

**Evaluation of metabolic capabilities
of *Cupriavidus necator* to produce
polyhydroxyalkanoates using low-
cost carbon sources**



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**UNIVERSIDAD DEL VALLE
FACULTAD DE INGENIERÍA
PROGRAMA DE DOCTORADO EN INGENIERÍA
ÉNFASIS EN INGENIERÍA QUÍMICA
SANTIAGO DE CALI
2026**

**Evaluation of metabolic capabilities of *Cupriavidus necator* to produce
polyhydroxyalkanoates using low-cost carbon sources**

**Thesis submitted in partial fulfillment of the requirements for the degree of:
Doctor of Engineering – Emphasis in Chemical Engineering**

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Abstract

Cupriavidus necator (*C. necator*) is a bacterium of great biotechnological interest due to its ability to synthesize polyhydroxyalkanoates (PHA), which are biodegradable polymers with industrial applications. The main objective of this study was to evaluate the metabolic capabilities of *C. necator* for PHA production by integrating different experimental conditions and computational analyses.

To achieve this, batch and pulse-fed batch cultures were carried out. For the latter, three different feeding strategies were applied. In the first strategy, two pulses (8.33 mL) of dextrose derived from bitter cassava starch (20 g/L) and ammonium sulfate at a C/N ratio of 73 were added at 24 and 48 h, extending the culture to 120 h. In the second strategy, a single pulse (3.15 mL) of only dextrose (20 g/L) was added at 48 h, with a total cultivation time of 96 h. And in the third strategy, a single pulse (6.14 mL) of only dextrose (20 g/L) was added at 60 h, also extending the culture to 96 h.

Batch cultures demonstrated the growth of *C. necator*, reaching a maximum biomass concentration of 5.17 g/L at 48 h and a polyhydroxybutyrate (PHB) accumulation of 47.1% at the end of the culture (72 h). In the pulse-fed batch cultures, the first strategy extended the exponential growth phase. This led to a gradual accumulation of PHB, reaching a PHB yield of 0.46 g/g and a productivity of 15 mg/(L*h) after 120 h of cultivation. However, the pulse-fed batch cultures, the first strategy resulted in an extended exponential growth phase and gradual PHB accumulation, reaching yields of 0.46 g/g and a productivity of 15.00 mg/(L*h) after 120 hours of cultivation. However, PHB accumulation was 20.57% and 25.12% lower compared to the accumulation observed in the batch culture. In contrast, the third strategy, with a single pulse at 60 h during the stationary phase, resulted in a higher PHB accumulation (52.40%) and productivity (23.22 mg/(L*h)), surpassing the values obtained in batch and the other pulse-feeding strategies.

Additionally, the *i*CNH2025A model represents a refined and highly curated metabolic reconstruction of *C. necator*, showing significant improvements compared to previous models. With 2,737 reactions, 1,803 unique metabolites, and 1,059 annotated genes, the model integrates key metabolic pathways, including the biosynthesis of PHB and poly(3-hydroxybutyrate-co-3-hydroxyvalerate) (PHBV), as well as central metabolic routes such as the Calvin-Benson-Bassham cycle (CBB), the Entner-Doudoroff (ED) pathway, the Embden-Meyerhof-Parnas (EMP) pathway, and the non-oxidative branch of the pentose phosphate pathway (PPP). The model was validated using MEMOTE, achieving a score of 89% and outperforming previous reconstructions such as RehMBEL1391 and *i*CN1361.

In silico growth simulations with fructose and dextrose showed a high correlation with experimental data ($R^2 = 0.76\text{--}0.78$), validating the model's predictive capacity. Moreover, it accurately captured the carbon flux toward PHB synthesis under various experimental

conditions, consistent with previous studies. Thus, the *iCNH2025A* model represents a significant advancement in the metabolic modeling of *C. necator*, offering a highly accurate and validated tool for analyzing its metabolism. Its ability to predict metabolic fluxes and growth rates makes it a valuable resource for industrial biotechnology, supporting future optimization strategies in biopolymer production.

Keywords: polyhydroxyalkanoates; dextrose from bitter cassava starch; *Cupriavidus necator*; low-cost substrates; mechanical and thermal properties, flux balance analysis.

Resumen

Cupriavidus necator (*C. necator*) es una bacteria de gran interés biotecnológico debido a su capacidad para sintetizar polihidroxialcanoatos (PHA), los cuales son polímeros biodegradables con aplicaciones industriales. El objetivo principal de este estudio fue evaluar las capacidades metabólicas de *C. necator* para la producción de PHA mediante la integración de diferentes condiciones experimentales y análisis computacionales.

Para lograr esto, se llevaron a cabo cultivos por lotes y cultivos alimentados por pulsos. Estos últimos se desarrollaron empleando tres estrategias diferentes: en la primera estrategia se suministraron dos pulsos (8.33 ml) de dextrosa proveniente de almidón de yuca industrial (20 g/L) y sulfato de amonio en una relación C/N de 73.0 a las 24 y 48 horas, extendiendo el cultivo hasta las 120 horas. En la segunda estrategia se suministró un solo pulso (3.15 ml) de dextrosa (20 g/L) a las 48 horas, y el cultivo tuvo una duración de 96 horas, y en la tercera estrategia, se suministró un solo pulso (6.14 ml) de dextrosa (20 g/L) a las 60 horas, extendiendo también el cultivo hasta las 96 horas.

Los cultivos por lote demostraron el crecimiento de *C. necator*, alcanzando una concentración máxima de biomasa de 5.17 g/L a las 48 horas y una acumulación de polihidroxibutirato (PHB) del 47.10% al final del cultivo (72 horas). Con relación a los cultivos lote alimentado por pulsos, para la primera estrategia se observó una extensión de la fase de crecimiento exponencial y una acumulación gradual de PHB, alcanzando rendimientos de 0.46 g/g y una productividad de 15.00 mg/(L*h) tras 120 h de cultivo. No obstante, la acumulación de PHB fue 20.57% y 25.12% menor comparado con la acumulación del cultivo por lote. Por el contrario, la tercera estrategia con pulso a las 60 h en la fase estacionaria permitió una mayor acumulación de PHB (52.40%) y productividad (23.22 mg/(L*h)), superando los valores obtenidos en lote y en las otras estrategias.

Además, el modelo *iCNH2025A* representa una reconstrucción metabólica refinada y altamente curada de *C. necator*, mostrando mejoras significativas en comparación con modelos anteriores. Con 2,737 reacciones, 1,803 metabolitos únicos y 1,059 genes anotados, el modelo integra rutas metabólicas clave, incluyendo la biosíntesis de PHB y de polihidroxibutirato-co-hidroxivalerato (PHBV), así como rutas centrales del metabolismo como el ciclo de Calvin-Benson-Bassham, la vía de Entner-Doudoroff (ED) y la vía de Embden-Meyerhof-Parnas (EMP). El modelo fue validado utilizando MEMOTE, obteniendo una puntuación del 89% y mostrando mejoras significativas en comparación con modelos anteriores como *RehMBEL1391* e *iCN1361*.

Las simulaciones de crecimiento *in silico* en presencia de fructosa y dextrosa demostraron una alta correlación con los datos experimentales ($R^2 = 0.76-0.78$), validando su capacidad predictiva. Además, el modelo reflejó con precisión el flujo de carbono hacia la síntesis de PHB bajo diferentes condiciones experimentales, en concordancia con estudios previos. Así, el modelo *iCNH2025A* representa un avance significativo en el modelado metabólico de *C. necator*, proporcionando una herramienta altamente precisa y validada para el análisis de su

metabolismo. Su capacidad para predecir flujos metabólicos y tasas de crecimiento lo convierte en un recurso valioso para la biotecnología industrial, facilitando futuras estrategias de optimización en la producción de biopolímeros.

Palabras clave: polihidroxialcanoatos; dextrosa a partir de almidón de yuca; *Cupriavidus necator*; sustratos de bajo costo; propiedades mecánicas y térmicas; análisis de balance de flujo.

Introduction

The increase in conventional plastic production, along with the rising global demand for plastics, highlights the need to seek alternatives that reduce dependence on synthetic and petroleum-based plastics. A promising solution is biodegradable plastics, which offer an environmentally friendly option, as their use could help decrease environmental pollution.

Within the group of bioplastics, there are PHA, which are biopolymers characterized by their biodegradability and biocompatibility, composed of fatty acid units. Microorganisms, such as bacteria, have become biological factories for producing PHA through the fermentation of organic components, including waste from the agro-industrial and agricultural sectors. These microorganisms synthesize PHA intracellularly and store it in the form of carbon, as reviewed by Behera et al. [1].

Within the PHA family, the most common is polyhydroxybutyrate (PHB), well known for its mechanical properties like those of conventional plastics. At the molecular level, it consists of repeating units of 3-hydroxybutyrate, a fatty acid responsible for its biocompatibility and biodegradability. These properties enable its use in various fields, including medicine, agriculture, and manufacturing, among others [2].

These bioplastics represent a promising alternative to traditional plastics in terms of sustainability. Although mass adoption faces challenges related to production costs, advances in research aimed at optimizing their production, utilizing renewable and low-cost carbon sources, and developing new technologies could make them more cost-competitive and efficient [3].

Regarding PHA production costs, it is important to note that the carbon source alone accounts for up to 50% of the total cost of the biopolymer [4]. Various studies have been conducted to reduce production costs by using carbon sources derived from agro-industrial and agricultural waste, such as fruit residues, used cooking oil, sugarcane molasses, starches, and more [3,5–9]. Other research efforts have focused on improving PHA yield by modifying the nutritional conditions and operational mode (batch or fed-batch). In parallel, several investigations aim to increase process efficiency through the application of genome-scale metabolic models (GEMs), which have become powerful tools for studying and optimizing PHA production[10,11]. This approach integrates experimental data with mathematical models to predict cellular metabolic behavior under different conditions. Through simulations such as flux balance analysis (FBA) [12], it is possible to identify metabolic pathways involved in PHA biosynthesis, optimize metabolic fluxes toward polymer production, and evaluate the impact of different cultivation conditions on process efficiency, and even detect bottlenecks in the polymer synthesis. Therefore, combining cultivation strategies with GEM tools could serve as a comprehensive approach to maximizing PHA production in *C. necator*.

In Colombia, several potential carbon sources such as starch are produced from different tubers, including yam, potato, sweet potato, and industrial cassava. By 2024, cassava production exceeded 289,000 tons, according to data from the Agronet information network of the Ministry of Agriculture [13]. In the Caribbean region, the main production areas include the departments of Magdalena, Atlántico, Córdoba, and Sucre [14].

Specifically, the department of Sucre accounts for 46.99% of the country's total industrial cassava production. In this region, Almidones de Sucre S.A.S. produces approximately 24,000 tons of starch annually. Of this production, around 15% is destined for international markets and 70% for the domestic market; however, approximately 15% remains uncommercialized. Notably, all starch produced in the department of Sucre is marketed in its native form, despite its significant agroindustrial potential for the development of value-added products.

Considering the availability of this substrate and the need to optimize its utilization in biotechnological processes, the application of GEMs becomes highly relevant, as these models enable a deeper understanding of *C. necator* metabolism and allow for the prediction of its behavior under different cultivation conditions. These tools make it possible to identify key metabolic pathways, optimize fluxes toward PHB biosynthesis, and detect intrinsic process limitations, thereby providing a rational basis for the design of more efficient biotechnological strategies. Consequently, the integration of GEMs in this work is essential to guide the selection of nutritional conditions and agroindustrial residue-derived substrates, with the aim of maximizing PHA production and contributing to the development of sustainable alternatives to conventional plastics.

Objectives

General objective

To evaluate the metabolic capabilities of *C. necator* in PHA production using dextrose bitter cassava starch as carbon source.

Specific objectives

1. Reconstruct a GEM of *C. necator* that represents the metabolic capacities for PHA production.
2. To establish operating conditions that favor the production of PHA from *C. necator* using bitter cassava starch as the main carbon source.
3. To explore scenarios of PHA production from *C. necator* using a GEM and constraint-based metabolic modeling tools.
4. To determine possible metabolic targets that may eventually be amenable to genetic modification as potential mechanisms for improving the bioprocess for PHA production.

Structure of the thesis

This document is organized into five chapters. In addition to the literature review (Chapter 1), the structure of the thesis details the development of each of the proposed research objectives, as described below: Chapters entitled *Production of polyhydroxybutyrate by batch cultivations of Cupriavidus necator using dextrose from bitter cassava starch as a low-cost carbon source* (Chapter 2) and *Production of polyhydroxybutyrate by fed-batch pulse-feeding cultivations of Cupriavidus necator using dextrose from bitter cassava starch as a low-cost carbon source* (Chapter 3) address the second specific objective. The chapter entitled *Reconstruction of a genome-scale model of Cupriavidus necator and exploration of PHB production scenarios using constraint-based metabolic modeling tools* (Chapter 4) addresses the first, third, and fourth specific objectives. Finally, general conclusions and future work are presented in Chapter 5.

List of contributions

Scientific papers

- **Integrating fed-batch pulse feeding and flux balance analysis for efficient PHB production by *Cupriavidus necator* from cassava-based dextrose.** Martha L. Ascencio-Galván, Víctor A. López-Agudelo, Andreas Dräger, Julio C. Caicedo, David Gómez-Ríos, Rodrigo Andler, and Howard Ramírez-Malule. (Accepted).
- **Valorization of cassava starch and sugarcane vinasse as polyhydroxyalkanoates by submerged cultures of *Cupriavidus necator*: a Physicochemical-biotechnological approach.** Isabel Dorado; Laura Pineda; Martha L. Ascencio-Galván; Victor A. López-Agudelo; Julio Caicedo; David Gómez-Ríos; Howard Ramírez-Malule. *ChemEngineering (ISSN 2305-7084)* 2024, 8, 73. <https://doi.org/10.3390/chemengineering8040073>
- **A bibliometric landscape of polyhydroxyalkanoates production from low-cost substrates by *Cupriavidus necator* and its perspectives for the Latin American bioeconomy.** Ascencio-Galván, M.; López-Agudelo, V.; Gómez-Ríos, D.; Ramirez-Malule, H. *J. appl. biol* 2024, 12.

Research project

- **Modelado y evaluación de la capacidad metabólica de *Ralstonia eutropha* para producir polihidroxialcanoatos (PHA) a partir de un sustrato de bajo costo derivado de yuca industrial de producción nacional.** Martha L. Ascencio-Galván, David Gómez-Ríos and Howard Ramírez Malule. Universidad del Valle. Código: CI 21191. 2022.
- **Biogenic waste valorization with decentralized bioenergy production** (International Mobility Project). MinCiencias-BMBF. CT-911-2020.

Scientific and academic presentations

- **“Reconstruction of a genome-scale model of *Cupriavidus necator* for PHA production”**. 7th BioProScale Symposium.. March 28th-31st, 2022. Berlin, Germany.
- **“Converting low-cost feedstock to PHA by a genome-scale model of *Cupriavidus necator*”**. VIII Congreso Internacional de Ingeniería Agroindustrial. Octubre 24-28, 2022. Chillán- Chile.
- **“*In silico* exploration of PHB production by a genome-scale model of *Cupriavidus necator*”**. 11th World Congress of Chemical Engineering. Junio 4 – 8, 2023. Buenos Aires-Argentina.
- **“Exploring PHB production by a genome-scale model of *C. necator*”**. XXXI Congreso Interamericano de Ingeniería Química. 30 de junio - 3 de julio, 2025. Cartagena-Colombia.

Chapter 1 A bibliometric landscape of polyhydroxyalkanoates production from low-cost substrates by *Cupriavidus necator* and its perspectives for the Latin American bioeconomy

1.1 Introduction

Bioeconomy has provided new technologies and business solutions to industries compared to the traditional and linear fossil-based production model [15]. Modern consumption habits have inevitably led to the growing generation of plastic waste and the production necessary to meet such demand. In Colombia, national plastic demand is notably high, exceeding 1.2 million tons per year, further reflecting the global trend of increasing dependence on synthetic polymers. Conventional plastics derived from petrochemical monomers stand out for their mechanical properties and biological and chemical resistance, but these advantages for the industry have unfortunately translated into a major environmental problem.. In 2021, the annual production of petrochemical-based polymeric materials was 390.7 million tons per year [16]. Due to the large oil refinery capacity worldwide, monomers required to produce synthetic plastic materials are considered low-priced commodities with high-volume and high-demand transactions. Likewise, their physical properties, such as high mechanical strength, thermal stability, and resistance to physicochemical and biological degradation mechanisms, have turned plastics into a major pollutant with serious consequences for the environment and health [17,18]. An extensive number of studies in the last twenty years have shown the effects of plastics pollution in marine environments and freshwater ecosystems such as rivers, lakes, estuaries and inland water [18,19]. The current generation of plastic waste exceeds approximately 1% the production since some production from previous years incorporates into the generation. At least 12% of plastic waste is mismanaged, leading to ocean inputs of 8 million tons per year, as reviewed by Albuquerque & Malafaia [20].

Bio-based biopolymers, especially biodegradable ones, emerge as alternatives in many fields where plastics are currently used as an essential material [21]. However, the global production and market share of these biopolymers are still incipient, with a volume of 2.217 million tons per year in 2022, of which only 51.5% corresponds to biodegradable bioplastics [22]. By 2027, the total capacity forecast will be 6.3 million tons, distributed as 56.5 and 43.5% for biodegradable and bio-based/non-biodegradable bioplastics, respectively.

In the field of bioplastics, biologically compatible polymers are of great interest [20,23,24]. The so-called biodegradable plastics can be assimilated by microorganisms in short periods (18 months - 2 years), under appropriate environmental conditions like temperature, pH, humidity, and nutrient supply [25]. According to reviews by Albuquerque & Malafaia [20], there are several types of commercially available biodegradable plastics, such as

polyhydroxyalkanoates (PHA) and polylactates (PLA), as well as various blends of biopolymers. Among the bioplastics, PHA stands as one of the most promising materials for reaching industrial production and massive commercialization in the mid-term due to its characteristics and high performance [26].

PHA are biopolymers produced intracellularly by various organisms such as archaea, bacteria, yeast, algae, plants, and their recombinant forms. These biopolymers are bioaccumulated under limited conditions of nitrogen and in excess of a carbon source diverting the metabolism towards carbon accumulation as energy reservoirs [27]. *C. necator* is a Gram-negative bacterium among the most studied and employed for PHA synthesis due to the ease of cultivation and its ability to accumulate higher amounts of PHA from renewable carbon sources such as glucose, fructose, and glycerol [28,29]. This bacterium has been given various names over time, including *Hydrogenomonas eutropha*, *Alcaligenes eutrophus*, *Ralstonia eutropha*, and *Wautersia eutropha* [30]. At present, more than 90 genera of microbial species with the capacity to accumulate PHA are reported in the literature, as reviewed by Albuquerque & Malafaia [20].

Currently, PHA industrial production is not profitable compared to that of synthetic polymers, as reviewed by Kumar et al. [31]. Several factors hamper the production cost of PHA, such as the nature and precedence of the carbon source, the cultivation cost, the productivity of the process, the efficiency of the metabolic machinery in the biosynthesis and the further downstream processing [32,33]. Among these factors, the most influential is the cost of the carbon source, which can account for up to 50% of the total production cost. The use of inexpensive and easily assimilable substrates, as well as microorganisms with high PHA synthesis capacity, may contribute to improving the gross margin of PHA production [31]. In this sense, (agro)industrial by-products/waste compounds such as glycerol, waste vegetable oils, animal fats, wastewater streams with high organic content, and sugar-rich materials such as molasses, lignocellulose, whey, and starch, as well as mixtures of these feedstocks, are envisioned as suitable low-cost substrates for PHA production [34–36]. Alves *et al.*, recently reviewed PHA-producing companies and their global production according to the substrates employed [37]. Several investigations have focused on the search for alternative substrates [24,38,39], identification of synthesis pathways [40], reconstruction of genome-scale models [10,11], and PHA recovery methods [41–43] that enable efficiently and economically feasible PHA production.

Given the high relevancy and worldwide interest in developing the large-scale production of PHA, there is a high volume of scientific publications related to PHA, which makes it difficult to obtain an appropriate glance at the main/recent findings in the topic. Bibliometric analysis is a useful tool for understanding the evolution, growth, and prospects of a research area. In addition, bibliometric analysis allows identifying the connection between subjects and even interdisciplinary collaborations over time, contributing the researchers to get

insights into topics of global interest. Furthermore, bibliometric analysis can be used to find innovative information that explain/support recent trends in new processes or applications. In the case of PHA production, some systematic reviews are available on specific topics such as genetic engineering tools for PHA production using inexpensive carbon sources for potential large-scale applications [44], continuous cultivation processes for PHA production [45], carbon waste streams from (agro)industrial for a cost-effective and sustainable PHA production [2], regulation of PHA synthesis [46], volatile fatty acids as carbon sources for PHA production [47], lignocellulosic feedstocks for PHA production [48], rapid quantification of intracellular PHA [49], among others. However, bibliometric analysis of studies on PHA production is still lacking.

