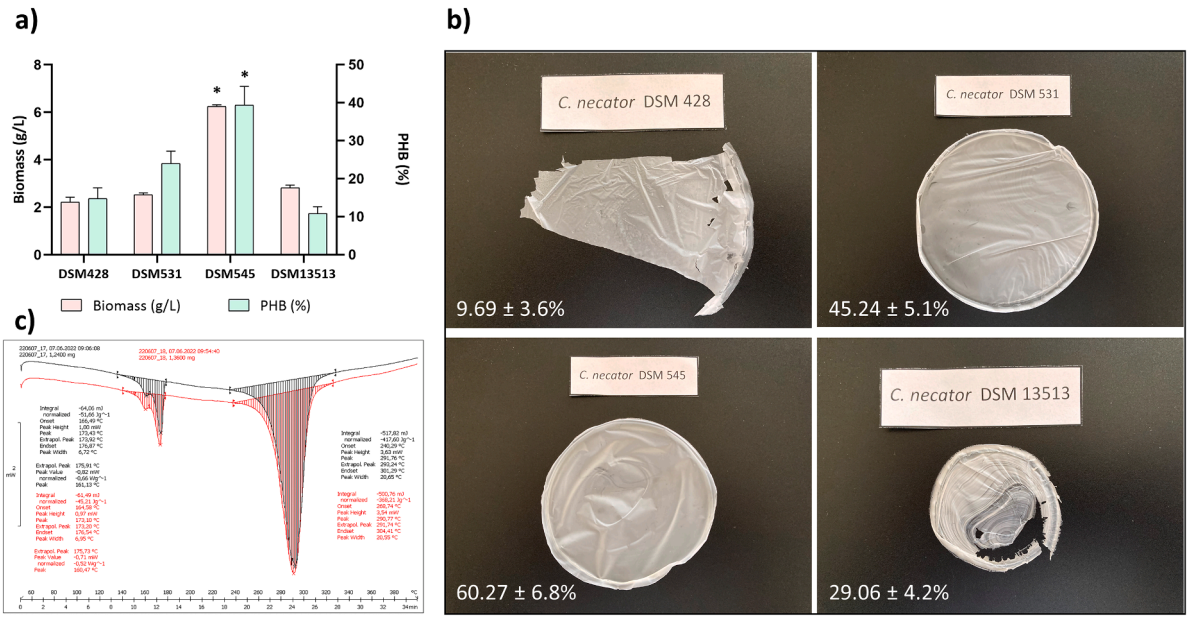

Fig. 1. Initial selection of the *C. necator* strain. a) Biomass (as dry cell weight) and PHB production after 72 h of batch cultivation with potato wastewater hydrolysate. Note that in this case, PHB quantification was performed by the colorimetric method. b) Biopolymer films (PHB) extracted by the conventional chloroform method from the four studied strains after 72 h of cultivation with potato wastewater hydrolysate. The polymer extraction yield (expressed as a percentage) is indicated in each image. c) Example of an integrated DSC heating scan of the polymer extracted from *C. necator* DSM13513. Data are expressed as mean±SD ($n = 3$). Asterisks indicate significant differences with respect to the other strains (* $p < 0.01$).

of DSM 428 and DSM 531 to metabolize fructose instead of glucose. This assumption is consistent with the analysis of the percentage of hexoses consumed, which showed that DSM 428, DSM 531 and DSM 13513 preferentially used fructose instead of glucose. Thus, in this study, *C. necator* DSM 428 metabolized 12.02 ± 1.19 % of the initial glucose and 88.78 ± 0.05 % of the fructose; DSM 531 consumed 11.18 ± 0.37 % of the glucose and 86.50 ± 0.92 % of the fructose, and DSM 13513 used 14.77 ± 0.47 % of the glucose and 88.78 ± 0.05 % of the fructose after 72 h of culture. However, *C. necator* DSM 545 was able to metabolize 94.41 ± 0.78 % of the initial glucose and only 47.71 ± 0.33 % of the fructose.