In this study, a bibliometric analysis was carried out to investigate the trends of the PHA production by *C. necator* aimed to provide a clear perspective on the current research landscape, highlighting renewable and low-cost carbon sources. In addition, the results were used to explore the PHA bioeconomy ecosystem in Latin America.

1.2 Materials and methods

1.2.1 Database selection

Data search and collection were performed in the Scopus and Web of science (WOS) databases since both are among the largest databases of peer-reviewed literature worldwide in the field of science, technology, etc. These databases allow for the use of Boolean operators, leading to the design of a comprehensive search query. In addition, Scopus and the WOS allow exporting bibliometric data in Excel® and text format, respectively, which are suitable for further analysis and systematic scientific mapping.

1.2.2 Search strategy and bibliometric indicators

The search query was designed in two search topics joined by the Boolean operator “AND”. The first topic was “PHA”. While the second topic was *H. eutropha*, *A. eutrophus*, *C. necator*, *W. eutropha* and *R. eutropha*, but all the terms were joined by the Boolean operator “OR”, to include all the names of *C. necator* over time.

The search strategy included terms in the article title, abstract, and keywords as described elsewhere [50,51]. The document type was constrained only to articles (i.e., other document types, such as letters, editorials, proceedings, and books were excluded) and VOSviewer 1.6.18 was used for visualization and data analysis [52]. All searches were done on March 14, 2023.

For the analysis of the carbon source used to produce PHA, the first one thousand author keywords with the greatest total link strength were analyzed, and the nodes related to *C. necator* and carbon sources were selected for bibliometric network mapping.

Search:

(TITLE-ABS-KEY (PHA) AND TITLE-ABS-KEY ("*H. eutropha*") OR TITLE-ABS-KEY ("*A. eutrophus*") OR TITLE-ABS-KEY ("*C. necator*") OR TITLE-ABS-KEY ("*W. eutropha*") OR TITLE-ABS-KEY ("*R. eutropha*")) AND (LIMIT-TO (DOCTYPE, "ar"))).

Database: Scopus. Timespan: 1992 - 2022. 532 records were retrieved.

Database: WOS. Timespan: 2000 - 2022. 2995 records were retrieved.

In all studies, a screening of retrieved articles was performed to exclude duplicates. Bibliometric networks were designed to contain between 40 and 60 nodes for better results visualization. The volume and growth of publications, subject areas, top 10 leading countries, institutions and cited papers, co-occurrence keyword network visualization, and co-occurrence keyword overlay visualization analysis were the bibliometric indicators evaluated in this study. Figure 1.1 represents the methodology described and used in this study.

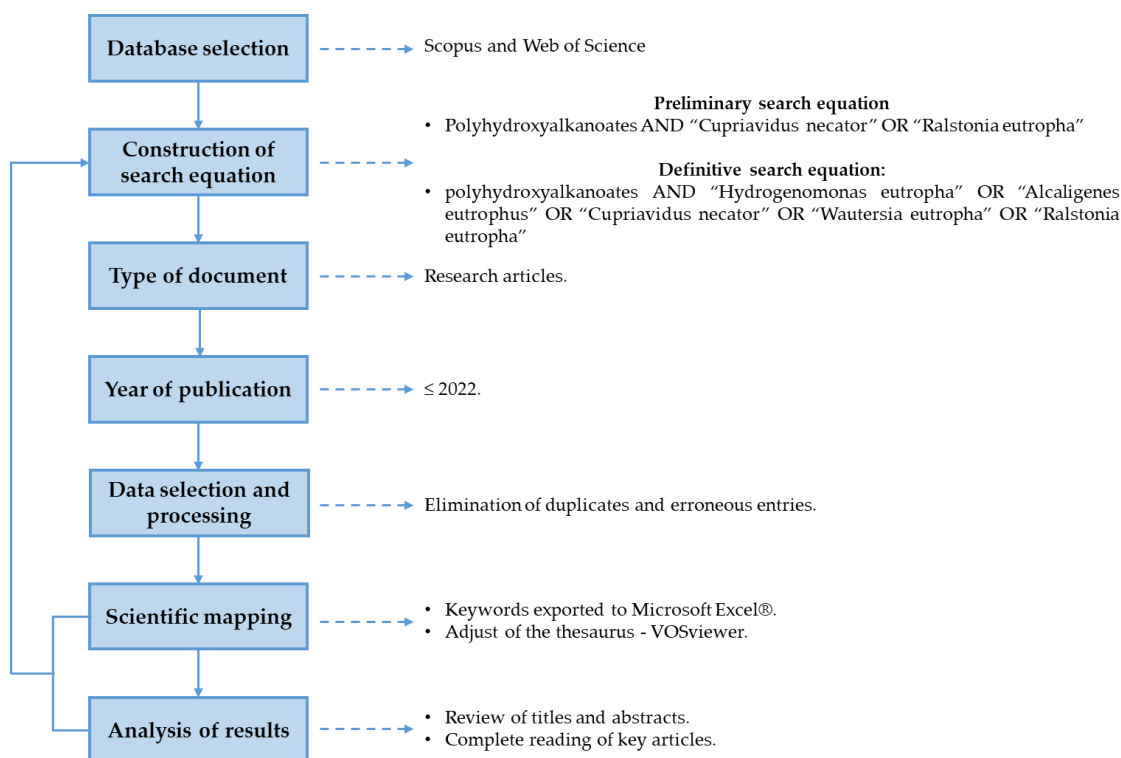


Figure 1.1 Methodology flowchart for bibliometric analysis.

1.3 Results and discussion

1.3.1 Evolution of research literature related to PHA production by *C. necator*

Fulfilling the search criteria for the selected databases, a total of 532 articles were identified in Scopus between 1992 and 2022, while 2995 articles were identified in WOS between 2000 and 2022. Figure 1.2 shows the evolution of the number of publications per year, showing an increase in the number of publications from 2010 onwards in both databases. The interest in bioplastics emerged from global concern about the environmental issues of plastic waste and the need for reducing petroleum dependence [24]. The increase in publications on the topic from 2010 onwards correlates with the appearance of the first policies on bioeconomy and the increase in the funding for bioeconomy-related topics [53]. In addition, the results indicated that the studies on this topic have been conducted mainly in the last two decades (from 2000 onwards), since the first reports on PHA production optimization and genetic engineering of *C. necator* date back to the early 2000 decade.

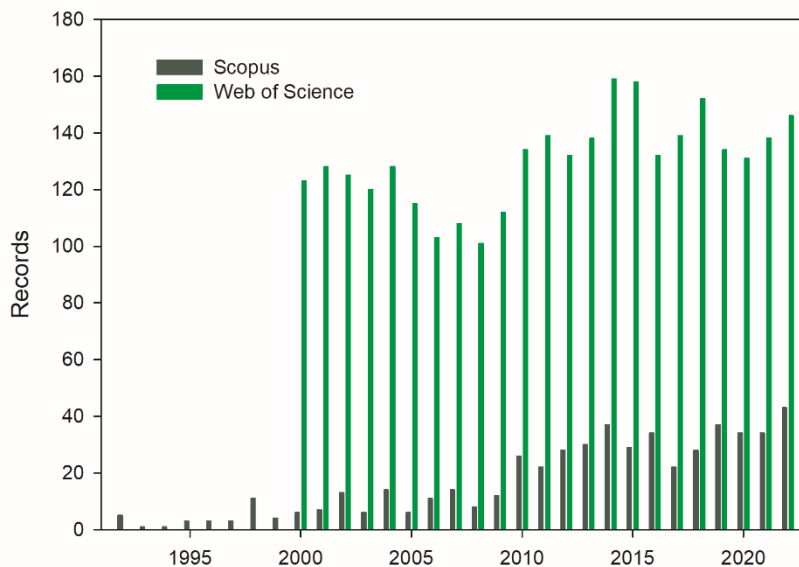


Figure 1.2 Evolution of research articles related to PHA production by *C. necator* during 1992–2022 and 2000–2022 by using the Scopus and the WOS databases, respectively.

1.3.2 Most productive countries and research institutions worldwide in scientific literature on PHA production by *C. necator*.

The Scopus and WOS databases show that the studies on PHA production by *C. necator* have been carried out in Asia (260 and 1181 papers), Europe (75 and 726 papers), North America (71 and 452 papers) and South America (30 and 98 papers), respectively. In addition, Japan and Germany lead the scientific output, with 83 and 563 papers (according to Scopus and WOS) equivalent to 15.6% and 18.7% of the world's scientific production for the period evaluated (Table 1.1), followed by the United States (13% and 15%), respectively. Of the total number of countries reported in the mentioned databases, most publications are by authors with affiliations from European and Asian countries (Table 1.2). The above is also consistent with the most cited papers in this field as shown in Table 1.3.

In the top 10 countries, Brazil is the only South American country publishing in this area of knowledge with 30 and 98 papers in Scopus and WOS, respectively (Table 1.1). For the period studied, other Latin American countries such as Argentina and Colombia are only in the 27th and 28th position with 5 papers each in Scopus, and 30th and 40th position with 31 and 9 papers, respectively, in WOS. This indicates that more work needs to be done in this research field, but above all that Latin American countries establish policies aimed at promoting and strengthening research.

Table 1.1 Top 10 most productive research countries worldwide on the topic of PHA

Database	Country/Region	Number of publications
Scopus	Japan	83
	United States	71
	South Korea	58
	Malaysia	48
	China	47
	Germany	46
	Brazil	30
	United Kingdom	29
	Russian Federation	26
	Germany	563
WOS	United States	452
	Japan	401
	China	282
	South Korea	203
	England	163
	India	159
	Malaysia	136
	France	113
	Brazil	98

Likewise, when reviewing the scientific production of PHA by *C. necator* from research institutions, it is evident that European and Asian institutions are the majority in the top 10. The exception is the Massachusetts Institute of Technology (USA), which occupied the fifth position (Table 1.2). This shows also that they are the countries with the highest investment in this scientific research field in the world.

1.3.1 Main research approaches on PHA production by *C. necator*

Figures 1.3 shows the top 10 areas of knowledge related to published studies on PHA synthesis using *C. necator*, in both databases. In this regard, and based on WOS, the main areas were (i) biotechnology applied microbiology, (ii) microbiology, and (iii) biochemistry molecular biology, and concentrated 43.2%, 24.6%, and 16.5% of the records, respectively. In the case of Scopus, the main areas were (i) biochemistry, genetics, and molecular biology (ii) immunology

and microbiology, and (iii) chemical engineering, and contributed 61.2%, 43.8%, and 40.7% of the indexed documents, respectively.

The two areas of knowledge with the most publications and cited studies in the databases consulted show that PHA production by *C. necator* is being worked on and developed in the basic sciences with approaches related with submerged cultures by using low-cost carbon sources and at the same time studies in the line of metabolic and genetic engineering (Tables 1.3 and 1.4).

Table 1.2 Top 10 most productive research institutions worldwide on the topic of PHA production.

Database	Research Institution	Number of publications
Scopus	Tokyo Institute of Technology	45
	Universiti Sains Malaysia	38
	Siberian Branch, Russian Academy of Sciences	23
	Biophysics Institute of the Siberian Branch of the RAS	23
	Massachusetts Institute of Technology	18
	Tsinghua University	18
	Westfälische Wilhelms-Universität Münster	16
	Riken	16
	Korea Advanced Institute of Science and Technology	15
	Tokyo Institute of Technology	138
WOS	University Of Munster	124
	Universiti Sains Malaysia	103
	Humboldt University of Berlin	99
	Riken	95
	Technical University of Berlin	95
	Helmholtz Association	76
	Centre National de la Recherche Scientifique CNRS	73
	Chinese Academy of Sciences	68
	Udice French Research Universities	61

All the papers listed in Table 1.3 are research articles that have been the basis for supporting recent research in the field of PHA by *C. necator*. The most cited paper in Scopus was authored by Cavalheiro *et al.* [54] and published in 2009. This work describes PHB production by *C. necator* DSM 545 strain using a byproduct from the biodiesel industry (glycerol) as a carbon source. The authors explored two strategies that seek to enhance PHA production: (i) high-density cultivation to increase the volumetric productivity of PHA and (ii) the use of low-cost carbon sources. In WOS, the second most cited paper was authored by Pohlmann and collaborates [55], and published in 2006. The authors reported the complete genome sequence of the two chromosomes of *C. necator* H16. This paper provides an analysis that has undoubtedly been the basis for researchers to continue to explore the biotechnological potential of this microorganism and at the same time to understand its metabolic diversity.

Table 1.3 Top 10 highest cited papers on the topic of PHA production according to Scopus.

Title	Year	Journal	Citations number	Reference
Poly(3-hydroxybutyrate) production by <i>Cupriavidus necator</i> using waste glycerol	2009	Process Biochemistry	326	[54]
Environmental analysis of plastic production processes: Comparing petroleum-based polypropylene and polyethylene with biologically-based poly- β -hydroxybutyric acid using life cycle analysis	2007	Journal of Biotechnology	310	[56]
Environmental life cycle comparison of polyhydroxyalkanoates produced from renewable carbon resources by bacterial fermentation	2003	Polymer Degradation and Stability	293	[57]
High yield production of polyhydroxyalkanoates from soybean oil by <i>Ralstonia eutropha</i> and its recombinant strain	2004	Polymer Degradation and Stability	260	[58]
Production of polyhydroxyalkanoates (PHAs) from waste materials and by-products by submerged and solid-state fermentation	2009	Bioresource Technology	230	[59]
Metabolic engineering of <i>Escherichia coli</i> for the production of polylactic acid and its copolymers	2010	Biotechnology and Bioengineering	224	[60]
Multiple β -ketothiolases mediate poly(β -hydroxyalkanoate) copolymer synthesis in <i>Ralstonia eutropha</i>	1998	Journal of Bacteriology Applied Microbiology and Biotechnology	189	[61]
Efficient production of polyhydroxyalkanoates from plant oils by <i>Alcaligenes eutrophus</i> and its recombinant strain	1998	Microbiology and Biotechnology	184	[62]
PHA synthase activity controls the molecular weight and polydispersity of polyhydroxybutyrate in vivo	1997	Nature Biotechnology	178	[63]
Microbial utilization and biopolyester synthesis of bagasse hydrolysates	2008	Bioresource Technology	168	[64]

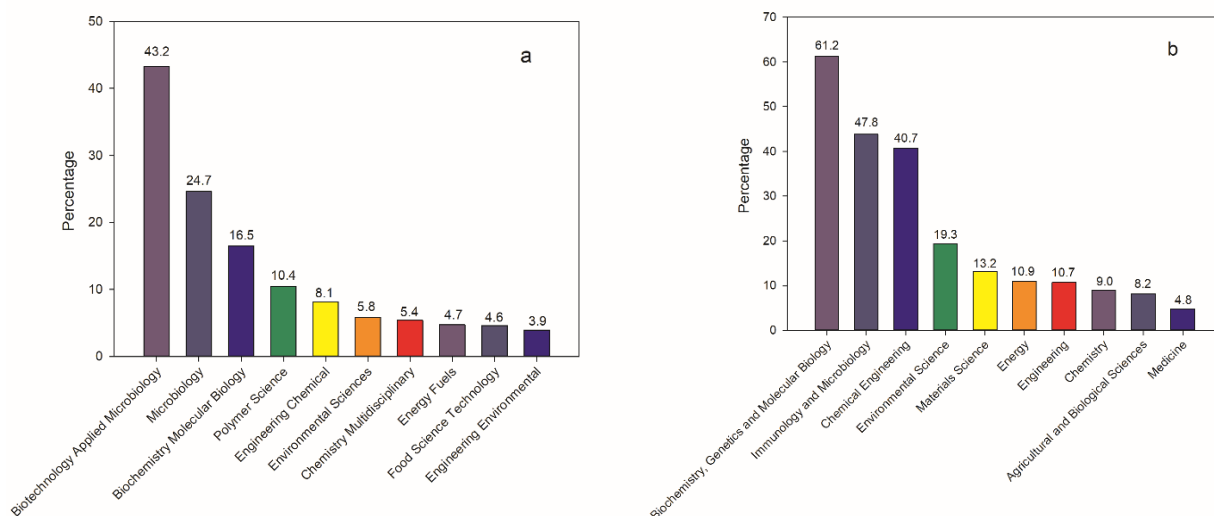


Figure 1.3 Contributions in polyhydroxyalkanoates production by *Cupriavidus necator* per area of knowledge (a) between 2000 and 2022 in Web of Science (b) between 1992 and 2022 in Scopus.

1.3.2 Bibliometric networks of production of PHA by *C. necator*

Figures 1.4 and 1.5 show the bibliometric networks of research topics related to PHA production by *C. necator* by using Scopus and WOS, respectively. The network visualization of Scopus contains 32 nodes grouped into 9 clusters (Figure 1.4a) while the WOS has 38 nodes grouped into 7 clusters (Figure 1.4a). Figures 1.4b and 1.5b show which topics have focused on the research over time, respectively. The clusters of Figure 1.4a (color-coded) can be classified as follow: cluster 1 (red color): fermentation, optimization, biodiesel; cluster 2 (green color): molecular weight, palm oil, *C. necator*, recovery, recombinant *Escherichia coli*; cluster 3 (blue color): bioconversion, biosynthesis, PHA, characterization; cluster 4 (yellow color): *Escherichia coli*, metabolic engineering, synthetic biology, *Pseudomonas putida*, PHB; cluster 5 (purple color): bioplastic, biorefinery, levulinic acid; cluster 6 (magenta blue color): copolymer, pha synthase, substrate specificity; cluster 7 (orange color): PHBV, volatile fatty acids; cluster 8 (brown color): biodegradable plastics and cluster 9 (pink color): β -oxidation.

The information retrieved from WOS displays the clusters as follows: cluster 1 (red color): PHBV, biodegradable plastics, activated sludge, volatile fatty acids, fed-batch fermentation; cluster 2 (green color): bacteria, biodegradation, bioremediation, hydrogen, hydrogenase; cluster 3 (blue color): *Bacillus megaterium*, glycerol, fermentation, optimization, characterization; cluster 4 (yellow color): molecular weight, palm oil, substrate specificity; cluster 5 (purple color): biodegradable polymer, PHBHHX, *Pseudomonas putida*; cluster 6

(magenta blue color): bioplastic, biorefinery, recovery; and cluster 7 (orange color): *Escherichia coli*, metabolic engineering, synthetic biology (Figure 1.5a).

Table 1.4 Top 10 highest cited papers on the topic of PHA production according to WOS.

Title	Year	Journal	Citations number	Reference
Water splitting-biosynthetic system with CO ₂ reduction efficiencies exceeding photosynthesis	2016	Science	571	[65]
Genome sequence of the bioplastic-producing "Knallgas" bacterium <i>Ralstonia eutropha</i> H16	2006	Nature Biotechnology	424	[55]
Bacterial respiration: a flexible process for a changing environment	2000	Microbiology-SGM	424	[66]
Metabolic engineering of <i>Saccharomyces cerevisiae</i> for the production of n-butanol	2008	Microbial Cell Factories	365	[67]
Molecular analysis of the copper-transporting efflux system CusCFBA of <i>Escherichia coli</i>	2003	Journal of Bacteriology	365	[68]
Taxonomy of the genus <i>Cupriavidus</i> : a tale of lost and found	2004	International journal of systematic and evolutionary microbiology	368	[69]
Producing microbial polyhydroxyalkanoate (PHA) biopolyesters in a sustainable manner	2017	New Biotechnology	308	[70]
Poly(3-hydroxybutyrate) production by <i>Cupriavidus necator</i> using waste glycerol	2009	Process Biochemistry	286	[54]
Production of isoprenoid pharmaceuticals by engineered microbes	2006	Nature Chemical Biology	277	[71]
The crystal structure of an oxygen-tolerant hydrogenase uncovers a novel iron-sulphur centre	2011	Nature	276	[72]

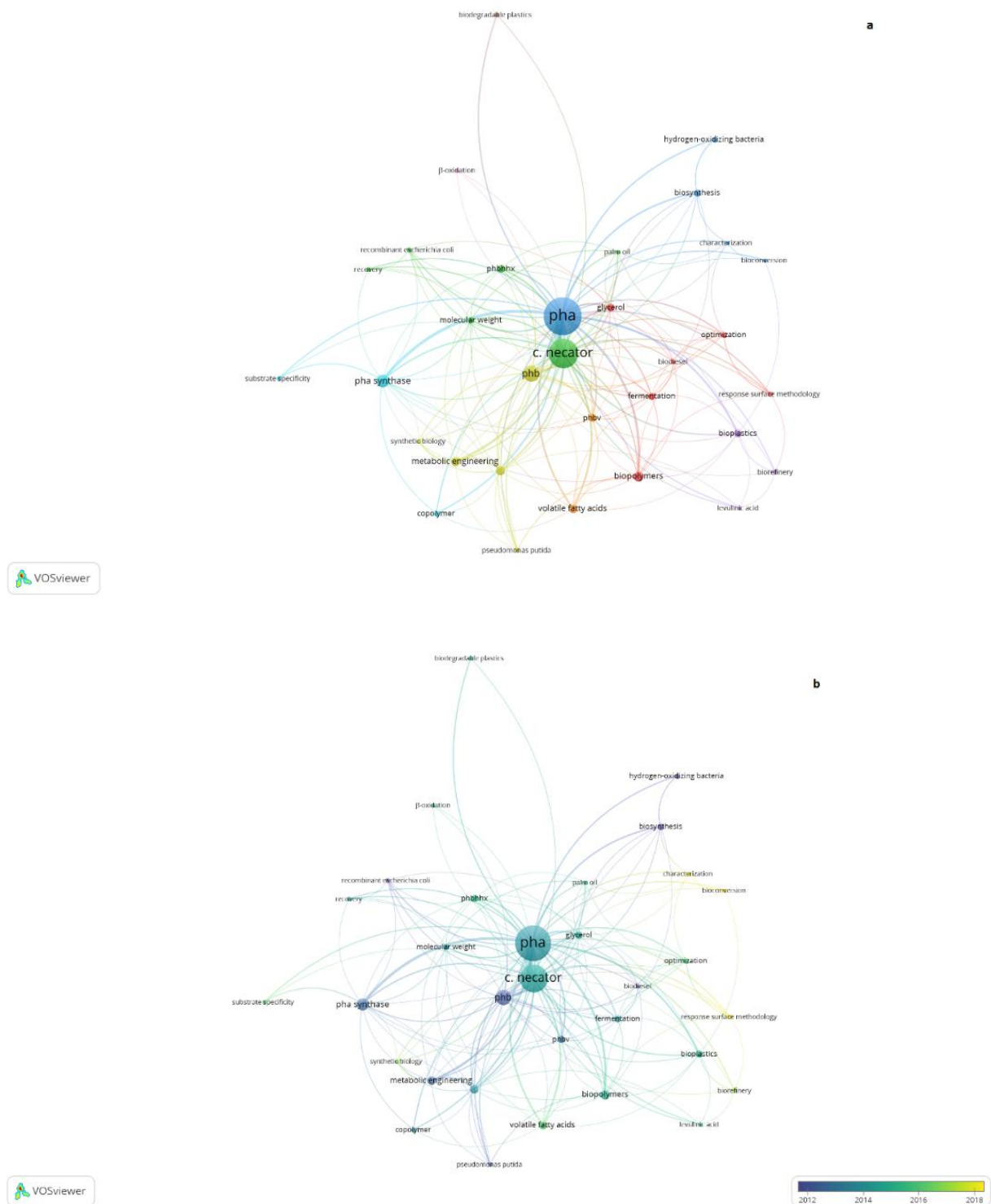


Figure 1.4 Bibliometric network of studies related to PHA production by *C. necator* between 1992 and 2022 in Scopus (a) Network visualization of the research-topic map (b) Overlay visualization of the research-topic map. Note: the minimum number of occurrences of a keyword is 5.

1.3.3 Carbon sources for PHA production

The main barrier to the large-scale production of PHA is the high cost of the carbon source used, representing up to 50% of the total cost of the bioplastic. Therefore, recent research has included the search for low-cost raw materials as a priority, including raw materials from (agro)industrial by-products, as well as renewable sources, which guarantee stable and cost-effective production over time [73].

Figure 1.6 shows the bibliometric networks of carbon sources used in the production of PHA by *C. necator* for the periods 1992-2022 and 2000-2022 by using Scopus (Figure 1.6a) and WOS (Figure 1.6b) databases, respectively. A review of the networks over time shows that the first carbon sources used to produce PHA by *C. necator* were invert sugars, organic acids, activated sludge, among others. These studies date back to the year 2000. For years 2010-2015, the production of PHA is centered on waste oil, oleic acid, waste glycerol, and from 2020, the trend is to use carbon sources such as vinasse, sugarcane molasse, fruit residues, CO₂, starchy and vegetable oils.

One of the great current efforts in the field of research on PHA production is the tireless search for rational alternatives to reduce the production costs of these biopolymers. Carbon sources from agro-industrial, domestic, and industrial waste are currently the subject of study in most of the research related to PHA, as reviewed by Guleria *et al.* [73]. In this section, the carbon sources most used in the production of PHA by *C. necator* are discussed, considering the results of the bibliometric analysis (Figure 1.6).

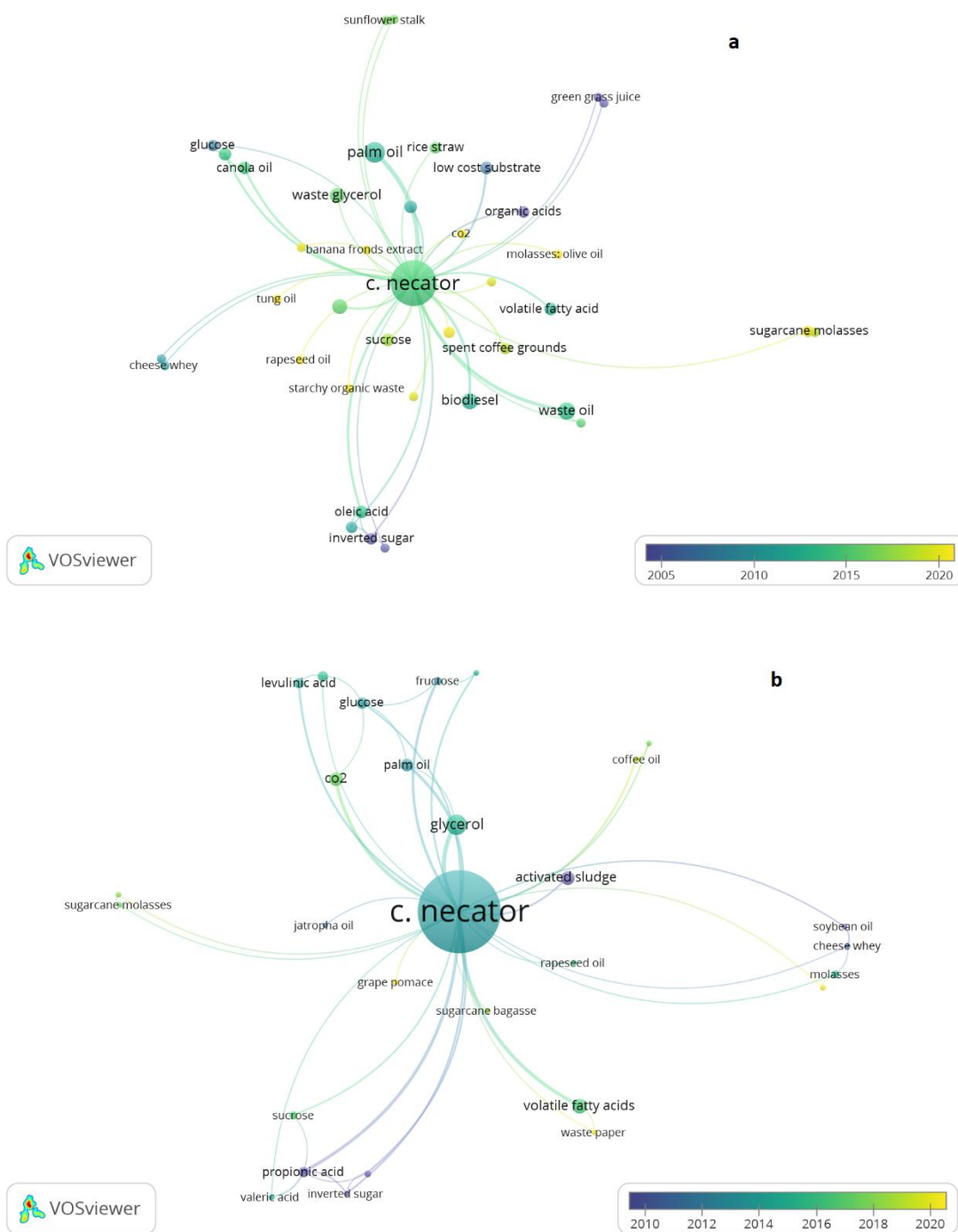


Figure 1.6 Overlay visualization of the research-topic map of studies of *C. necator* related to carbon source to produce PHA (a) Scopus and (b) Web of Science. Note: the minimum number of occurrences of a keyword is 1.

1.3.4 Fat and oils residues

As the research shows, there is currently a great interest in the use of waste fats and oils as raw materials to obtain new value-added products. The generation of waste from the meat industry worldwide represents 20% of the total waste generated by the food industry. As a result, animal

fat is being valorized to produce various products, including PHA, as a result of the waste management policies of many countries and the interest of the scientific community in optimizing resources and reducing environmental impact [74].

Fats obtained from different by-products of the meat industry were used to obtain the copolymer poly(hydroxybutyrate-co-hydroxyhexanoate) [P(HB-co-HHx)], using *R. eutropha* Re2058/pCB113. Initially, the by-products of this industry were subjected to either mechanical or enzymatic pre-treatment. Then, the fat was obtained by means of a thermo-pressure hydrolysis process at a temperature of 130-180°C and pressure of 3-20 bars, followed by a separation phase. To obtain polymer, they used pork fat, fish fat, pork fat/protein emulsion, pork mineral-fat-mixture, pork fat-greaves and canola oil as carbon sources. The authors report an accumulation of 90% of the polymer with a HHx content of 12-26 mol% [75]. Gutschmann *et al.* [76], using the same strain, employed animal fat to produce P(HB-co-HHx). For the use of fat as a carbon source they carried out thermal liquefaction, using a 150 L pilot scale double jacketed feeding system, employing continuous feeding. The laboratory scale cultures yielded results of 80% PHA accumulation and 17 mol% HHX content. Riedel *et al.* [77] evaluated of production of PHA using low-quality and poorly consumable animal fats after an emulsification strategy. The authors conducted cultivations of the wild-type *R. eutropha* strain H16 for PHB production and also cultivations of the recombinant strain Re2058/pCB113 for the production of P(HB-co-HHx) copolymer. An accumulation of 79–82% PHB was achieved with wild-type strain while recombinant strain reached 49–72% (w/w) of PHA per CDW with a HHx content of 16–27 mol%.

Our search also showed that waste oils are low-cost sources of carbon to produce PHA. Indeed, many research reporting the use of olive oil waste effluent used cooking oil, palm oil, coffee waste oil, jatropha oil, and other oils [73,78–81]. Coffee waste is also an inexpensive substrate containing fatty acids. [P(HB-co-HHx)] was produced in engineered strain *C. necator* Re2133 overexpressing (R)-specific enoyl coenzyme-A hydratase (*phaJ*) and PHA synthetase (*phaC2*) with deletion of acetoacetyl Co-A reductases (*phaB1*, *phaB2*, and *phaB3*) from coffee waste oil. PHA copolymer yield was 69% and was obtained with a coffee oil concentration of 1.5% and C/N ratio of 20. In addition, the PHA accumulation consisted of HB (78 mol%) and HHx (22 mol%) [78].

Glycerol is another residual carbon source from oil processing, which is used in the synthesis of PHA. Indeed, PHA of different compositions was synthesized by using glycerol as a carbon substrate. In that work, PHB was effectively synthesized by *C. eutrophus* B-10646 (a patented engineered strain of *C. necator*) in fed-batch cultivations with purity degrees ranging 82-99%, yielding a maximum of 78% polymer accumulation [82]. Furthermore, tung oil, a natural oil obtained from the seeds of the tung tree, which is used industrially to treat wood because of its high strength, was used as a carbon source using the *R. eutropha* Re2133/pCB81 strain to produce the terpolymer P(3HB-co-3HV-co-3HHx)[83]. Furthermore, the authors also reported

that the oil coating on the surface of the PHA films had antioxidant activity. This bioactive PHA may be expanded to medical field applications.

1.3.5 Sugar-rich residues and derivatives

The world sugar industry produces roughly 70 million tons of molasses per year. Despite it being considered a byproduct of sugar production, its world trade is consistently decreasing due to the valorization activities on site. Bioplastics production is also an interesting alternative for sugar waste valorization. Currently, research on engineered PHA producers from sugar by products is still low. *C. necator* has a deficient utilization of simple sugars as a carbon source for PHA production. Therefore, some engineered strains have been constructed to allow the assimilation of simple sugars as a substrate in the production of PHA. Recombinant strains of *C. necator* expressing genes *phaC1437* from *Pseudomonas* sp. 6-19, *pct540* from *Clostridium propionicum*, *sac* from *Mannheimia succiniciproducens* and *ldhA* from *E. coli* can produce poly(3-hydroxybutyrate-co-lactate) using sucrose as the sole carbon source [84].

The pathway for sucrose utilization was introduced in an engineered *C. necator* by introducing the *sacC* gene encoding the β -fructofuranosidase from *M. succiniciproducens*. The enzyme hydrolyzes sucrose into glucose and fructose and PHB yield reached 73% in cultivations with 20 g/L sucrose concentration. The same strain was cultivated using sugarcane molasses pre-treated with activated carbon as the carbon source to produce PHB and poly(3-hydroxybutyrate-co-lactate). The final PHB content achieved was 82.5% in batch fermentations [85].

Sugar-rich hydrolysates as inexpensive carbon sources have been also used for PHA production. Strains of *C. necator* have been used to synthesize PHB from wheat bran hydrolysates [86]. Likewise, recombinant strains of this same bacterium have been engineered to metabolize arabinose, by heterologous expression of a set of *E. coli* genes [87].

C. necator H16 and *C. necator* 5119 strains were co-cultured with *Bacillus subtilis* since it is a sucrose hydrolyzing organism resulting in 45% PHA production and propionic acid, which is a precursor for the hydroxyvalerate monomeric unit. Thus, the microbial consortia produced 66% PHBV having 16 mol% hydroxyvalerate, showing that co-culture strategy allows overcoming the requirement of a previous strain engineering for sucrose utilization in PHA production [88].

Vinasse was also used as a basal medium for PHB production with *C. necator* L359PCJ, which uses glycerol from vinasse as a carbon source rather than residual sugars. Experiments in CSTR bioreactors using a medium concentrated vinasse based produced a PHB content of 66.3% [89]. Recently, *C. necator* B-10646 was grown on sugar beet molasses previously hydrolyzed with β -fructofuranosidase, converting 89% of the sucrose (88.9%) into glucose and fructose. In that case, the maximum PHB content reached was 80% [90].

Starch is a high-quality glucose source with high availability and low cost. *C. necator* DSM 545 has been reported as unable to grow on starch. To overcome this limitation, a recombinant strain was constructed expressing glucodextranase *Gld* from *Arthrobacter globiformis* I42 and the α -amylase *amyZ* from *Zunongwangia profunda* SM-A87. PHA yields were 34, 52 and 63% with purple sweet potato waste, broken rice, and raw corn starch, respectively [91]. Similar results were obtained in the *R. eutropha* DSM 545 strain using simultaneous saccharification and fermentation along with the addition of commercial amylases [92]. Furthermore, PHA production using cassava starch hydrolysate using *C. necator* KKU38 cultivated under N-limited conditions produced 61.60% of PHB. Cassava flour hydrolysates were also used in the cultivation of *C. necator* H16 yielding a PHA accumulation of 64% [93].

1.3.6 Perspectives for the Latin American Bioeconomy

Currently, green innovativeness is considered a significant component of economic progress, environmental sustainability and improving wellness levels [94]. For the period studied, the results showed the global collaboration network of the PHA scientific ecosystem. Clearly, Germany, the USA, China, Japan, South Korea, France, England/United Kingdom, Malaysia, Belgium and India, among others, show the strongest collaboration and higher number of contributions (Figure 1.7). Most of the EU countries are also part of intense collaboration, which remarks the compromise of the economic community in advancing towards a sustainable bioeconomy and particularly, in the substitution of petrochemical-based materials. As depicted in Figures 1.7a and 1.7b, countries that are not part of the most industrialized economies but are important in the primary sector and biomass resources such as Malaysia, India and Latin America (with Brazil as the main contributor), also contribute significantly to the knowledge in PHA production.

Research in topics such as biomaterials and green chemistry is emerging and growing fast since those areas are expected to play an important role in a low-carbon economy and the generation of innovative bioindustries [15]. Concerning the large-scale production of bio-based chemicals, the OECD proposes that around 35% of industrial chemicals are substituted by bio-based chemicals by 2030. Latin America contributes approximately 4% of the global virgin plastics production, equivalent to 14.4 million tons, destined mostly for the domestic markets. As for 2022, the world's bioplastics production was 2.217 million tons, which is less than 1% of the total demand for plastics [22]. The share of bioplastics production relative to fossil-derived materials in Latin America follows a similar trend and currently accounts for only 1.3%. Nevertheless, when considering only the bioplastics market Latin America contributes 9% of the total bioplastics production, equivalent to 189 thousand tons [95].

The bioplastics industry is growing fast, with a projected growth rate of 3.7% for 2023, which means for Latin America a value of 2 billion USD [95]. In the current global plastics market, only Mexico is an important player, with 5% of the total polymer exports given its important refining capacity in the region. On the opposite side, Brazil is expected to become a big player

in the biodegradable polymers industry with an important share in the market for bioplastics, considering its leadership in the sugar and oleaginous exportations with 20% and 30% of the global trade, respectively [96]. Apart from Brazil, Argentina is the 5th world exporter of oleaginous seeds, while Mexico, Colombia and Guatemala are in the top 20 sugar exporters [96]. As it has been previously shown, those materials are the source of prospective inexpensive substrates for PHA production. The geographical and meteorological conditions favor the crops, plantations and husbandry activities, which allows the continuous production of biomass during the whole year. These conditions might become Latin America an interesting hub for the production of PHA, bioplastics and, in general, many different bioproducts required for the transition towards a sustainable bioeconomy.

The world production of bioplastics is forecasted to triplicate in 2026 the production of 2021 up to 7.6 million tons, in which at less 70% would be biodegradable and the rest biobased non-biodegradable [22]. Nevertheless, this is still a very low percentage (2%) of global plastics production; hopefully, the growth would be higher as new regulations and restrictions on the use of non-biodegradable plastics are introduced worldwide. Since Brazil is the world leader in the sugarcane industry and oleaginous seeds production, it has comparative and competitive advantages that can be also irradiated to its neighbor countries with similar geographic conditions. This also depends on effective economic integration between the South American countries and infrastructure developments that increase their competitiveness.

In the last years, Brazil has experienced the fastest industrial growth in bioplastics production in Latin America due to the very developed agro-industrial activity and big bioethanol distilleries that produce different kinds of biowaste with the potential for transformation into added-value bioproducts. Certainly, Brazil is called to lead the transition towards bioplastics in the continent; currently, this country accounts for more than 50% of the total bioplastics production in South America. An important pool of multinationals such as Amcor, Arkema, BASF, BioAmber, DowDuPont, Mitsubishi Chemical Corporation, NatureWorks LLC, Novamont, Solvay and many others have a presence in the country, in addition to the local Braskem, which is the largest petrochemical company in Latin America. All of them have established plans in the country with the aim of developing bioplastic production facilities. Indeed, the UN Economic Commission for Latin America and the Caribbean (ECLAC) recognized in 2020 the production of a bioplastic developed by Braskem as one of the most outstanding cases of sustainable development in the continent.

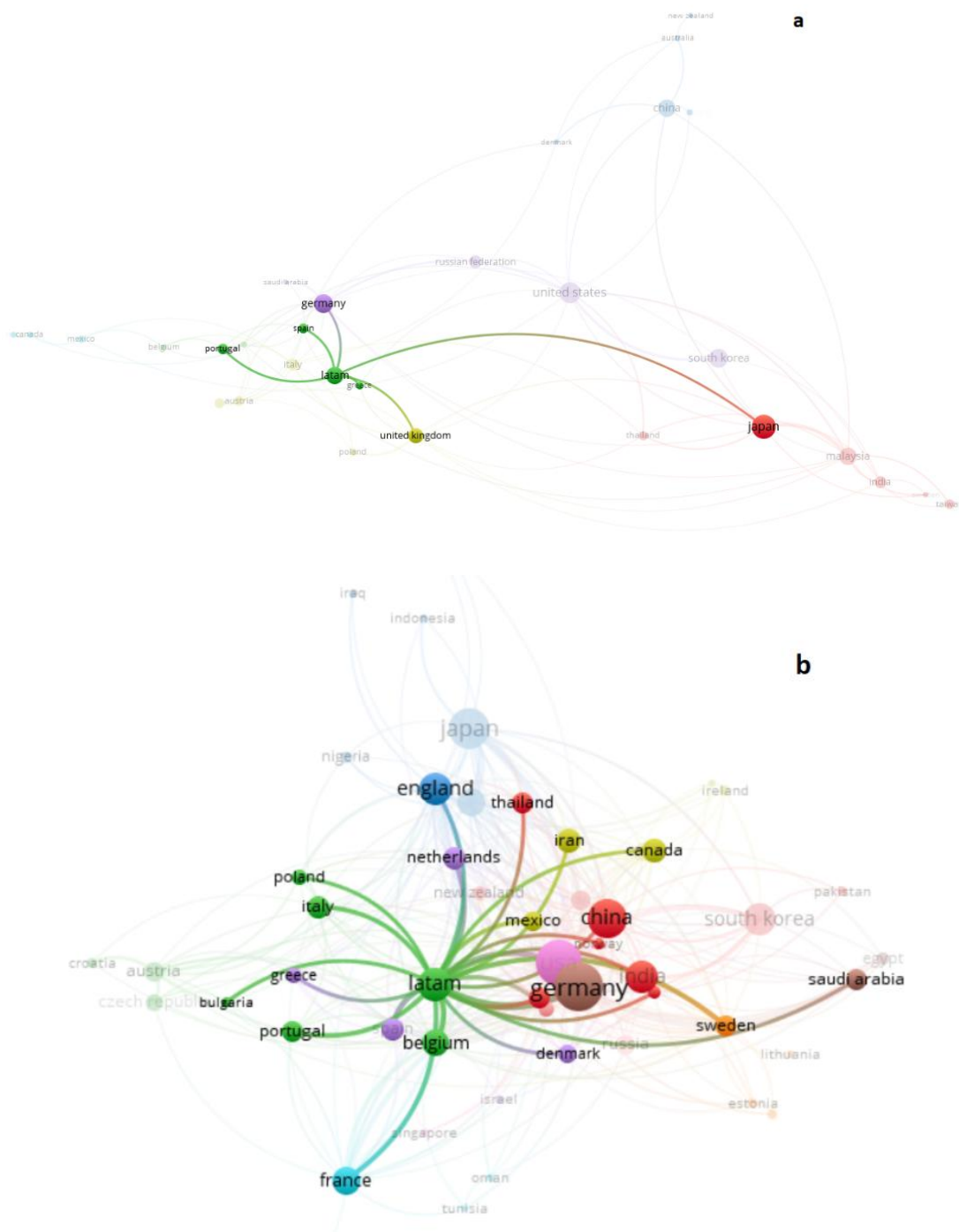


Figure 1.7 Latin American collaborations network of studies related to production PHA by *C. necator*, a) Scopus and b) Web of Science. Note: the minimum number of occurrences of a keyword is 5.