The polymer extraction yield was significantly higher for DSM 545 (60.27 ± 6.83 %), as expected since this strain is the most productive of the strains analyzed; however, the second most productive strain, DSM 531, did not show significant differences in the polymer yield compared to DSM 13513 or DSM 428 (45.24 ± 5.13 %) ($p < 0.05$) (Fig. 1). No significant differences were found in the melting temperature, or in decomposition temperature of any of the polymers recovered by the chloroform method (Table 2). The melting temperature of the biopolymer produced by the different strains was higher than that of the reference standard, however the standard consisted of a heteropolymer of PHBV, whereas the biopolymer produced by *Cupriavidus* strains could be preferentially PHB. The decomposition temperature, a good indicator of thermal stability, was very similar among the different strains, the values obtained being within the range described in the literature (López-Abelairas et al., 2015). The degree of crystallinity was very similar for the polymer extracted from DSM 428, DSM 531 and DSM 545 (ranging from almost 50–57 %), but was much lower for DSM 13513 (Table 2). Accordingly, *C. necator* DSM 545 was selected to carry out the optimization of the conditions for PHB production using potato wastewater hydrolysate.

3.3. Optimization of culture conditions with C. necator DSM 545

Under the initial conditions selected to test the ability of the strains to produce PHA, *C. necator* DSM 545 was able to produce up to 57.54 ± 7.81 % PHB (2.56 ± 0.56 g/L PHB) (note that these and the following PHB quantification values are obtained by GC), resulting in a biomass of 4.41 ± 0.34 g/L after 72 h of fermentation. It should be noted that these values for PHB accumulation were higher than those found in the literature for the same strain and very similar feedstock (Brojanigo et al., 2020; Ertan et al., 2021). Nevertheless, to improve this PHB accumulation during the fermentation of potato wastewater hydrolysate with *C. necator* DSM 545, an initial RSM using a Box–Behnken design was carry out to determine the most appropriate values for hexose concentration (considering glucose and fructose), sodium nitrate concentration and initial pH. The design rendered a variable PHB content, ranging from 36.13 % to 86.12 % of PHB (Table 3). A mathematical model was constructed from these data (Eq. (14)) that explained 67.13 % of the variation (see supplementary material).

$$\begin{aligned}[\mathrm{PHB}](\%) = {} & -1187 + 15.12 \times [\mathrm{hexoses}] - 46.8 \times [\mathrm{NaNO_3}] + 352 \times \mathrm{pH} \\ & - 0.0758 \times [\mathrm{hexoses}] \times [\mathrm{hexoses}] + 7.6 \times [\mathrm{NaNO_3}] \times [\mathrm{NaNO_3}] \\ & - 24.7 \times \mathrm{pH} \times \mathrm{pH} - 0.162 \times [\mathrm{hexoses}] \times [\mathrm{NaNO_3}] - 1.77 \\ & \times [\mathrm{hexoses}] \times \mathrm{pH} + 3.9 \times [\mathrm{NaNO_3}] \times \mathrm{pH} \end{aligned} \quad (14)$$

In any case, although the mathematical model was overfitted, the estimated regression equation (Eq. (14)) allowed to calculate the optimal hexose and sodium nitrate concentration and initial pH conditions that would result in the highest PHB accumulation. For this initial optimization approach, the best parameters to meet this requirement would be 27.12 g/L hexoses, 0.5 g/L $NaNO_3$, and pH 6.19, which would result in a theoretical PHB content of 89.27 %. This model was experimentally validated and rendered a PHB accumulation of 88.68 ± 1.98 %, a value not significantly different from the calculated theoretical value ($p = 0.7155$). Despite the overfitting of the model, the RSM employed was accurate enough to estimate PHB content (89.27 % estimated vs. 88.68 % experimental).