In this interesting panorama, the PHA production is expected to multiply 10-fold the current small production of 56 thousand tons, by 2027 [22]. Latin America is a region with a high potential for biomass production given the availability of cultivable land, fertile soils and water

supply that favor the consolidation of production chains in food production, fibers and forestry, bioproducts and bioenergy [97]. In addition, the availability of large amounts of waste biomass generated in the primary sector of the economy still lacks adequate waste management in many geographical areas.

Since the most prospective low-cost substrates, namely fats and oils, sugars, starch and their derivatives are readily available in Latin America from different sources, positive perspectives are envisioned for the establishment of processes for PHA and other bioplastics production in the continent. Some estimations indicate that Latin America could host up to 45-50% of the global bioplastics production. However, the funding of research in the region is considerably low when compared to USA, Asia and Europe. The mean investment in science, technology and innovation in the region is less than 1% of the GDP; this condition limits the scientific capacities of the Latin American countries, turning them into low-specialized economies that import technology, act as raw materials suppliers, or serves as a location for industrial outsourcing.

Recently, Moshood et al. [94] examined the biodegradable plastic product innovation across biodegradable plastic firms and non-governmental organizations (NGOs) emphasizing energy savings, public policies, and reductions in materials and pollution control as strategic points for business leaders, academics, and policymakers. The ECLAC has identified some common issues to be solved to promote the bioeconomy-related industries in Latin America: (i) absence of adequate regulatory frameworks, (ii) complexity of national regulatory processes, (iii) weak capacities to comply with external regulations in the target markets for bioproducts, (iv) incompatibility of regulations between conventional products and similar bioproducts and (v) difficulty in enforcing existing regulations [97]. The knowledge of the different kinds of biomass available, the formulation of precise public policies, the increase of cooperation for the development of scientific and technological capabilities and the identification of the market potential, products, and applications would accelerate the adoption of bioplastics in the region and towards the external markets.

1.3.7 Business landscape in Colombia

Colombian biomass residues account for about 20 million metric tons annually. However, the Colombian's government estimates this potential up to 43 million tons of biomass residues. Here, the agricultural sector plays an important role in Colombia's bioeconomy, for example, 33 megatons of sugarcane were produced in 2019 [98]. The following are some of the productions in this sector (in metric tons): Coffee (720,000), corn (1.3 million), potatoes (3.1 million), palm oil (5.8 million), bananas (3.7 million), pineapples (900,000), avocado (325,000) [99]. Thus, the potential of Colombia, and other neighboring countries such as Ecuador, Peru and Brazil, could contribute to low-cost carbon sources to produce PHA.

1.4 Conclusions

A clear scientific interest in the use of low-cost feedstocks (such as starch, waste fats and oils, molasses, and cellulose hydrolysates) for PHA production by *C. necator* arose during the last decade. The geographical and meteorological conditions of Latin America may open the opportunity to establish productive processes of PHA and bioplastics production in the region thanks to the high availability of biomass, products, and byproducts from agroindustry during the whole year. From regulatory and cooperation standpoints, efforts are required to establish a clear framework in Latin America for the development of bioeconomy initiatives. It is also necessary to intensify the research and strengthen the collaboration on this topic, especially in those regions that may act as potential hubs for the PHA and bioplastic production in the near future. Further studies and more intense scientific collaboration may contribute to achieving the objective of feasible and profitable PHA production as a real substitution alternative for conventional plastics.

Implications for the Experimental and Methodological Design of This Thesis

This first chapter of the thesis is based on the bibliometric study described above, in which the global landscape of polyhydroxyalkanoate (PHA) production by *Cupriavidus necator* from low-cost substrates was analyzed, with particular emphasis on perspectives for the Latin American bioeconomy. Beyond its descriptive nature, the results of this analysis were used as a conceptual and methodological foundation for the design of the present doctoral thesis. The main implications for the subsequent chapters are summarized below:

- *Selection of low-cost, regionally relevant carbon sources:* the bibliometric analysis revealed a global trend toward the use of renewable and low-cost substrates for PHA production. This gap justified the selection of dextrose derived from bitter cassava starch, an abundant regional resource, as the main carbon source experimentally evaluated in Chapters 2 and 3.
- *Justification for the use of *C. necator* as a model organism:* The analysis confirmed *C. necator* as the most frequently studied and metabolically versatile microorganism for PHB production, particularly in studies aiming at process optimization. This evidence supported its selection as the biological platform for both experimental cultivations and metabolic modeling throughout Chapters 2 to 4.
- *Integration of metabolic modeling as a complementary tool:* The bibliometric analysis highlighted a gap between experimental studies and the systematic use of genome-scale metabolic models to interpret and predict PHA production. This need provided the rationale for the reconstruction and validation of the *i*CNH2025A metabolic model and its application in Chapter 4.

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Chapter 2 Production of polyhydroxybutyrate by batch cultivations of *Cupriavidus necator* using dextrose from bitter cassava starch as low-cost carbon source

2.1 Introduction

PHA is the biopolymer that has stood out the most in recent years due to its high biodegradability, resistance, and versatility. Therefore, it is estimated that PHA could become a direct competitor of petrochemical plastics [100].

Additionally, PHB is one of the most well-known and studied PHA and is the only homopolymer in this family. PHB shares similar physical properties with polypropylene [101], including a similar melting point, crystallinity, molecular weight, and tensile strength. Concerning mechanical properties, PHB demonstrates high moisture resistance but is rather brittle and rigid. Nonetheless, due to its biodegradability, these properties may alter over time [102]. Furthermore, PHB is the most environmentally friendly polymer, considering its biocompatibility and biodegradability. These characteristics enable its use as an implant material in the human body and as a carrier for the extended release of antibiotics [2]. These properties, combined with the homogeneous, dense, and nanostructured formation achieved through a biotechnology method, position the PHB as a suitable biocompatible material for metallic implants.

According to Morlino et al. [103], *C. necator* is one of the most studied microorganisms for PHB production due to its versatile metabolic capacity. *C. necator* can grow both as a chemoautotroph and a heterotroph in aerobic and anaerobic environments, with the ability to utilize a variety of carbon sources for PHB synthesis. Currently, PHA production is limited by the high production costs, which are three to four times higher than those of synthetic polymers (between 0.60 and 0.87 USD/lb), hindering its industrialization and commercialization [104]. In the PHA production process, the carbon source could represent up to 50% of the final cost [4,14,105,106].

As reviewed by Wang et al. [107] and Bathia et al. [108], the use of waste as a carbon source in submerged cultures of *C. necator* for PHA production could be an alternative to reduce the final production costs. Currently, and over the last decade, many works have been carried out on the biosynthesis of PHA using various sugar-containing wastes as alternative substrates that allow for cost reductions [91,107,109–111].

Bitter cassava is one of the foremost prospective crops for added-value product generation in the bioeconomy, due to its high starch content (76.7% average [112]), low requirements for cultivation, and non-competition with food production [13]. Specifically, bitter cassava

production in Colombia has grown significantly in recent years, with varieties characterized by high yields (25 ton/ha), becoming an important source of income for local farmers and contributing to the country's economic development [113]. Colombia produces about 269,000 tons of bitter cassava per year, used mainly in the food industry to produce glucose and fructose syrups, flour, concentrated cattle feed, and bioethanol [13,114]. Therefore, the use of bitter cassava to obtain starch hydrolysate (e.g. dextrose), a potential carbon source, to produce high-value compounds such as PHA, has been proposed.

As a strategy to take advantage of the properties provided by cassava starch, this chapter evaluated dextrose from bitter cassava starch as a low-cost carbon source to produce PHB through submerged batch cultures of *C. necator*, as a prospective alternative for bioplastic production.

2.2 Materials and methods

2.2.1 Microorganism, culture media and cultivation conditions

C. necator DSM 428 was obtained from the Leibniz Institute DSMZ collection (German Collection of Microorganisms and Cell Cultures GmbH) as a glass ampoule in a vacuum-packed double vial with a lyophilized tablet of a single strain of microorganisms. For its activation, the lyophilized cells from the ampoule were rehydrated and grown in 5 mL of liquid nutrient broth (NB). NB medium (PanReac AppliChem, ITW Reagents, Monza, Italy) was composed as follows: meat extract 3 g/L, meat peptone 5 g/L. The cultures were incubated at $35^{\circ}\text{C} \pm 2^{\circ}\text{C}$ for 24 to 48 h, and the microbial growth obtained was aseptically added to a liquid medium composed of 20% glycerol and NB in 1.5 mL vials for subsequent storage at -20°C .

Initially, 1.5mL of cryopreserved cells were reactivated in 50 mL of NB as seed medium, disposed in 250 mL shake flasks at 35°C and 150 rpm for 24 h. Two pre-cultures stages were carried out for strain adaptation prior to production cultures. The first pre-cultures, containing 45 mL of modified mineral saline medium (MSM), were inoculated with 5 mL of the cultivated seed broth. MSM medium contained (per liter): dextrose from bitter cassava starch (commercially obtained) 20 g, $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ 6.7 g, KH_2PO_4 1.5 g, $(\text{NH}_4)_2\text{SO}_4$ 1.0 g, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.2 g, iron and ammonium citrate 60 mg, $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ 10 mg, and element trace solution 1 mL. Element trace solution (per liter): H_3BO_3 0.3 g, $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ 0.2 g, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ 0.1 g, $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ 30 mg, $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$ 30 mg, $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$ 20 mg, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 10 mg [115].

Then, the second pre-cultures were prepared identically and inoculated with 5mL of broth from the first pre-cultures. The PHB production cultures were performed in modified MSM medium. Production cultures were inoculated at 10% v/v of second pre-cultures and the cells were grown for 72 h at 35°C and 150 rpm. The pH was adjusted to 6.8 with NaOH 2M. Cultures were performed in triplicate.

2.2.2 Biomass and sugars quantification

Biomass concentration was quantified as cell-dry weight in 1.0 mL samples. Samples were centrifuged at 5,000 rpm for 10 min. Supernatants were used for sugar determination, and wet cells were dried at 70 °C for 24 h for further weight determination.

Reducing sugars were determined using the 3,5-Dinitrosalicylic acid (DNS) method [116] and High-Performance Liquid Chromatography (HPLC).

2.2.3 PHA detection and extraction

The relative intracellular formation of the polymer was determined by lipophilic staining with Sudan black B, where the formation of PHA is detected with the presence of black granules inside the cells. For the PHB extraction, the remaining cultures were centrifuged at 5000 rpm for 10 min. The obtained pellets were dissolved in 5 mL of sodium hypochlorite and 5 mL of chloroform. Then, cells were agitated in a vortex and kept at room temperature for 20 h in agitation, before being centrifuged at the aforementioned conditions. As a result, three separate phases were obtained: an inorganic phase (upper), corresponding to the sodium hypochlorite, the broken cellular material (middle), and the organic phase of chloroform with the dissolved biopolymer (lower). The PHA present in each sample was precipitated by the addition of isopropyl alcohol. The precipitate was dried at 70°C for 24 h, and the PHB dry weight was quantified [117].

Calculations

The substrate yield on biomass ($Y_{X/S}$) and PHB ($Y_{PHB/S}$), specific substrate consumption rate (Q_S) and specific production rate (q_{PHB}) were determined using the following equations:

$$Y_{X/S} = \frac{X_f - X_0}{S_0 - S_f}$$

$$Y_{PHB/S} = \frac{PHB_f - PHB_0}{S_0 - S_f}$$

$$Q_{PHB} = \frac{dPHB_f}{dt}$$

where X_f corresponds to the final biomass concentration ($g \cdot L^{-1}$), X_0 is the initial biomass concentration ($g \cdot L^{-1}$), S_0 and S_f are the initial and final substrate concentrations ($g \cdot L^{-1}$), respectively, and PHB_f is the final PHB concentration ($g \cdot L^{-1}$).

2.2.4 Material characterization

The crystallographic analysis of the PHB was performed using a panalytical X-ray diffractometer with Cu-K α radiation (wavelength $\lambda = 1.5405 \text{ \AA}$) operating at 45 kV and 40 mA.

A parallel beam optical system was implemented, comprising a parabolic mirror in the incident beam, a 0.18° parallel plate collimator, and a flat graphite monochromator in the diffracted beam. X-ray scanning was conducted in the range of 5 to 90 degrees 2θ , in step scanning mode, with increments of 0.03° (2θ) and a counting time of 2 s. The functional groups and moieties present in the PHB powder samples were determined using Fourier transform infrared Spectroscopy (FTIR) (IR Affinity-1, Shimadzu Scientific Instruments, Columbia, MD, USA). The FTIR spectra were recorded in the range from 4000 to 500 cm^{-1} . For chemical composition evaluation, an energy-dispersive X-ray (EDS) analysis was performed using a Philips XL 30 FEG with a high-purity Ge EDS detector (Philips N.V, Eindhoven, The Netherlands). ZAF correction was applied to the stoichiometric analyses due to the low reliability of EDS under nitrogen concentrations. The structural analysis of the PHB was carried out via scanning electron microscopy (SEM) using a Philips XL 30 FEG, operating at 15 keV with a backscattered electron detector (EDAX-EDS). The thermal stability of the extracted PHB, along with standard samples, was analyzed via thermogravimetric analysis (TGA). The temperature range was from 30°C to 600°C , at a heating rate of $10^\circ\text{C}/\text{min}$ in a nitrogen atmosphere (N_2 flow rate = $40\text{ mL}/\text{min}$). The degradation rate of PHB samples was performed using temperature data of T5%, T10%, and T50% obtained from the TGA analysis. The melting point (T_m) and glass transition temperature (T_g) of the PHB samples were determined via differential scanning calorimetry (DSC) analysis (DSC-1 series, Mettler-Toledo, Columbus, OH, USA) with a heating and cooling rate of $10^\circ\text{C}/\text{min}$ in a N_2 environment with a gas flow of $20\text{ mL}/\text{min}$. For DSC analysis, 3.5 mg of the PHB sample was loaded in an aluminum pan and heated in the temperature range of -10°C to 200°C at a heating rate of $10^\circ\text{C}/\text{min}$. The point of inflection in the DSC curve between onset and offset temperatures corresponds to the glass transition temperature and the melting point, measured as the peak temperature of an endothermic event.

2.3 Results and discussion

2.3.1 Kinetic evaluation of *C. necator* in dextrose medium and PHB production in shake flask cultures

Figure 2.1 shows the evolution of glucose consumption, biomass, and PHB production over time in shake flask cultivations of *C. necator* using dextrose from bitter cassava starch as a low-cost carbon source. During the first 12 h, a short adaptation phase characterized by a reduced growth rate and limited dextrose consumption was observed. Subsequently, an exponential phase was observed, ranging from 12 to 48 h of cultivation, followed by a stationary phase of 12 h. The maximum biomass concentration achieved was $5.17\text{ g}/\text{L}$ at 48 h. At the end of the cultivations, the glucose concentration was $10.75\text{ g}/\text{L}$, which corresponds to 53.7% of the initial concentration. PHB production and accumulation were evaluated at the end of the cultures (72 h). PHB concentration was $2.01\text{ g}/\text{L}$, which corresponded to a PHB content of 47.10%. The yields of biomass and PHB per gram of substrate were $0.31\text{ g}/\text{g}$ and $0.18\text{ g}/\text{g}$, respectively. Moreover, the volumetric productivity of PHB reached $27.91\text{ mg}/(\text{L}\cdot\text{h})$ (see Table 2.1). The accumulations of PHB content were visually confirmed through microscopy utilizing the Sudan

black staining method (see Figure 2.2), which was monitored after 24, 48, and 72 h of cultivation. This qualitative analysis allows for the observation of the progressive increase in polymer intracellular accumulation as cultivation advances.

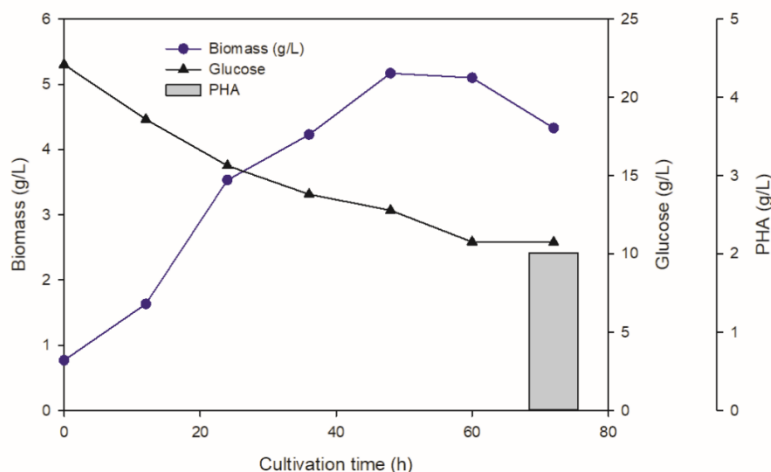


Figure 2.1 Glucose consumption, biomass, and PHB production over time in shake flask cultivations of *C. necator* using dextrose from bitter cassava starch hydrolysate as a carbon source.

In this culture, the growth of *C. necator* in carbon sources rich in glucose, such as dextrose from bitter cassava starch, was demonstrated. Additionally, it was observed that there was no depletion of the carbon source by the end of the culture. This behavior mirrors that reported by [118] when employing the same strain (*C. necator* DSM 428) in cultures with concentrations ranging from 5 to 20 g/L of glucose, where substantial amounts were reported at the end of the culture (between 1.4 g/L and 17.4 g/L). The same author reports that only with 2 g/L glucose, the growth phase was limited by the total consumption of the carbon source. Other authors [119] reported a decrease in biomass quantity when the glucose concentration was 20 g/L, attributing this concentration to the inhibition of growth in *C. necator* DSM 545. Furthermore, they indicated that with glucose concentrations higher than 10 g/L, complete glucose depletion does not occur at the end of the cultivation. In our study, glucose concentrations of 20 g/L were utilized; however, biomass production at 48 h exceeded the data reported by [119]. Despite the absence of glucose depletion in the medium, it is likely that nitrogen source depletion (ammonium sulfate) occurred, enabling PHB accumulation.

Table 2.1 Biomass growth and polyhydroxybutyrate biosynthesis in batch cultivation of *C. necator*

Fermentation mode	Time (h)	X(g/L)	PHB(g/L)	PHB (%)	$Y_{X/S}$ (g/g)	$Y_{PHB/S}$ (g/g)	Q_{PHB} mg/(L*h)
Batch	72	4.33	2.01	47.10	0.31	0.18	27.91

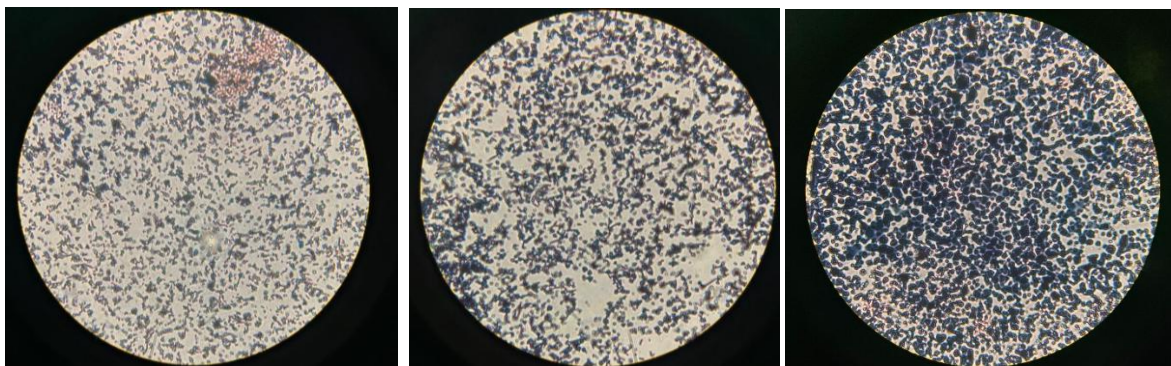


Figure 2.2 Detection of PHB by 100x microscopy using Sudan black staining (a) 24 h, (b) 48 h (c) 72 h.

Furthermore, the production of PHB through microbial cultivation of *C. necator* has been extensively studied, and other works reported the utilization of similar carbon sources for biopolymer production [91,120,121]. In this process, the PHB content is higher than reported by [122] for *C. necator* after 58 h in culture in a study utilizing broken rice as a carbon source (38% PHB). Similarly, Oliveira et al. [123] reported PHB accumulation of 33.3% when employing soybean and 2.5% molasses in solid-state fermentation with *C. necator*. Likewise, a study reported an accumulation of 42.2% at 31 h of cultivation of *C. necator* DSM 545 using fructose as the carbon source [124]. In contrast, higher accumulations (84.3% and 92%) were reported at 72 h of cultivation for strains *C. necator* IBP/SFU-1 and *C. necator* B-10646 respectively, using glucose as the carbon source [111,125]. Therefore, glucose is a promising carbon source for PHB production using *C. necator*. Even though fructose is the only sugar capable of being metabolized by hydrogen-oxidizing bacteria, they can easily mutate, enabling them to metabolize glucose through the ED to form pyruvate, which is transformed by dehydrogenase into Acetyl-CoA, one of the precursors of PHA, as demonstrated in this study using *C. necator* DSM 428 [111,126].

2.3.2 Biopolymer characterization

XRD analysis

Figure 2.3 presents the XRD pattern of the PHB obtained via microbial cultivation of *C. necator* using dextrose from bitter cassava starch. From these results, an Orthorhombic phase with a 19-P212121 space group belonging to PHB was evidenced by the reflections of the crystallographic planes (020); (110); (130); (202); and (410) located at angles $2\theta = 13.52^\circ$; 16.91° ; 27.28° ; 31.62° and 45.341° , respectively, indexed through the reference file JCPDF 00-001-0182. Similar XRD patterns of PHB were reported by [127,128]. The intense (202) peak indicates the crystalline nature, and it has been proposed that the polymer matrix adopts a regular helicoidal conformation with two antiparallel chains in the Orthorhombic unit cell within the crystalline domain. In this sense, it is possible to observe the PHB obtained by *C.*

necator with a unit cell, which consists of an orthorhombic crystalline structure system [128,129]

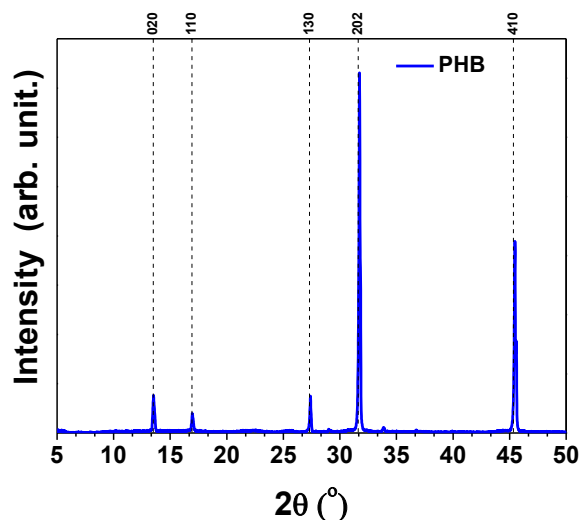


Figure 2.3 XRD of PHB produced in submerged cultures of *C. necator* using dextrose from bitter cassava starch as a carbon source.

FTIR Spectroscopy

The vibration modes of the bonds present for the PHB were analyzed using the FTIR spectra presented in Figure 2.4. In addition, the FTIR spectra were fitted by Gaussian functions (deconvolutions) to analyze the internal signals present in the material. Figure 2.4 shows the FTIR spectrum belonging to PHB, where it was possible to determine four high-intensity bands located at 520 cm^{-1} , 1278 cm^{-1} , 1721 cm^{-1} , and 3442 cm^{-1} , respectively. On one hand, those bands are associated with the vibrational state of C=O, C=H, and CH₃ bonds of PHB obtained in our study [130,131]. On the other hand, it was possible to determine a bond corresponding to a wave number of 536.1 cm^{-1} associated with the tensile vibrational state of C–H from the stretching methyl group, while the band at 1074.1 cm^{-1} , corresponds to the ester bounding, and the band at 1271.8 cm^{-1} corresponding to symmetric bending H–C–H from CH₃ groups. The 1721 cm^{-1} signal was associated with C=O ester carbonyl group, 2947.8 cm^{-1} , and the band at 2947.8 cm^{-1} reveals the presence of alkyl (CH₃) halides, which confirms that the polymer is PHB [128,129]. Furthermore, the band at 3270.8 cm^{-1} corresponds to the asymmetrical deformation of the C–H bond in the CH₂ group. Finally, a band located at 3450.1 cm^{-1} is attributed to the energy absorption caused by the stretching of the O–H bonds [132]. These results also coincided with those obtained by [124,128,133,134].

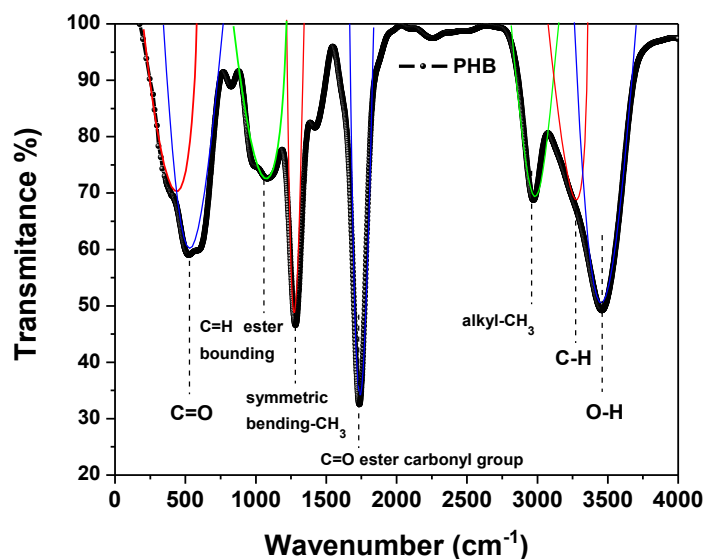


Figure 2.4 FTIR characterization of PHB produced in submerged cultures of *C. necator* using dextrose from bitter cassava starch as a carbon source.

EDS analysis

To investigate the chemical composition of the formed biopolymer, an EDS analysis was conducted on PHB synthesized by *C. necator* (see Figure 2.5). This analysis shows carbon and oxygen to be principal components, with 50.74 wt% and 38.77 wt%, respectively [135,136]. The results indicate a very low level of impurities, which correspond to sodium and chlorine traces from the PHB extraction method employed in the current research (Table 2.2). Other studies reported residual elements such as sodium hypochlorite, an essential component during the extraction process [136,137]. To remove these types of impurities, PHB can undergo an effective purification process that enables its safe commercial application.

Table 2.2 Chemical composition of the PHB produced by *C. necator* using dextrose from bitter cassava starch as a carbon source.

Spectrum	In stats.	C	O	Na	Cl	Total
Average	Yes	50.74	38.77	4.61	5.88	100.00

The above is consistent with the presence of sodium and chlorine in Figure 2.5.

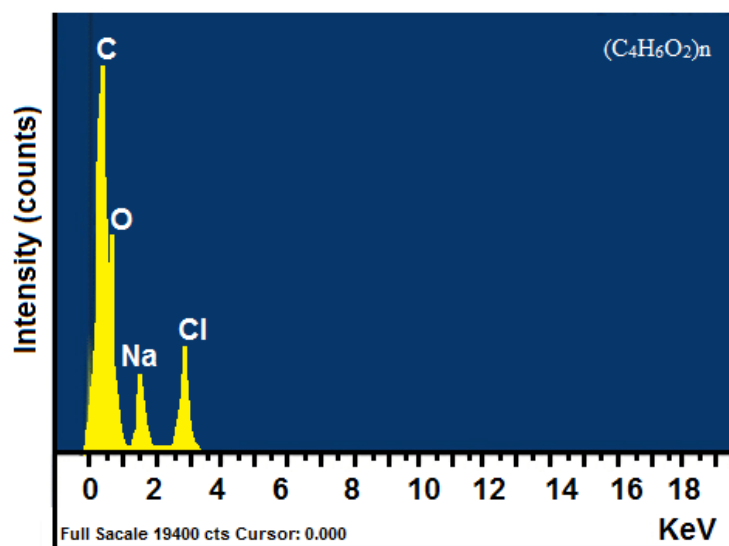


Figure 2.5 EDS characterization of PHB produced in submerged cultures of *C. necator* using dextrose from bitter cassava starch as a carbon source.

SEM analysis

The SEM micrograph analysis obtained for the PHB shows a distribution of micro- and nano-pellets with a spindle-shaped morphology (Figure 2.6a). In Figure 2.6b, a magnification of the obtained pellets is analyzed, showing an irregular surface with a varied morphological distribution from angular rounded geometries with flake shapes [135]. A similar semi-crystalline morphology with a brittle appearance characteristic of PHB was also reported by [137]. The morphological nature analyzed by SEM allows us to identify, from the morphology of the PHB pellets, that they are optimal for subsequent extrusion or 3D printing processes.

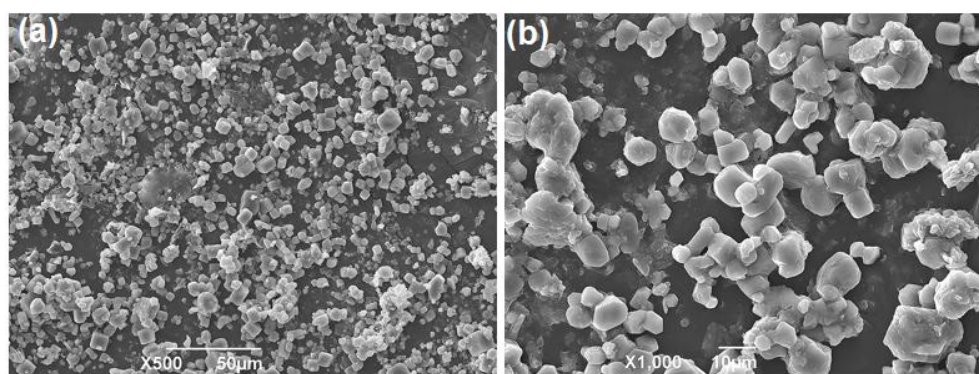


Figure 2.6 SEM micrograph of PHB produced in submerged cultures of *C. necator* using dextrose from bitter cassava starch as a carbon source: (a) distribution of micro- and nano-pellets and (b) magnification of the obtained pellets showing an irregular surface.

TGA results

The thermal stability of PHB was analyzed by TGA as shown in Figure 2.7. It can be seen from the TGA curve that PHB exhibited the first one-step thermal degradation with the onset at 85.6 °C and a minimum centered at 104.5 °C, while the second and most important process in the

one-step thermal degradation was onset at 173.2 °C and its minimum was centered at 220.5 °C. Finally, the third change in the thermogram begins one-step thermal degradation with an onset at 407.5 °C, and its minimum was centered at 468.7 °C. Additionally, Figure 2.7 depicts the TGA and the derivate weight profiles of PHB produced by *C. necator* using dextrose from bitter cassava starch as a carbon source. The TGA curve shows the weight loss of synthesized PHB in three stages. The first or initial stage of mass loss occurred in the temperature range of 85.6 °C to 173.2 °C. This mass loss can be associated with the loss of ambient humidity acquired by the hydrophilic characteristic of the PHB polymer. Moreover, this mass loss was approximately 1.0% of the total mass, which occurs due to the evaporation of physically adsorbed solvents such as sodium hypochlorite and chloroform on the polymer. Furthermore, the second and most relevant step in the degradation of the PHB was observed in the temperature range of 173.2 °C to 220.5 °C and can be related to a reduction in molecular weight, which includes chain scission and hydrolysis. The faster thermal degradation of PHB that occurs in this stage is obtained via destruction of H-C-H from CH₃ groups, C=O ester carbonyl group, and alkyl (CH₃) halides such as was observed in FTIR results (Figure 2.4), and the destruction of crystalline regions associated with orthorhombic unit cell, such as was observed in XRD results (Figure 2.3). The third step of weight loss was observed in the temperature range of 407.5 °C to 468.7 °C and occurs with a further temperature rise, as hydrolysis also contributes to degradation, along with chain scission with subsequent formation of crotonic acid. Finally, it can be observed that the maximum degradation temperature for PHB obtained by *C. necator* is 451.8 °C. Comparing our results with other reports in the literature, it is observed a decomposition temperature 49 and 33% higher than the PHB obtained in other studies [138,139]. Therefore, the degradation temperature for the current polymer showed high thermal stability compared with other biopolymers [128]; this will allow it to be used in applications where high temperatures are required.

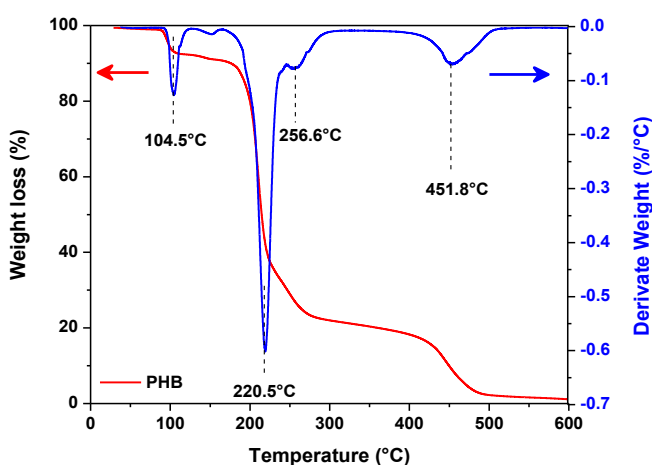


Figure 2.7 TGA of PHB produced in submerged cultures of *C. necator* using dextrose from bitter cassava starch as a carbon source.

DSC analysis

DSC analysis was carried out to study the thermal nature of PHB produced in this study (Figure 2.8); the second heating scan shows a melting temperature (T_m) of the polymer to be 173.4 °C. This value is similar to the standard PHB (Sigma Aldrich, St. Louis, MI, USA), which has a (T_m) of 177.0°C, and to the report in other studies for PHB (172.6°C) [140]. From DSC analysis, the crystallinity degree (X_c) of PHB was calculated by comparing the area of the melting peak (ΔH_m) with the melting enthalpy of 100% crystalline PHB. From the technical references, the melting enthalpy of 100% crystal PHB is 146 Jg⁻¹ [141]. By using the reference value of the melting enthalpy together with the value of the melting enthalpy obtained for this biopolymer (55.6 Jg⁻¹), it can be observed that the degree of crystallinity for PHB produced in this study was 38%. Considering the last analysis of the X_c , the thermal degradation temperature, which can be associated with differences in structure, and the X_c observed from XRD results, the glass transition temperature (T_g) obtained for PHB ranges from -5°C to 7°C. So, according to the crystallographic analysis using XRD (Figure 2.3), the vibration modes of the bonds observed by FTIR spectroscopy (Figure 2.4), the compositional and morphological nature observed from SEM-EDS results (Figures 2.5 and 2.6), and thermal stability observed from TGA (Figure 2.7) the random, relatively crystalline nature of PHB, hinders the initial movement of the polymer chain resulting the current glass transition temperature (-5°C to 7°C), melting enthalpy (55.6 Jg⁻¹), and X_c (38%)

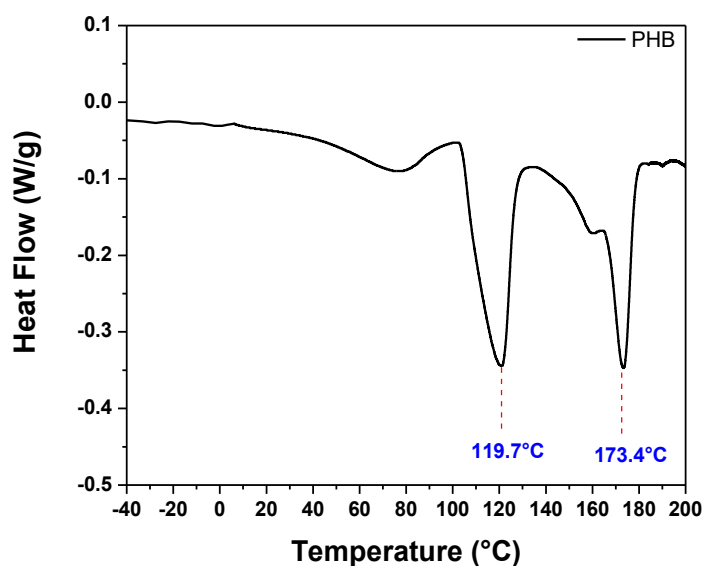


Figure 2.8 DSC characterization of PHB produced in submerged cultures of *C. necator* using dextrose from bitter cassava starch as carbon source (curve for heating cycle).

2.4 Conclusions

The current chapter presents a potential process to produce PHB by submerged cultures of *C. necator* using only dextrose from Colombian bitter cassava starch as a substrate.

The conditions used for PHB production in this study resulted in a yield of 2.01 g/L of PHB, with an accumulation of 47.1%.

The PHB was characterized by XRD, FTIR, SEM-EDS, TGA, and DSC. The experimental results showed crystallographic characteristics, vibrational modes of the bonds, compositional nature, morphology, and thermal stability. This demonstrates that the random and relatively crystalline nature of this polymer hinders the initial movement of the polymer chain, generating a glass transition temperature range of (-5°C to 7°C), a melting enthalpy of 55.6 Jg⁻¹, and a degree of crystallinity (XC) of 38%.

This approach is undoubtedly a potential valorization of industrial cassava starch to produce biodegradable bioplastics such as PHB.

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Chapter 3 Production of polyhydroxybutyrate by fed-batch pulse-feeding cultivations of *Cupriavidus necator* using dextrose from bitter cassava starch as low-cost carbon source

3.1 Introduction

The search for biodegradable and environmentally friendly polymers is an industrial and social need. Therefore, the development of novel and alternative processes, such as polymer production by microbial synthesis with low-cost substrates, emerges as a promising alternative to produce biodegradable polymers. The production of PHA is currently one of the most recurrent research topics [124,142,143], owing to a growing trend towards the use of biodegradable plastics like these to replace conventional petroleum-derived plastics.

PHA production is carried out using microorganisms, including bacteria, algae, archaea, plants, and their recombinant forms. These biopolymers accumulate intracellularly under limited oxygen, nitrogen, or phosphorus, in the presence of excess carbon substrates. Several factors have been identified as crucial for achieving sustainable production of this bioplastic, such as the selection of the microorganism, the carbon source nature, availability, and cost, in addition to the bioprocess operating conditions and downstream processing [27]. The Gram-negative bacterium *C. necator* can accumulate high levels of PHA, particularly PHB, a linear polyester with thermoplastic and mechanical properties comparable to those of polypropylene. [101,144]. Additionally, PHBV can be synthesized through controlled manipulation of the growth medium. [144,145]. The polymer composition, including mechanical and thermal properties, is determined by the producing bacterial strain and the fermentation conditions [111]. As for the carbon source, multiple reports indicate that its cost is one handicap for PHA production scaling up, since it may represent up to 50% of the total cost of the biomaterial [146,147].

Another approach explored in this research to increase PHB production and reduce costs involves improving fermentation processes using *C. necator*, with emphasis on increasing cell concentration and intracellular PHB accumulation. One way is through the refinement of the culture medium and operating conditions [124,148]. One of the most used strategies to enhance productivity is fed-batch fermentation. Some studies report that accumulations reach 0.52 to 1.08 times higher in fed-batch compared to batch systems [149,150].

Hence, investigating the use of inexpensive substrates and residues is crucial for building a sustainable production pathway. Currently, several studies have focused on the search for alternative substrates to reduce costs, evaluating the use of waste materials [151,152]. These materials include molasses, olive oil, cooking oil waste, lactic acid, and starch [8,9,152,153]. These latter materials are emerging as an alternative carbon source for PHA production due to

their low cost [154]. Additionally, they are highly available and a sustainable source of fermentable glucose syrups [155].

In Colombia, starch is obtained from various tubers, including yam, potato, sweet potato, and industrial cassava. By 2022, the latter reached a production of more than 242 thousand tons, according to data from the AGRONET information network of the Ministry of Agriculture, highlighting the production of industrial varieties that already exceed 15% of the total crop (AGRONET, 2024). National consumption of industrial cassava surpasses 269 thousand tons, of which 69 thousand tons are used for starch and 200 thousand tons for other uses. Starch is a high-quality glucose source with high availability and low cost [13].