A second RSM with Box-Behnken design was applied to try to increase PHB production and improve the residue utilization (see supplementary material). The mathematical model constructed using these data (Eq. (15)) explained 93.85 % of the variation and allowed the estimation of optimal conditions: 45.0 g/L for hexose concentration, 0.1

g/L for $NaNO_3$ concentration, and pH 7.0, which would result in 99.04 % of PHB accumulation, a value validated experimentally in triplicate, yielding a PHB accumulation of 98.57 ± 3.00 % ($p = 0.8458$).

$$[\mathrm{PHB}](\%) = -901 + 5.88 \times [\mathrm{hexoses}] - 87.4 \times [\mathrm{NaNO_3}] + 336 \times \mathrm{pH} + 0.1359 \times [\mathrm{hexoses}] \times [\mathrm{hexoses}] + 6.30 \times [\mathrm{NaNO_3}] \times [\mathrm{NaNO_3}] - 25.53 \times \mathrm{pH} \times \mathrm{pH} - 0.454 \times [\mathrm{hexoses}] \times [\mathrm{NaNO_3}] - 0.338 \times [\mathrm{hexoses}] \times \mathrm{pH} + 8.68 \times [\mathrm{NaNO_3}] \times \mathrm{pH} \quad (15)$$

To check the real improvement of the optimized conditions, *C. necator* DSM 545 was cultivated in triplicate for 72 h under: i) Initial conditions (see Section 2.2); ii) First estimated optimum and iii) Second estimated optimum. Through the two rounds of culture optimization, PHB production was significantly increased with respect to the initial conditions, going from 57.54 ± 7.81 % to 83.74 ± 2.37 % ($p = 0.0063$) and 95.28 ± 3.31 % ($p = 0.0009$) for the first and second optimums, respectively (Table 4). This data was consistent with the increase in the carbon-to-nitrogen ratio, as it changed from 20 g/L under the initial conditions to 38.8 g/L and 80.7 g/L under the conditions defined by the first and second optimums, respectively. However, the biomass increased significantly only in the first optimum, reaching 6.09 ± 0.38 g/L ($p = 0.0028$). As can be seen in Table 4, the kinetic parameter μ_{max} was not improved under the optimal conditions evaluated, while the culture parameters were significantly increased. In view of these results, the estimated conditions for the second optimum allowed an increase in PHB productivity and an improvement in culture yields regarding the initial conditions, but they did not promote an advantage with respect to the first optimum. Moreover, a higher consumption of wastewater hydrolysate was employed under the conditions of the first optimum than under the conditions of the second optimum (57.57 ± 3.27% vs. 19.76 ± 1.39 % of consumed hexoses, respectively). Therefore, the first optimum was selected as the best conditions that, from a technical and environmental point of view, resulted in the highest PHB accumulation by *C. necator* DSM 545 from potato wastewater hydrolysate.

With respect to the culture conditions calculated in the two rounds of RSM, the estimated optimums define culture parameters that yield values in the highest range of PHB accumulation obtained at the laboratory scale, despite the overfitting of the model. In many cases, these values are far from the ranges described in the literature. For example, different authors have described PHA synthesis from starchy substrates by *C. necator* DSM 545. Such is the case of Rusendi and Sheppard (1995), who used half the concentration of glucose used in the present study (13.6 g/L glucose), coming from potato waste hydrolysate, and they obtained 5.0 g/L biomass and 76.9 % PHB with the same microbial strain. In 2008, Haas and colleagues obtained higher results than those reported here, using acidified waste potato starch (Haas et al., 2008). They used *Rasltonia eutropha* NCIMB 11599, a glucose-utilizing mutant, similar to DSM 545, which produced 179 g/L biomass and 94 g/L PHB, large amounts especially compared to the data presented in this work. It should be noted that these authors carried out potato fermentation in a fed-batch reactor, which is different from the shake flasks used in this study. However, considering the percentage of PHB accumulation from this study, these data are in the high range of the values usually obtained from starchy substrates (Brojanigo et al., 2020; Dalcanton et al., 2010; Poomipuk et al., 2014; Rusendi and Sheppard, 1995) (Table 5). The conditions defined in this study open the door to future developments to scale-up the process with the goal of industrial application.