This chapter used dextrose of bitter cassava starch as a low-cost carbon source for PHB production in fed-batch pulse feeding cultivations of *C. necator*. Here, three pulse feeding strategies were evaluated as alternatives to increase cell density and PHB accumulation. Additionally, the biomaterial accumulated by *C. necator* was characterized using XRD, FTIR, SEM, EDS, TGA, and DSC.

3.2 Materials and methods

3.2.1 Microorganism, culture media and cultivation conditions

Cryotube cell suspensions (1.5 mL) of *C. necator* DSM 428 conserved at -80°C in glycerol solution (20%) were reactivated and grown under sterile conditions in 50 mL of nutrient broth (PanReac AppliChem, ITW Reagents, Monza, Italy) as seed medium, disposed in 250 mL shake flasks. Cells grew at 35°C, pH of 6.8, and 150 rpm for 24 h.

MSM medium for precultures and production was slightly modified with 20.0 g/L of dextrose from bitter cassava starch (commercially obtained) and 1.0 g/L of ammonium sulfate as carbon and nitrogen source, respectively (C/N ratio 44). The other components of the MSM were as follows (g/L): Na₂HPO₄·7H₂O 6.7, KH₂PO₄ 0.1, MgSO₄·7H₂O 0.2, sodium citrate 0.059, FeSO₄ 0.035, CaCl₂·2H₂O 0.01, and trace element solution. The trace element solution contained (in g/L) H₃BO₃ 0.3, CoCl₂·6H₂O 0.2, ZnSO₄·7H₂O 0.1, MnCl₂·4H₂O 0.03, NaMoO₄·2H₂O 0.03, NiCl₂·6H₂O 0.02, CuSO₄·5H₂O 0.01 [156].

Two pre-cultures stages were conducted for strain adaptation prior to inoculation of production cultures. For the first preculture, 250-mL shake flasks were filled with 45 mL of modified MSM medium and inoculated with 5 mL of seed broth under the same operation conditions as the seed medium. Subsequently, the second preculture was identically operated, being inoculated with 5 mL of the first preculture broth.

Production cultures were inoculated at 10% v/v using the modified MSM medium. Pulsed fed-batch cultivations were conducted by triplicate in 250-mL shake flasks, starting with 50 mL of

culture broth. Identical operating conditions, such as seed and precultures, were used. Three different pulse-feedings were performed as shown in the following table (see Table 3.1)

Table 3.1 Pulse feeding strategies for PHB production by *C. necator*.

Strategy 1	Strategy 2	Strategy 3
Two pulses (8.33 ml) of dextrose from bitter cassava starch (20 g/L) and ammonium sulfate in a C/N ratio of 73.0 were fed at 24 and 48 h, extending the culture up to 120 h.	One pulse (3.15 ml) of only dextrose (20 g/L) was fed at 48 h. The culture lasted 96 h.	One pulse (6.14 ml) of only dextrose (20 g/L) was fed at 60 h, also lasting the culture up to 96 h.

3.2.2 Biomass and sugars quantification

Biomass was quantified as cell dry weight in g/L. Samples of culture broth (1.0 mL) were centrifuged at 5,000 rpm for 10 minutes, after which the supernatants were used for sugar analysis, and the wet cell pellet was dried at 70°C for 24 h to determine its dry weight. Dextrose concentration was determined by HPLC (Agilent Technologies 1200, Santa Clara, CA) employing an Aminex HPX-87H ion exchange column, using sulfuric acid 0.008N as mobile phase at 0.6 mL/min and 35 °C [157].

3.2.3 PHA detection and extraction

For qualitative intracellular polymer detection during the cultures, cells were stained with Sudan black B, which revealed PHA formation as black granules under a microscope. Polymer extraction involved centrifuging the culture broth at 5,000 rpm for 10 minutes to collect cell pellets, which were then dissolved in 5 mL of sodium hypochlorite and 5 mL of chloroform. This mixture was vortexed, left at room temperature under agitation for 20 h, and centrifuged again. Three phases were obtained: the top aqueous layer, a middle layer of cellular debris, and the bottom organic phase containing chloroform with the dissolved biopolymer. PHA was precipitated by adding isopropyl alcohol, and the resulting precipitate was dried at 70°C for 24 h for PHA dry weight quantification [117].

Calculations

The substrate yield on biomass ($Y_{X/S}$) and PHB ($Y_{PHB/S}$), specific substrate consumption rate (Q_S) and specific production rate (qPHB) were determined using the following equations:

$$Y_{X/S} = \frac{X_f - X_0}{S_0 - S_f}$$

$$Y_{PHB/S} = \frac{PHB_f - PHB_0}{S_0 - S_f}$$

$$Q_{PHB} = \frac{dPHB_f}{dt}$$

where X_f corresponds to the final biomass concentration ($g \cdot L^{-1}$), X_0 is the initial biomass concentration ($g \cdot L^{-1}$), S_0 and S_f are the initial and final substrate concentrations ($g \cdot L^{-1}$), respectively, and PHB_f is the final PHB concentration ($g \cdot L^{-1}$).

3.2.4 Characterization of PHA

The characterization of the biopolymer was carried out exactly as described in Chapter 2, Section 2.2.3.

3.3 Results and discussion

3.3.1 Effect of pulsating feeding on PHB production by *C. necator*

The temporal profiles of dextrose, biomass, and PHB in *C. necator* batch cultures using bitter cassava starch-derived dextrose are shown in Figure 3.1. During the first 12 h, a short adaptation phase characterized by a reduced growth rate and limited dextrose consumption was observed. Exponential growth was observed between 12 and 48 h. The maximum biomass concentration reached was 5.2 g/L (48 h), with a remanent carbon source concentration of 10.75 g/L at 72 h, an adequate uptake of 51%. A PHB concentration of 2.01 g/L was reached at 72 h, yielding 46.42% of biopolymer accumulation, a PHB yield ($Y_{PHB/S}$) of 0.18 g/g, and a volumetric productivity (Q_{PHB}) of 27.91 mg/(L*h) (see Table 3.2). Dextrose was not completely consumed during cultures, probably due to nitrogen exhaustion, which affects biomass accumulation but simultaneously favors PHB accumulation [118]. PHB accumulation often begins in the exponential phase and peaks in the stationary phase, as reported by other researchers using various carbon sources such as rice, soybean, and molasses [91,120,121,157]. Usually, fructose is the preferred carbon source for PHA production by *C. necator*; however, the strain can utilize glucose, leading to acetyl-CoA formation, as a precursor for PHA synthesis [111,125,156].

Table 3.2 Effect of the mode fermentation on cell growth and PHB production in *C. necator* cultures.

Fermentation mode	Time (h)	X(g/L)	PHB(g/L)	PHB (%)	$Y_{X/S}$ (g/g)	$Y_{PHB/S}$ (g/g)	Q_{PHB} mg/(L*h)
Batch	72	4.33	2.01	47.10	0.31	0.18	27.91
Fed-batch - Strategy 1	48	3.80	0.51	13.3	0.41	0.07	10.62
	72	4.80	0.86	17.92	0.34	0.07	11.94
	120	5.35	1.80	33.55	0.33	0.13	15.00
Fed-batch- Strategy 2	24	3.25	0.46	14.15	0.36	0.08	19.16
	96	4.50	1.12	25.12	0.25	0.08	11.66
Fed-batch- Strategy 3	24	3.20	0.46	14.38	0.35	0.08	19.16
	96	4.25	2.23	52.40	0.21	0.15	23.22

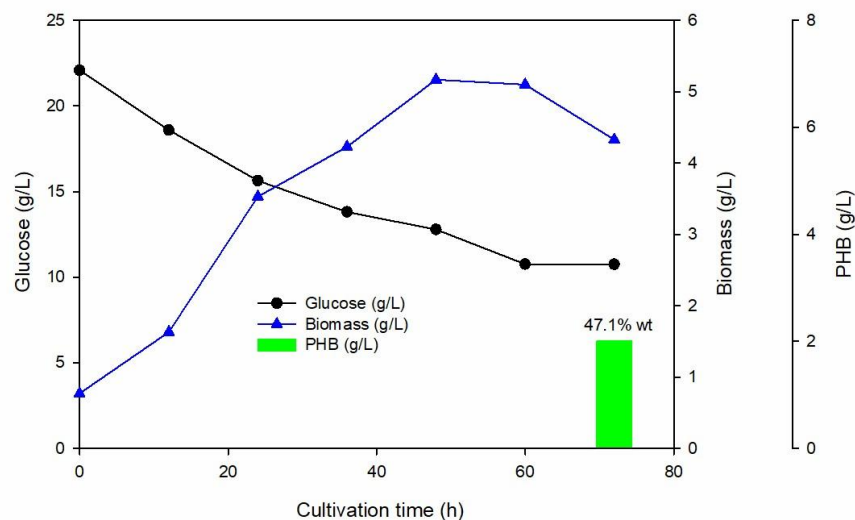


Figure 3.1 Glucose consumption, biomass and PHB dynamics in *C. necator* cultivations. Initial C/N ratio of 44.

The first pulsating feeding strategy consisted of two pulses at 24 and 48 h, containing dextrose and ammonium sulfate in a C/N ratio of 73 (Figure 3.2). The exponential growth phase began at 12 h and extended to 24 h (1.70 to 3.45 g/L). At this point, 4.12 g/L of sugar had been consumed. Subsequently, a first pulse (8.33 ml - C/N of 73) was added, resulting in an instantaneous dextrose concentration of 21.2 g/L. During the next 24 h, the biomass grew at a lower but stable rate, increasing from 3.45 to 3.80 g/L, showing a biomass yield ($Y_{X/S}$) of 0.41 g/g at 48 h (see Table 3.2). After 48 h, an identical second pulse was added to the cultures, diluting biomass and restoring carbon source concentration to 23.6 g/L.

A notable increase in biomass of 26.3% from 3.80 g/L at 48 h before the second pulse, up to 4.80 g/L at 72 h, was recorded, with a biomass yield of 0.34 g/g (see Table 3.2). Then, at 108 h, the biomass had increased by 14.5%, with a yield of 0.34 g/g, evidencing an extension of the exponential growth phase after the second pulse. Adding a feed medium during the acceleration phase (at 24 h) slowed down the growth rate. This is likely attributed to the high concentration of glucose and ammonium sulfate. In this regard, it has been reported that the specific growth rate of *C. necator* decreases significantly at glucose concentrations over 20 g/L, as was observed in this study [158]. Such inhibition persisted until 36 h, when a second, but lower acceleration phase was recorded. This growth trend was maintained during the next 72 h, supported by the second substrate pulse added at 48 h. The PHB to biomass ratios (%) observed were 13.3, 17.9, and 33.5 at 48, 72, and 120 h, respectively. At 120 h of cultivation, the PHB yield was 0.13 g/g, and the volumetric productivity was 15.00 mg/(L*h) (see Table 3.2). However, the PHB accumulation was 20.57% lower compared to that observed in the batch cultivations. This is likely due to the additional time of the pulses, which avoided reaching the nitrogen limitation condition required for unleashing the PHA production, thus leading to lower biopolymer accumulation than in batch cultures [159].

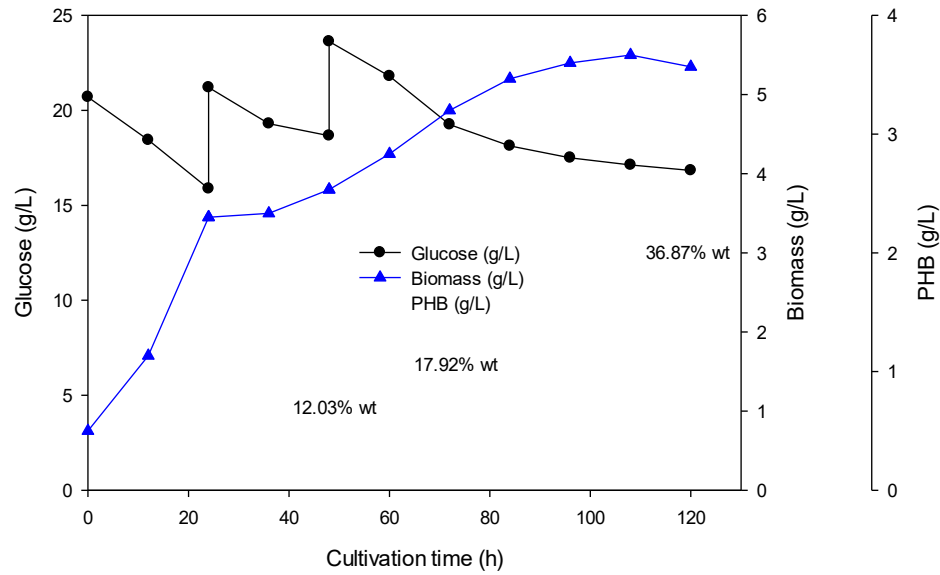


Figure 3.2 Glucose consumption, biomass, and PHB dynamics in *C. necator* cultivations in a two-pulsating fed-batch (24 and 48 h) with a C/N ratio of 73 and an initial C/N ratio of 44.

Given the behavior observed in the two-pulses strategy and the lower PHA accumulation attained in the cultures, a second pulsating strategy was tested, adding only one substrate pulse at 48 or 60 h of cultivation. A pulse of carbon source only was applied at 48 h (3.15 ml of dextrose (20 g/L)), as shown in Figure 3.3. The exponential phase was recorded immediately after inoculation and extended to 24 h of cultivation when the PHB accumulation started due to the probable early nitrogen limitation. At 48 h, the glucose concentration was 13.8 g/L before adding the pulse, and the biomass of 4.0 g/L. At 60 h, the biomass remained at 3.95 g/L, indicating that the dilution caused by the pulse was approximately equal to the growth rate. However, from 60 h onwards, biomass production increased by 13.9% (4.50 g/L at 96 h) with a biomass yield of 0.25 g/g (see Table 3.2). In this case, biomass production at 96 h was 16.6% lower than the biomass obtained in the two-pulse strategy since the expected limiting conditions were reached in the one-pulse strategy. Regarding PHB accumulation, 14.15 and 25.12% were attained at 24 and 96 h, respectively. These accumulations were 58.34% lower than those obtained in the batch cultivation and 25.12% lower than the two-pulsating fed-batch cultivations. This behavior contradicts previous studies claiming higher PHA accumulations in fed-batch cultivations [149,150,160]. Nevertheless, earlier studies reported that implementing fed-batch strategies with supplementation may lead to lower productivity than batch cultivations [142].

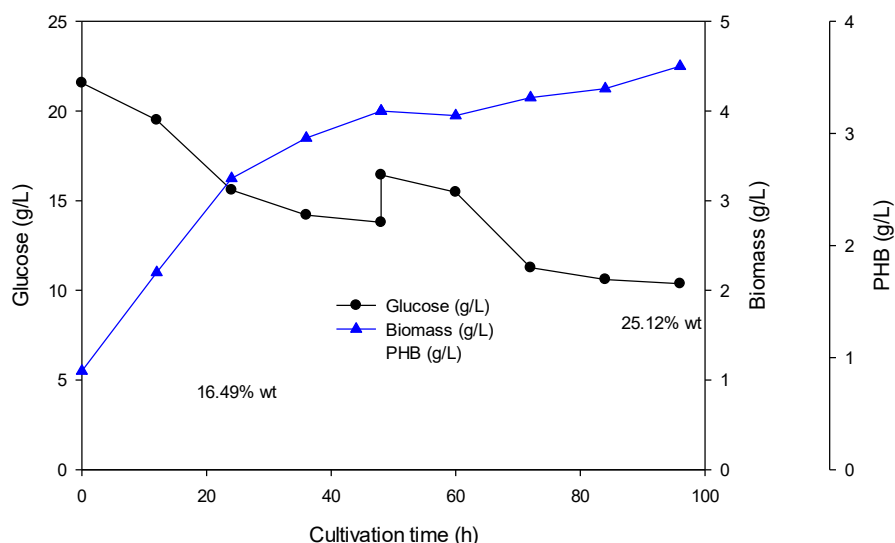


Figure 3.3 Glucose consumption, biomass, and PHB dynamics in *C. necator* cultivations in a one-pulsating fed-batch (48 h) of bitter cassava dextrose. Initial C/N ratio 44.

The carbon source pulse was displaced to 60th h, thus adding 6.14 ml of dextrose (20 g/L) during the stationary phase (see Figure 3.4). At this point, the glucose concentration was 15.4 g/L, and the biomass concentration was 4.0 g/L. An increase of 4.9% in biomass was observed from 60 to 96 h of cultivation. A decrease of 25.4% in the glucose uptake was observed from 72 h of cultivation onwards, leading to a remanent of glucose of 10.7 g/L at 96 h. In this case, the PHB accumulation reached 52.40%, PHB yield of 0.15 g/g, and volumetric productivity of 23.22 mg/(L*h) at the end of the cultures (see Table 3.2), which is higher than the batch, the two-pulse (24 and 48 h) and the one-pulse (48 h) operations. These results reveal that adding carbon at the beginning of the stationary phase favors higher biomass production but lower PHB accumulation by avoiding (probably) the limiting conditions that trigger biopolymer accumulation as an energy reservoir. Conversely, the one pulse feeding applied in the advanced stage of the stationary phase, which is probably characterized by the nutritional limiting conditions, led to lower biomass production but higher PHB accumulation. Moreover, nitrogen starvation has likely occurred at this time of cultivation, and the availability of carbon increased the intracellular PHB accumulation [142,161]. The specific PHB accumulation obtained in this latter fed-batch strategy was 12.88% higher than in the batch system, as has been previously reported for other fed-batch operations with *C. necator* [149,150].

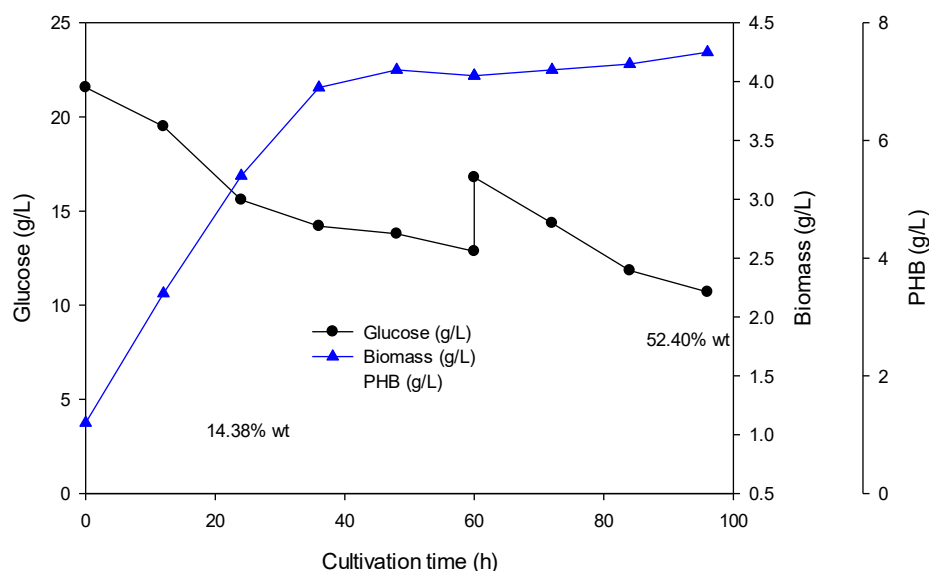


Figure 3.4 Glucose consumption, biomass, and PHB dynamics in *C. necator* cultivations in a one-pulsating fed-batch (60 h) of bitter cassava dextrose. Initial C/N ratio of 44.

3.3.2 Biopolymer characterization

The higher PHA accumulation obtained in our study (52.40%), i.e., the strategy of one pulse feeding at 60 h, was chosen for polymer characterization. Figure 3.5a shows the X-ray diffraction pattern of PHB produced by fed-batch pulse feeding cultivations of *C. necator* using dextrose derived from bitter cassava starch. The diffraction pattern reveals an orthorhombic phase with a P212121 space group, indicated by reflections from the crystallographic planes (130), (202), and (410) at 2θ angles of 27.16° , 31.78° , and 45.53° , respectively. These reflections were indexed using the JCPDF reference file 00-001-0182. The prominent (202) peak suggests a high degree of crystallinity, which aligns with the proposed polymer matrix structure, where regular helicoidal conformations are formed by two antiparallel chains within the orthorhombic unit cell. This confirms that the PHB produced by pulsed fed-batch cultivations of *C. necator* exhibits an orthorhombic crystalline structure system [128,129].