C. necator DSM 545 is a high PHB producer and a versatile microorganism, able to adapt to substrates of different constitution, as it could be checked in Table 5. There are several challenges to overcome in this field, but the possibility of carrying out efficient processes of simultaneous saccharification and fermentation, or eliminating the hydrolysis pretreatment step by genetic modification of the microorganisms, or even using alternative feedstocks with high nitrogen content as a nitrogen supplement, could make industrial bioplastic production in an efficient, cost-effective and sustainable manner a near reality.

3.4. PHB extraction methods

Conventional chloroform extraction resulted in a biopolymer yield of 63.85 ± 5.62 %, a purity of 102.75 ± 3.81 % and a recovery of 72.48 ± 6.14 % (Table 6). The PHB yield and recovery values are lower than those previously reported by Gahlawat and Soni (2017), who showed a PHA yield of 95 % and a PHA recovery of 90–95 % when polymer extraction was performed using chloroform from *C. necator* DSM 545 biomass. In the same vein, Fiorese and coworkers reported a 96 % recovery of PHB after chloroform extraction of the polymer produced by the same microorganism (Fiorese et al., 2009). These differences were attributed to the method used in the present study, particularly the avoidance of the alcohol precipitation step. However, the PHB purity in the present study was higher than that obtained by Gahlawat and Soni (2017), who described up to 91 % PHBV purity, or by Fiorese et al. (2009), who showed 95 % of PHB purity.

Chlorinated solvents have some associated problems, mainly related to human and animal health, and also related to economics due to their high cost on an industrial scale. They also have some drawbacks in terms of their impact on the environmental footprint of their use. Therefore, more ecofriendly and less expensive solvents sought to enable a more economical and efficient industrial process. In the present study, an inexpensive and reusable alkaline solvent that showed no environmental impact at low doses were evaluated. PHB extraction using NaOH rendered a significantly higher PHB yield of 78.78 ± 0.57 %. In terms of PHB purity and recovery, no improvement over the halogenated method was observed, resulting in a PHB purity of 99.83 ± 4.95 % and a recovery of 87.01 ± 4.61 % (Table 6). PHB purity is very similar to the value reported by the authors who originally described the alkaline extraction method (Rodrigues et al., 2022); however, the current PHB recovery percentage is slightly lower than the previously reported values. It should be noted that the conditions described by these authors were originally used to treat mixed microbial culture and may behave harshly with pure culture as the ones used in the present study. In addition, this difference could be related to technical limitations of the centrifugation equipment, which did not allow complete precipitation of the polymer, making separation of the solid and liquid phases difficult. Other authors have described a similar procedure for PHA extraction using NaOH and have reported similar values. As previously reported, Mohammadi and coworkers obtained PHA from *C. necator* after

Table 4
Growth and culture parameters recorded for *C. necator* DSM 545 in different PHA producing media. The conditions tested are: Initial conditions (20 g/L hexoses, 2.82 g/L $NaNO_3$, pH 7.0); First estimated optimum (27.12 g/L hexoses, 0.50 g/L $NaNO_3$, pH 6.19) and Second estimated optimum (45.00 g/L hexoses, 0.10 g/L $NaNO_3$, pH 7.00). Table summarizes data for hexose consumption (%), biomass (as DCW, g/L), PHB content (%), maximum growth rate (μ_{max}), volumetric productivity (r_p), growth yield ($Y_{X/S}$), and polymer yield ($Y_{P/S}$) after 72 h of incubation. Data are presented as mean±SD ($n = 3$). Different letters in the same column indicate a significant difference between the culture conditions ($p < 0.01$).

	Consumed hexoses (%)	Biomass (g/L)	PHB (%)	PHB (g/L)	μ_{max} (h^{-1})	r_p ($gL^{-1}h^{-1}$)	$Y_{X/S}$	$Y_{P/S}$
Initial conditions	79.22 ± 1.06 [a]	4.41 ± 0.34 [a]	57.54 ± 7.81 [a]	2.6 ± 0.6 [a]	0.362 ± 0.008	0.036 ± 0.008 [a]	0.350 ± 0.022 [a]	0.203 ± 0.041 [a]
First optimum	57.57 ± 3.27 [b]	6.09 ± 0.38 [b]	83.74 ± 2.37 [b]	5.1 ± 0.2 [b]	0.363 ± 0.009	0.071 ± 0.002 [b]	0.431 ± 0.010 [b]	0.361 ± 0.008 [b]
Second optimum	19.76 ± 1.39 [c]	4.56 ± 0.26 [a]	95.28 ± 3.31 [b]	4.3 ± 0.2 [b]	0.326 ± 0.098	0.060 ± 0.001 [b]	0.637 ± 0.004 [c]	0.607 ± 0.019 [c]

Table 5
Production of biomass and bioplastics by culture of *C. necator* DSM 545 using carbon/nitrogen sources coming from a variety of feedstocks and in different modes of operation. n.d.: not determined.

Feedstock	Pretreatment	Biomass (g/L)	PHA	Mode of operation	References
Sugarcane molasses	H_2SO_4 (pH 1.1) and thermal pretreatment	approx. 9.8	1.30 g/L	Batch (shake flask)	(Baei et al., 2011)
Cane molasses	–	23.06	39.00 %	Batch (shake flask)	(Beaulieu et al., 1995)
White sweet potato	Enzymatic hydrolysis	11.10	27.67 %	Batch (shake flask)	(Brojanigo et al., 2020)
Purple sweet potato	Enzymatic hydrolysis	10.86	31.05 %	Batch (shake flask)	(Brojanigo et al., 2020)
Waste grade glycerol	–	not determined	24.6 %	Fed-batch (2 L reactor)	(Cavalheiro et al., 2012)
Waste grade glycerol	–	82.5	62 %	Fed-batch (2 L reactor)	(Cavalheiro et al., 2009)
Rice starch hydrolysate – soy oil supplemented	2.7 M HCl and therma pretreatment	11.64	43.00 %	Batch (shake flask)	(Dalcanton et al., 2010)
Molasses	Enzymatic hydrolysis	9.1 (residual biomass)	11.7 g/L (equivalent to 56 %)	Fed-batch (7.5 L reactor)	(Dalsasso et al., 2019)
Vinasse and molasses	Enzymatic hydrolysis	12.4 (residual biomass)	12.6 g/L (equivalent to 58 %)	Fed-batch (7.5 L reactor)	(Dalsasso et al., 2019)
Glucose	–	3.30	33.97 %	Batch (shake flask)	(Ertan et al., 2021)
Oil from spent coffee grounds	n-hexane extraction	8.90	89.6 % (PHB/PHV)	Batch (3 L reactor)	(Ingram and Winterburn, 2021)
Orange fruit wastes	2.9 % Citric acid, thermal and enzymatic pretreatment	1.23	n.d.	Batch (shake flask)	(Locatelli et al., 2019)
Passion fruit wastes		0.83	n.d.	Batch (shake flask)	(Locatelli et al., 2019)
Cassava starch	Enzymatic hydrolysis	4.28	73.88 %	Batch (shake flask)	(Poomipuk et al., 2014)
Slaughterhouse residues (swine)	Freeze-thaw and filtration	2.5	18.7 % (0.5 g/L)	Batch (shake flask)	(Rodríguez G. et al., 2021)
Potato processing waste	Enzymatic hydrolysis	5.00	76.90 %	Batch (reactor with 1 L working volume)	(Rusendi and Sheppard, 1995)
Potato starchy wastewater	Enzymatic hydrolysis	6.09	83.74 % (5.1 g/L)	Batch (shake flask)	This study

Table 6
PHA yield, purity and recovery and DSC properties of biopolymer extracted from *C. necator* DSM 545 using two extraction methods. Data are shown as mean±SD ($n = 3$). T_m: melting temperature; ΔH_f: enthalpy of fusion; T_d: decomposition temperature; X_c: degree of crystallinity.