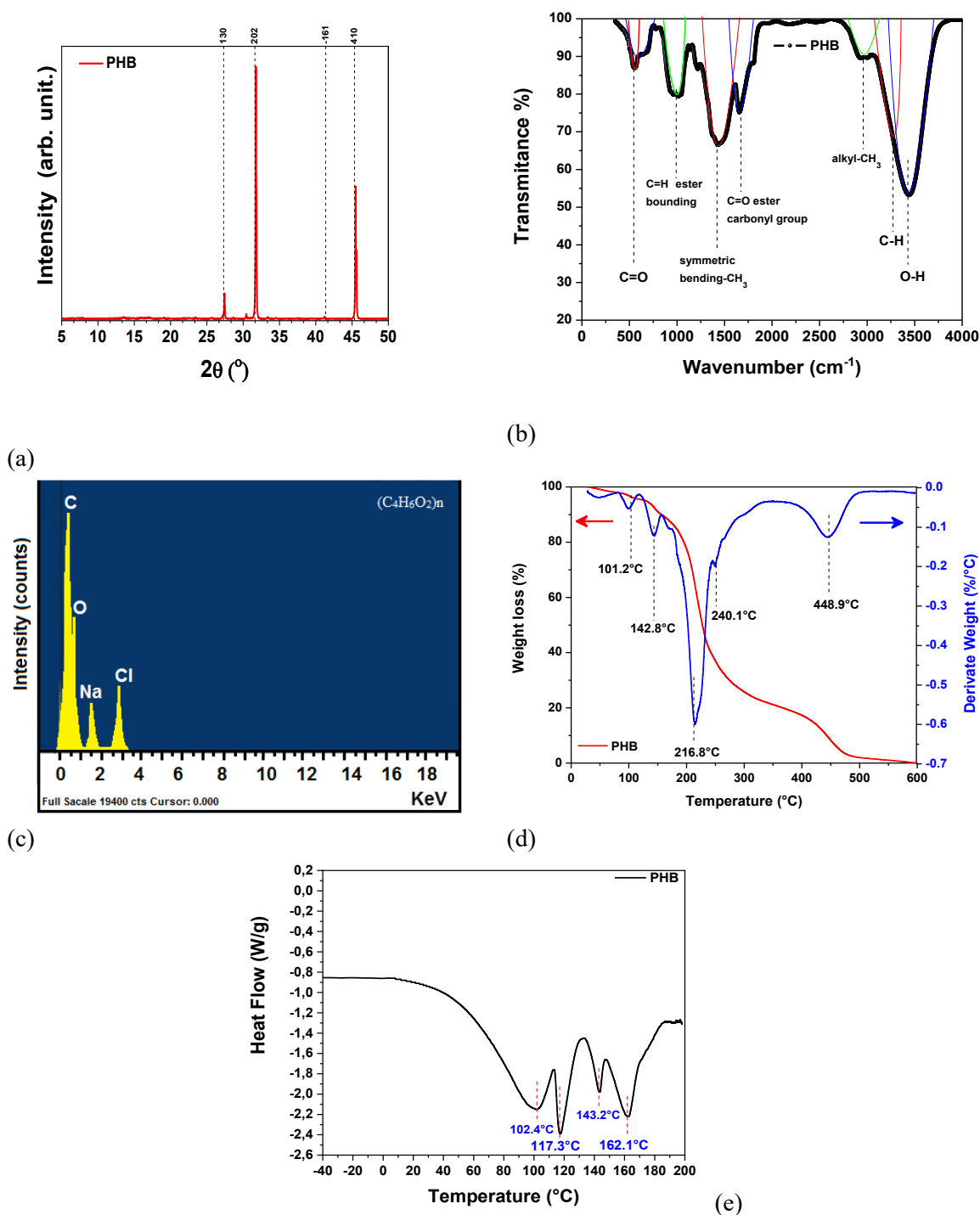


Figure 3.5 Characterization of PHB produced in one-pulse (60 h) fed-batch cultivations of *C. necator* with dextrose from bitter cassava starch as carbon source. (a) XRD, (b) FTIR spectra, (c) EDS, (d) TGA, (e) DSC.

The FTIR spectra are shown in Figure 3.5b, where the vibration modes of the bonds in PHB were analyzed. The spectra were deconvoluted using Gaussian functions to investigate the materials internal signals further. Figure 3.5b reveals four prominent bands at 520 cm^{-1} , 1278 cm^{-1} , 1721 cm^{-1} , and 3442 cm^{-1} , corresponding to the vibrational states of C=O, C-H, and CH_3 bonds in PHB produced by pulsed fed-batch cultivations of *C. necator* using dextrose from

cassava starch as a low-cost substrate [130]. A band at 536.1 cm^{-1} was also identified, associated with the tensile vibrational state of C–H from stretching of the methyl group. The band at 1074.1 cm^{-1} corresponds to ester bonding, while the 1271.8 cm^{-1} band is linked to the symmetric bending of H–C–H from CH_3 groups. The signal at 1721 cm^{-1} was attributed to the C=O ester carbonyl group, and the band at 2947.8 cm^{-1} indicates the presence of alkyl (CH_3) halides, confirming the polymer as PHB [128,129]. The band at 3270.8 cm^{-1} corresponds to asymmetrical deformation of the C–H bond in CH_2 groups, while the band at 3450.1 cm^{-1} is attributed to energy absorption due to the stretching of O–H bonds [132].

Figure 3.5c presents the EDS analysis of the PHB. EDS analysis was performed to determine the chemical composition of the biopolymer produced by *C. necator* using dextrose from bitter cassava starch. The results indicate that carbon and oxygen are the primary components, constituting 50.74 wt% and 38.77 wt%, respectively [135]. Additionally, the trace amounts of sodium and chlorine are likely remnants from the extraction and purification process, which involved using sodium hypochlorite and chloroform, leading to residual sodium content of 4.61% and chloride of 5.88%.

The thermal stability of PHB was evaluated using TGA, as illustrated in Figure 3.5d. The TGA curve reveals three distinct stages of thermal degradation for PHB. The initial degradation occurs between 90.54°C and 103.9°C , where a weight loss of approximately 1.0% is observed. This loss is attributed to the evaporation of moisture and the release of solvents like sodium hypochlorite and chloroform that are physically adsorbed on the polymer due to its hydrophilic nature. Then, the most significant thermal degradation occurs between 197.9°C and 247.7°C . This phase is linked to a molecular weight reduction resulting from chain scission and hydrolysis. The rapid thermal degradation during this stage is likely due to the breakdown of H–C–H bonds in the CH_3 groups, the C=O ester carbonyl groups, and alkyl (CH_3) halides. These transformations align with the FTIR results (Fig. 3.5b) and indicate the destruction of the crystalline structure, as evidenced by the XRD findings (Fig. 3.5a). The final degradation stage is observed between 413.9°C and 468.7°C , where further temperature increase promote hydrolysis and chain scission, leading to the formation of crotonic acid. Thus, the maximum degradation temperature recorded for the PHB polymer is 468.7°C , indicating that PHB produced by pulsed fed-batch cultivations of *C. necator* has high thermal stability compared to other biopolymers [128].

The thermal behavior of PHB was investigated through DSC, as depicted in Figure 3.5e. The fourth heating scan revealed a melting temperature (T_m) of 162.1°C for the polymer. From this analysis, the degree of crystallinity (X_c) of the PHB was calculated by comparing the melting enthalpy (ΔH_m) with that of fully crystalline PHB, which has a reference value of 146 J/g [141]. Using this reference, along with the experimental melting enthalpy of 61.32 J/g obtained for the synthesized biopolymer, the crystallinity degree of PHB produced by *C. necator* using dextrose from bitter cassava starch was 42%. The crystallinity degree, in conjunction with the

thermal degradation temperature, reflects variations in structure and crystallinity observed in the XRD results. The PHB's glass transition temperature (T_g) also ranged between -5°C and 7°C .

Figure 3.6 presents the SEM micrograph of PHB. The image reveals a distribution of micro- and nano-pellets with spindle-shaped morphology (Fig. 3.6a). Upon closer magnification, the pellets exhibit an irregular surface with varied morphological features, ranging from angular to rounded geometries and flake-like shapes (Fig. 3.6b) [135]. The morphological characteristics observed through SEM indicated that the structure of the PHB pellets is well-suited for subsequent extrusion or 3D printing processes.

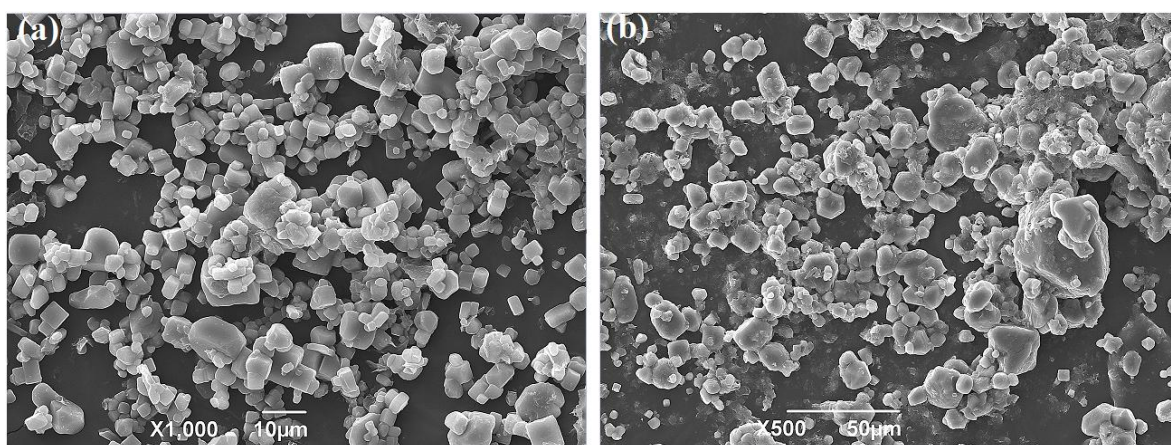


Figure 3.6 SEM micrograph of PHB produced in fed-batch shake flask cultivations of *C. necator* (one pulse at 60 h), using dextrose from bitter cassava starch as a carbon source. (a) distribution of micro- and nano-pellets and (b) magnification of the obtained pellet showing an irregular surface.

By integrating the findings from XRD crystallographic analysis, vibrational bond modes from FTIR spectroscopy, compositional and morphological data from SEM-EDS, and thermal stability from TGA, we can conclude that the polymer's random, yet semi-crystalline structure restricts the movement of polymer chains. This structural arrangement explains the measured glass transition temperature (-5°C to 7°C), melting enthalpy (61.32 J/g), and crystallinity degree (42%) of the biopolymer. This confirms that using pulses increases the process efficiency and the crystallinity degree of the polymer obtained, compared to the degree of crystallinity obtained in PHB by batch cultures (38%) [7]. Therefore, by increasing the crystallinity degree, the thermal and mechanical properties of the polymer material can be increased.

3.4 Conclusions

The implemented pulse feeding strategies demonstrated a decisive influence on the balance between cell growth and PHB accumulation in fed-batch cultures of *C. necator*. Early feed addition (at 24 and 48 h) favored prolonged cell growth but limited PHB accumulation, likely due to the continuous availability of nitrogen, which prevented the establishment of the nutrient-

limiting conditions necessary to trigger biopolymer biosynthesis. In contrast, the application of a single carbon pulse during the late stationary phase (60 h) promoted a significantly higher PHB accumulation (52.40%), even surpassing the values observed in batch cultures. These findings highlight that the timing of supplemental feeding is key to directing cellular metabolism either toward biomass production or biopolymer synthesis, and that strategies designed to induce specific nutritional limitations can significantly enhance PHB yield and productivity in submerged cultures. The physical and thermal characterization of the produced PHB (XRD, FTIR, SEM, EDS, TGA, and DSC) confirms its potential for industrial applications. This process represents a cost-effective and environmentally friendly alternative for large-scale biopolymer production.

Publication: This chapter was partially submitted as Martha L. Ascencio-Galván, Víctor A. López-Agudelo, Andreas Dräger, Julio C. Caicedo, David Gómez-Ríos, Rodrigo Andler, and Howard Ramírez-Malule. Integrating fed-batch pulse feeding and flux balance analysis for efficient PHB production by *Cupriavidus necator* from cassava-based dextrose.

Chapter 4 Reconstruction of a genome-scale model of *Cupriavidus necator* and exploration of PHB production scenarios using constraint-based metabolic modeling tools

4.1 Introduction

A GEM computationally describes a complete set of stoichiometry-based, mass-balanced metabolic reactions in an organism using Gene-Protein-Reaction (GPR) that are formulated based on genomic and experimental data [162]. These models approach the cellular metabolism through a set of linear equations obtained from the mass balances per each biochemical reaction according to the GPRs. Thus allowing the *in silico* representation of the complete metabolic reaction network of a cell or organism [163]. Relevant applications that have benefited from advances in the use of GEM include, but are not limited to, strain development to produce bio-based chemicals and materials, drug targeting in pathogens, prediction of enzymatic functions, modeling interactions between multiple cells or organisms, understanding human diseases, and bioplastic production [164].

GEM applications include strain design to produce bio-based compounds, identification of drug targets in pathogens, prediction of enzymatic functions, modeling of interactions among multiple cells or organisms, the study of human diseases, and bioplastic production [164]. Two GEMs have been reported so far for *C. necator* as model microorganisms for bioplastic production. The RehMBEL1391 model [10] stands out as the first extensively curated model for this species, and iCN1361 [11] as a more recent version with improvements in annotation and experimental validation, enabling high-accuracy predictions of cell growth and PHB synthesis under various conditions according to the authors.

Both models have served as platforms to explore metabolic optimization strategies; however, they exhibit limitations related to outdated genomic annotations, a lack of integration of new metabolic pathways, and insufficient validation under current experimental conditions. The limited availability of updated models underscores the need to reconstruct more comprehensive and specific GEMs that can accurately simulate diverse physiological scenarios useful for bioprocessing optimization and design of rationally engineered strains for PHB production.

Once a GEM has been carefully curated and validated, several analyses can be conducted by solving the LP problem under different restrictions and objective functions. In this context, tools such as Phenotypic Phase Plane (PhPP) analysis and *in silico* strain engineering pipelines like OptKnock [165] further expand the utility of GEMs by enabling the evaluation of culture conditions and the identification of genetic targets for enhanced metabolite production [166]. In the case of *C. necator*, variations in nutrient uptake, the balance between growth and polymer

accumulation and effects of gene deletions on metabolic flux distribution can be investigated by incorporating GEM analysis [167]. Moreover, such integration of experimental and *in silico* data supports the rational design of metabolic engineering strategies, thereby increasing the industrial applicability of GEM-based predictions [168].

This chapter presents the reconstruction and validation of the novel *i*CNH2025A model for *C. necator*, including its phenotypic characterization under different conditions and the identification of metabolic targets predicted to enhance PHB synthesis. The combined use of experimental data and computational simulations provides a robust foundation for advancing metabolic engineering efforts in this industrially relevant bacterium.

4.2 Materials and methods

4.2.1 Reconstruction of the metabolic network of *C. necator*

The pipeline followed in the reconstruction and curation of the *i*CNH2025A model for *C. necator*, is summarized in Figure 4.1.

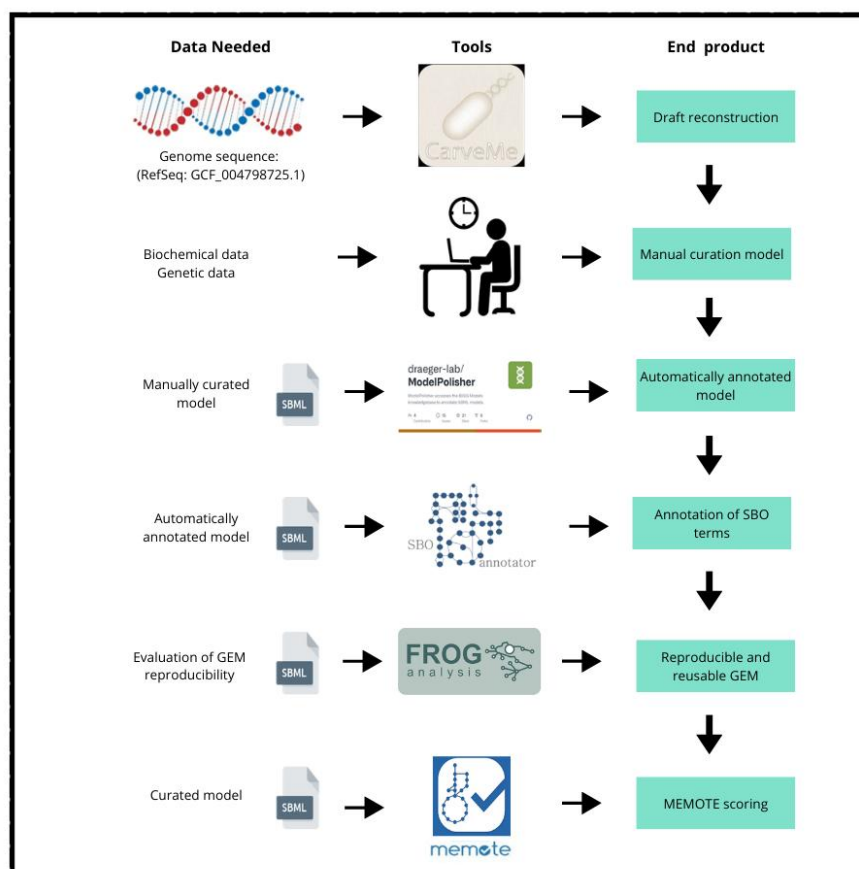


Figure 4.1 Steps for the reconstruction and curation of the metabolic network of *C. necator*.

CarveMe: was used to generate the draft reconstruction of *C. necator* [169]. This reconstruction was performed by using the KEGG (Kyoto Encyclopedia of Genes and Genomes) and protein databases (NCBI), which have the assembled sequence of the *C. necator* genome (RefSeq: GCF_004798725.1 of 2019).

Manual curation model: biochemical information of the *C. necator* strain was collected by using databases, literature, and experimental data (BiGG Models, Metacyc, Rhea, Uniprot) [170]. Relevant reactions, enzymatic information, and metabolic pathways necessary in the synthesis of PHA or other important specialized metabolites were identified and added, thus improving the initial reconstruction.

Automatically annotated model: The tool was used ModelPolisher version 2.0.1 to extensively annotate the initial draft of the reconstruction [171]. ModelPolisher compares the identifiers of the model entities with the BiGG Models database [172].

Addition of SB terms: SBO terms are often used to annotate a mathematical model, providing supplementary information about model entities, such as metabolites or reactions [173]

Reproducible and reusable GEM: FROG was used to ensure the reproducibility and traceability of the GEM by integrating genomic and biochemical data into a FAIR-compliant model, useful for simulating and optimizing PHB production [174].

MEtabolic MOdel TEsting (MEMOTE): The command line version of MEMOTE metabolic model testing test version 0.13 [175] was used, and the GEM score was monitored in MEMOTE each time it was further annotated for improvement it.

4.2.2 Refinement of the reconstruction using evidence from literature

After the initial draft was curated and annotated, the following steps were followed to perform manual refinement.

Mass and charge balance and improved gene annotation: the entire set of biochemical reactions was manually curated, conducted, addressing missing reactions ('gaps'), disconnected reactions, and blocked reactions. This process relied on databases and scientific literature. The curation involved adding reactions, metabolites, and including their correct molecular formulas to ensure a correct charge and mass balance in each reaction. Furthermore, model Gene-Protein-Reaction (GPR) associations were scrutinized, with missing genes and associations being incorporated as necessary.

Identification of thermodynamic cycles (TICs): Integration of thermodynamic data into GEM is very useful for verifying the energetic feasibility and directionality of reactions [176]. Any new model must be thermodynamically checked and curated to avoid the generation of internal thermodynamic cycles that cause inconsistent fluxes in the simulation. Thermodynamic cycles in metabolic networks are those reactions that do not exchange energetic metabolites with the environment and therefore violate the second law of thermodynamics [176–178]. The script used can be found on our GitHub repository: <https://github.com/mascencio27/GSM-iCNH2025A/tree/main>

Identification of unlimited reactions: one of the methodologies used for identification and curating TICs in metabolic networks has been described by [179] and [180]. First, the set of reactions involved in one or more TICs was identified by solving a series of optimization problems known as Flux Variability Analysis (FVA), which had as medium constraints finite values of consumption fluxes (1.0 mmol/gDW/h). The reactions involved in TICs were identified as intracellular reactions if their values with the solution matched the lower bound and/or upper initial constraint of FVA [181,182].

Identification of the basic set of TICs: Schellenberger and colleagues [180] demonstrated that the set base of TICs in a metabolic network can be identified by calculating the basis vectors of the null space of the stoichiometric matrix. Thus, the set of unbounded reactions forming part of the TICs was used to define a stoichiometric matrix. Its null space was used to identify TICs formed by two or more reactions. The script used can be found on our GitHub repository: <https://github.com/mascencio27/GSM-iCNH2025A/tree/main>

Curation of TICs: The manual curation of TICs was performed through (i) elimination of reversible reactions linearly dependent, and (ii) constraint of directionality of reaction through the calculation of Gibbs free energy reaction (ΔGr). (i) To correct TICs formed by linearly dependent reactions, the use of NADHP/NADP⁺ in anabolic reactions and NADH/NAD⁺ in catabolic reactions were forced, as recommended by Pereira and colleagues [183]. (ii) TICs formed by wrong directionality assignments were corrected by calculating ΔGr using eQuilibrator to calculate changes in the Gibbs free energy under conditions (pH: 6.8, Ionic strength: 0.3M) to define the directionality of the reactions [184].