Extraction method	PHA yield (%)	PHA purity (%)	PHA recovery (%)	Tm (ºC)	ΔH_f (J/g)	T_d (ºC)	X_c
Chloroform extraction	63.85 ± 5.62 [a]	102.75 ± 3.81 [a]	72.48 ± 6.14 [a]	177.17 ± 0.11 [a]	−79.12 ± 2.40 [a]	295.16 ± 1.59 [a]	0.54 ± 0.20 [a]
NaOH extraction	78.78 ± 0.57 [b]	99.83 ± 4.95 [a]	87.01 ± 4.61 [a]	172.37 ± 0.72 [b]	−71.46 ± 1.23 [b]	236.21 ± 2.19 [b]	0.49 ± 0.01 [b]

treatment with 0.05 M NaOH for 3 h at 4 °C with 96.6 ± 1.6 % PHA purity and 96.9 ± 1.5 % PHA recovery (Mohammadi et al., 2012). Moreover, López-Abelairas and colleagues, using a similar concentration of alkali agent, obtained similar values of PHB purity and recovery (approximately 92 % and 80 %, respectively) even when performing the extraction from a different *Cupriavidus* strain (López-Abelairas et al., 2015). These results point out that alkali concentration and time of contact with microbial biomass may be critical factors in determining polymer recovery yield results. Further studies will be required to determine the best conditions for the extraction of PHB using NaOH with the highest recovery yield.

Regarding the thermal properties of the biopolymer obtained by the two tested methods, differences in melting temperature, enthalpy of fusion, decomposition temperature and degree of crystallinity were observed (Table 6). The melting temperature of the polymer obtained by the alkali method was within the range of other PHB polymers obtained by the chloroform method (Fiorese et al., 2009). It is interesting to note that the decomposition temperature of the polymer obtained by the NaOH treatment was significantly lower (236.21 ± 2.19 °C) than that of the polymer extracted by the chloroform method (295.16 ± 1.59 °C). This may be explained by different factors. First, the NaOH method for PHB extraction under such conditions was not the best for ensuring the integrity of the polymer. The ester bonds of PHA are susceptible to hydrolysis, and NaOH is known to degrade PHA, reducing molecular weight and crystallinity (Anis et al., 2013; Mohammadi et al., 2012). Secondly, the cells may be more susceptible to these NaOH conditions than the cells used in the original protocol. In this study, the microbial cells came from a pure culture, whereas Rodrigues and coworkers employed a mixed microbial culture with a heterogeneous community. This should be considered for future applications, or even for optimizing the best parameter for NaOH extraction, as it represents the temperature range in which the polymer could be melted without losing stability and degrading. Very similar thermal values were previously obtained by López-Abelairas et al. (2015) for the biopolymer recovered from *C. necator* H16 by 0.5 N NaOH treatment. These authors also reported a decomposition temperature of the NaOH-extracted polymer that was lower than that of the chloroform-extracted polymer. However, the melting temperatures of the polymer extracted by Rodrigues et al. (2022), using a very similar treatment, were remarkably lower than the present values. This difference could be attributed to the different nature of the polymer, while in the present study only PHB homopolymer was produced, the cited authors extracted a copolymer of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate). Melting temperature and decomposition temperatures for chloroform recovered polymer are within the range described in the literature (López-Abelairas et al., 2015; Marudkla et al., 2018; Rebocho et al., 2020).

4. Conclusions

The present study shows for the first time the highest data on bioplastic production in shake flasks by *C. necator* DSM 545 after culturing in potato wastewater hydrolysate. This work:

- Proposes an optimized enzymatic hydrolysis that uses the lower enzyme dose and time.
- Establishes microbial culture conditions regarding carbon source concentration (potato wastewater hydrolysate), nitrogen source concentration and pH that result in the highest PHB accumulation.

- Suggests the recovery of intracellular PHB by using a non-halogenated agent, more economical and eco-friendly than traditional treatments.

In conclusion, this study highlights the valorization of agroindustrial by-products into PHB, promoting the circular economy.

E-supplementary data for this work can be found in e-version of this paper online.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2023.120766.

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