Analysis of in silico metabolic scenario and validation of the iCNH2025A model

In silico simulations and constraint-based analyses were performed using MATLAB software version 2021b, Systems Biology Markup Language (SBML) version 3.0, Constraint-Based

Reconstruction and Analysis Toolbox (COBRA) version 3.0, and GNU Linear Programming Kit (GLPK) package. An FBA was performed to simulate the growth of *C. necator* under varying culture media. For this, the *iCNH2025A* model validations were performed by comparing the predicted fluxes at a pseudo-steady state against experimental data of *C. necator* in batch cultures [185]. The FBA simulations were performed by maximizing the specific growth rate under the minimization of the Manhattan norm of fluxes by using the “optimizeCbModel” function along with the “minNorm” option, both included in the COBRA toolbox [186]. The experimental growth rate in batch cultures was compared with the model’s predictions. According to the experimental data, exchange fluxes of glucose, NH₄, O₂, P_i, and SO₄ were constrained and used as lower and upper (CO₂) bound constraints. In this work, a two-stage approach was used: biomass maximization in FBA followed by flux minimization, as presented in Equation (4.1) and (4.2). FVA was evaluated to avoid unbounded fluxes for the nutrients.

$$\min \sum v \quad (4.1)$$

$$\begin{bmatrix} Sv = 0 \\ l_b \leq v \leq l_u \\ f^T v = Z_{max} \end{bmatrix} \quad (4.2)$$

where *S* is the stoichiometric matrix of coefficients for *m* metabolites and *n* reactions, *v* is the flux vector of dimension *n*, *Z_{max}* represents the biomass maximization, *f* is a vector of weights, and *l_b* and *u_b* are the lower and upper bounds, respectively. Thus, in the first stage the FBA standard problem for biomass maximization is solved, whereas the second stage deals with the minimization of the vector of fluxes.

PhPP Analysis

Phenotypic Phase Plane (PhPP) analysis is based on the systematic exploration of the flux space defined by two key variables, considered independent, to identify regions that represent different physiological states of the organism. Each point in this space corresponds to the solution of an FBA problem with fixed constraints on these fluxes, optimizing a specific objective function. This approach enables the characterization of metabolic transitions and the assessment of how environmental, or cultivation conditions influence the resulting metabolic phenotype [187].

In this study, PhPP analysis was applied to calculate all possible variations in ammonium and glucose uptake rates, as well as in ammonium uptake and PHB production rates, in relation to

cell growth. For each point in this space, an FBA problem was solved in which v_x and v_y were fixed (imposed as constraints) within a defined range, as expressed in Equation (4.3):

$$\begin{aligned}
 (v_x, v_y) &\in \text{defined range} \\
 \max_v Z &= c^T v \\
 \text{Subject to: } S * v &= 0 \\
 l_b &\leq v \leq u_b \\
 v_x &= v_x^* \text{ fixed} \\
 v_y &= v_y^* \text{ fixed}
 \end{aligned} \tag{4.3}$$

where:

- S is the stoichiometric matrix,
- v is the flux vector,
- c is the vector defining the objective function (e.g., growth rate, PHB production),
- l_b and u_b are the lower and upper bounds for each reaction,
- v_x^* and v_y^* are the fixed values for each coordinate of the phenotypic phase plane.

The selected objective functions were: ammonium uptake rate, glucose uptake rate, and PHB production. Ammonium uptake rates (x-axis) and glucose uptake or PHB production rates (y-axis) ranged from 0 to 3 mmol/(g DCW·h), while the *in silico* cell growth was represented on the z-axis.

4.2.3 Identification of potential metabolic targets susceptible to genetic modification

Considering the flux distributions under different environmental conditions, potential genetic modifications were proposed by performing *in silico* single-gene knockouts and using methodologies such as OptKnock. This is a bi-level optimization framework designed to identify gene deletion strategies that couple microbial growth with the overproduction of a target metabolite. The outer optimization problem simulates genetic interventions by constraining or removing specific reactions, while the inner optimization problem solves a standard FBA to maximize biomass yield under the imposed constraints. This approach exploits the inherent metabolic trade-offs, forcing the organism to redirect fluxes toward the desired product as a requirement for growth. By integrating stoichiometric models with optimization theory, OptKnock provides a rational, *in silico* strategy for metabolic engineering that predicts interventions capable of enhancing bioprocess productivity [165].

This optimization tool incorporates two simultaneous strategies: one that calculates the flux distribution based on the maximization of the biomass reaction (biomass yield) and another,

linked to the reactions from the previous optimization problem, which maximizes the flux of the desired metabolites.

Mathematically, OptKnock can be formulated as a bi-level programming problem, see Equation (4.4):

$$\begin{aligned}
 & \max_y Z_{prod}(v) \\
 & \text{subject to } y \in \{0, 1\}^n \\
 & v_j \leq M * y_j \quad \forall_j \in \text{reactions} \\
 & \text{Where } v \text{ solves:} \\
 & \max_v Z_{biomass} = c^T v \\
 & \text{subject to } S * v = 0 \\
 & l_b \leq v \leq u_b
 \end{aligned} \tag{4.4}$$

Where: y_j is a binary variable indicating whether reaction j is active (1) or deleted (0), M is a large constant used to disable flux in deleted reactions, v is the flux vector, S is the stoichiometric matrix, and c is the vector defining the biomass objective function. The outer problem maximizes the production of the target metabolite (Z_{prod}) by selecting reaction deletions, while the inner problem performs FBA to maximize cell growth ($Z_{biomass}$) under these genetic constraints. For this optimization approach, the PHB production flux was set as the optimization objective, as previously employed by Aminian and colleagues [188].

4.3 Results and discussions

4.3.1 General properties and validation of the iCNH2025A model

The curated GEM of *C. necator*, hereafter referred to as iCNH2025A, contains 2,737 reactions, 1,803 unique metabolites, and 1,059 annotated genes, including transport reactions, exchange reactions, and internal cytoplasmic reactions. After curation, a total of three reactions associated with PHB metabolism were included. Additionally, mass-balancing was applied to 1.07% of the reactions, and 7.40% of them required charge-balancing (see supplementary material S1 and S2 (<https://github.com/mascencio27/GSM-iCNH2025A/tree/main>)). MC³ [189] highlighted only 1.34% dead-end metabolites and 1.3% zero flux reactions. The reactions' SBO terms were chosen as precisely as possible using SBO annotator [173], a tool that automatically assigns SBO terms, available in GitHub <https://github.com/draeger-lab/SBOannotator/>. Table 4.1 shows the SBO terms added. The tool ModelPolisher [171] was also used for extensive annotation of metabolites, reactions, and subsystems. Within them, the subsystems of amino acid metabolism, PHA biosynthesis, fatty acid biosynthesis, cofactor biosynthesis, and vitamin biosynthesis are related (Figure 4.2). The model also includes the complete Calvin Benson-

Bassham (CBB) cycle, the Entner-Doudoroff pathway (ED), the Embden-Meyerhof-Parnas pathway (EMP), and the non-oxidative branch of the pentose phosphate pathway (PPP). To ensure the reproducibility of our model, the FROG analysis [174] was applied. The generated reports include comprehensive analyses of FVA, reaction deletion, objective function, and gene deletion, enabling a thorough assessment of GEM performance.

Table 4.1 SBO terms were added in the *i*CNH2025A model according to the type of reaction.

SBO Terms	Name	#
SBO:0000629	Biomass reaction	1
SBO:0000630	ATP maintenance	1
SBO:0000185	Transport reaction	768
SBO:0000627	Exchange reaction	309
SBO:0000632	Sink reaction	7
SBO:0000176	Biochemical reaction	1651

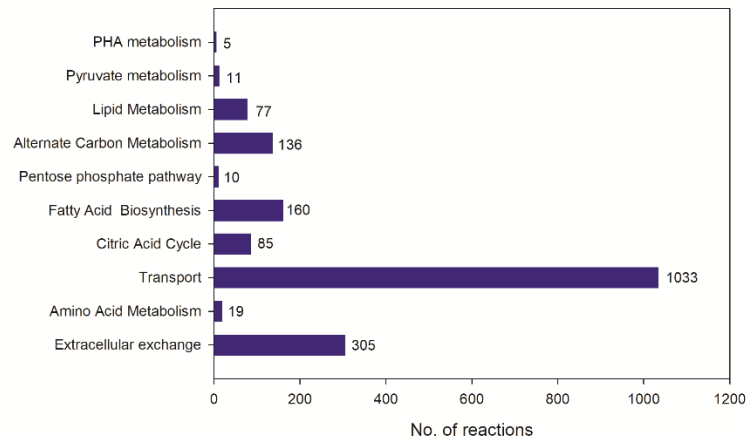


Figure 4.2 The distribution of reactions in the *i*CNH2025A model sorted by metabolic subsystems.

4.3.2 Comparison of *i*CNH2025A with other GEMs of *C. necator*

Here, the properties of the proposed metabolic network are compared with those of other published GEM of *C. necator*. In the scientific literature, two models have been reported to describe the metabolic behavior of this bacterium. The first metabolic model of *C. necator*, RehMBEL1391 [10], published in 2011, is a model with 1,391 reactions, including 229 transport reactions and 1,171 metabolites, which was used to design strategies for producing PHB under different pH values and carbon/nitrogen uptake ratios. That model was built using non-conventional identifiers for metabolites and reactions. The GEM RehMBEL1391 scored

60% in MEMOTE [175] (Figure 4.3a), showing the presence of stoichiometric inconsistencies (40%) and mass (46.7%), and charge (53.2%) reaction imbalances, indicating a low level of manual curation (see supplementary material S3 <https://github.com/mascencio27/GSM-iCNH2025A/tree/main>). The other metabolic model, *i*CN1361, published in 2022 [11], comprises 1,292 reactions, including 98 transport reactions and 1,265 metabolites. This model was applied to investigate system-level metabolic changes in *C. necator* H16 under nitrogen-limited conditions, and provided new insights into alterations of metabolic fluxes under these conditions. The MEMOTE report for *i*CN1361 showed a score of 90% (Figure 4.3b), indicating that it is a model that meets the standards in terms of consistency (99%), mass balance (99.7%), and charge balance (100%) (see supplementary material S4 <https://github.com/mascencio27/GSM-iCNH2025A/tree/main>). Our GEM *i*CNH2025A was also tested in MEMOTE (<https://memote.io/>, accessed on February 18, 2024) and validated in SBML. The score was 89% (see Figure 4.3c) with a consistency of 98% in stoichiometric terms, mass balance, and metabolite connectivity reaching 100%, and a charge balance of 91.9% (see supplementary material S5 <https://github.com/mascencio27/GSM-iCNH2025A/tree/main>). These percentages were obtained by the model after being refined and curated with the help of various databases, achieving percentages higher than 70% in annotations of metabolites, reactions, and terms, indicating that *i*CNH2025A meets the standards of the metabolic modeling community in terms of annotation, consistency, and completeness. The *i*CNH2025A model is encoded in SBML Level 3 Version 1 [190] with extension packages for flux balance constraints and groups [191,192]. The scripts used to reconstruct and validate MEMOTE are publicly available via GitHub (<https://github.com/mascencio27/GSM-iCNH2025A/tree/main>).

Furthermore, the RehMBEL1391 and *i*CN1361 models represent the metabolic capabilities of *C. necator* to produce only PHB from sugars. Our model *i*CNH2025A contains two pathways associated with PHA synthesis. (i) The pathway biosynthesis of PHB using sugars via the glycolytic pathway by three key enzymes: *Ketothiolase*, *Acetoacetyl-CoA reductase*, and *PHB synthetase* (Figure 4.4a), and (ii) the pathway biosynthesis of copolymer PHBV from glucose and propionic acid, which is catalyzed by two key enzymes: the 3-hydroxybutyl-CoA (3HBCoA) and the 3-hydroxyvaleryl-CoA (3HVCoA) (Figure 4.4b).

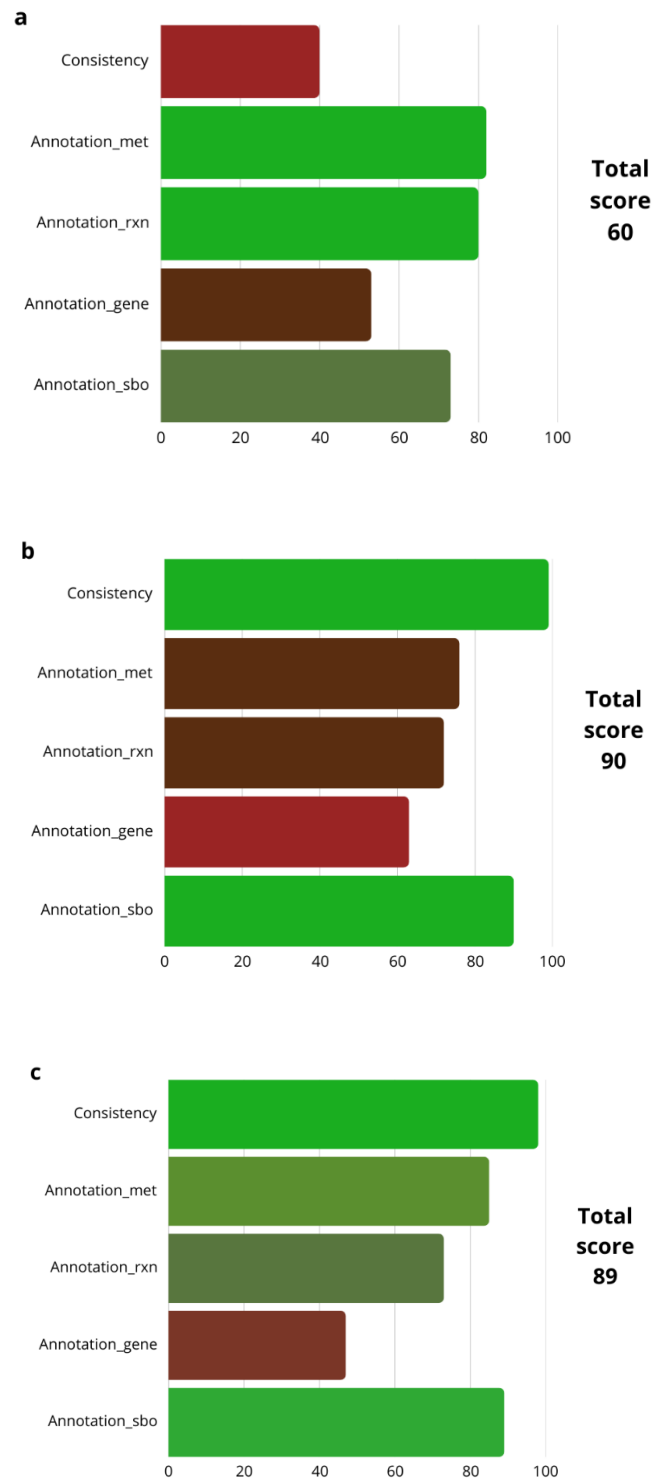


Figure 4.3 MEMOTE reports for different GEMs of *C. necator* a) GEM RehMBEL1391-2011 b) GEM *i*CN1361 – 2022 c) GEM *i*CNH2025A – 2025 (this study).

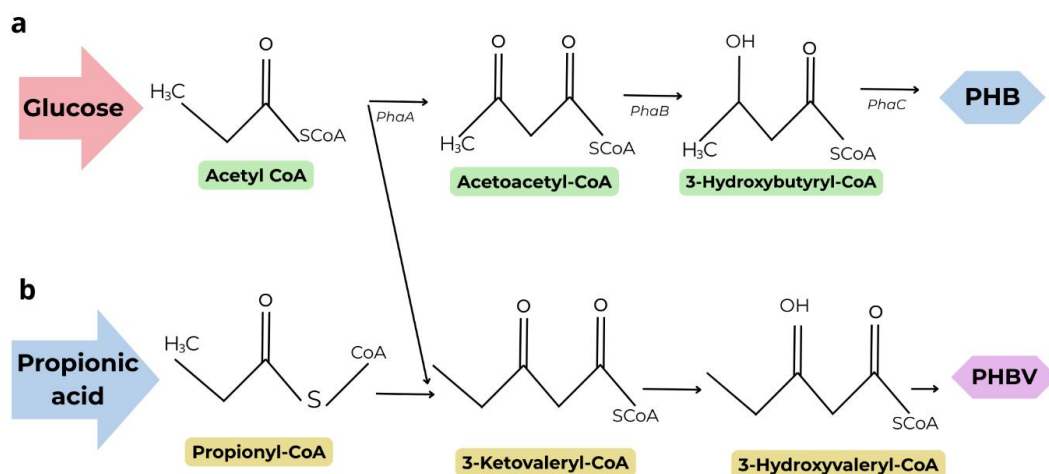


Figure 4.4 Pathway biosynthesis of a) polymer PHB and b) copolymer PHBV.

4.3.3 Thermodynamic properties

Identification of thermodynamic cycles and curation.

TICs (See Supplementary material S6 <https://github.com/mascencio27/GSM-iCNH2025A/tree/main>) were identified. The prominent TICs were associated with subsystems such as transport, citric acid cycle, fatty acid metabolism, membrane lipid metabolism, and purine metabolism. Also, 997 unbounded reactions in the metabolic network were identified. One of the TICs identified in *iCNH2025A* was within the citric acid cycle, catalyzed by isocitrate dehydrogenase; these reactions are essential for carbohydrate metabolism, participating in the citric acid cycle that catalyzes the oxidative decarboxylation of isocitrate to form 2-oxoglutarate. Also, these reactions are identical except that one is dependent on NAD^+ (R_ICDHx) and the other on NADP^+ (R_ICDHyr) (Fig 4.5a). Pereira and colleagues [183] recommend using $\text{NADPH}/\text{NADP}^+$ in anabolic reactions and NADH/NAD^+ in catabolic reactions to correct TICs formed by linearly dependent reactions. Therefore, the model was modified by retaining the R_ICDHyr and eliminating the NAD^+ -dependent reaction (R_ICDHx). Other TICs identified were within fatty acid metabolism, catalyzed by the enzyme 3-cis-2-trans-enoyl-CoA, involved in the positional and geometric isomerization process of unsaturated fatty acyl-CoA intermediates (Fig. 4.5b). In this case, $\Delta_r G'^\circ$ for R_ECOAH7 ($\Delta_r G'^\circ = -0.4 \text{ kJ/mol}$) and R_HACD7 ($\Delta_r G'^\circ = -15.6 \text{ kJ/mol}$) were calculated. These values indicate that the reactions are irreversible in the forward direction. Therefore, directionality assignments were corrected. Thus, 93.14% of the TICs and 23.6% of the non-limited reactions were cured.

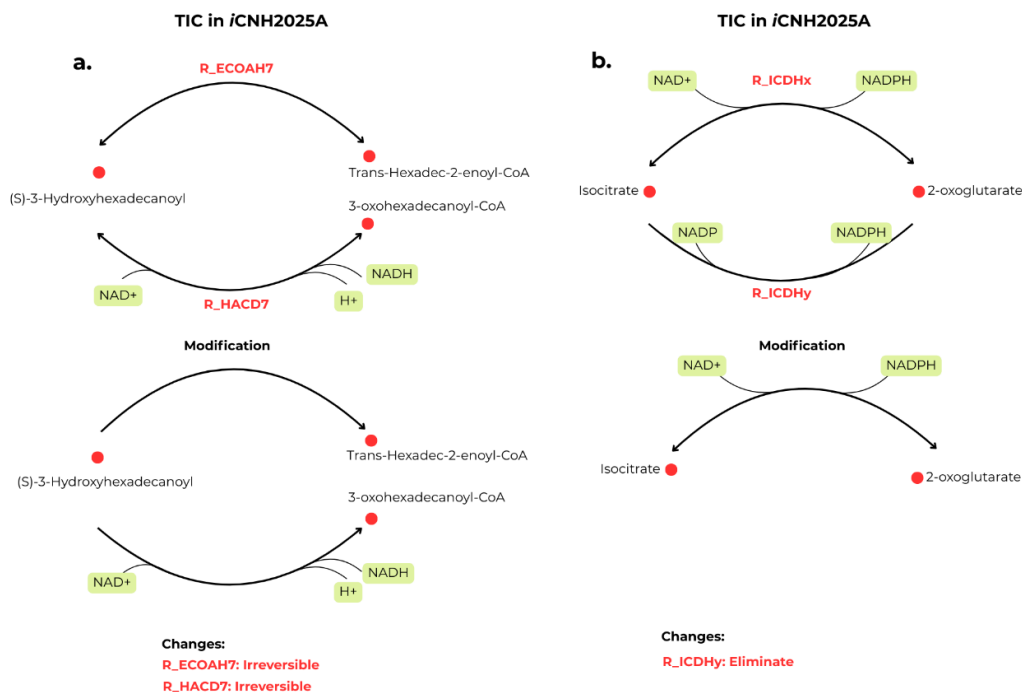


Figure 4.5 TICs found in iCNH2025A with proposed modifications; (a) TIC at citric acid cycle, catalyzed by isocitrate dehydrogenase; (b) TIC at fatty acid metabolism, catalyzed by the enzyme 3-cis-2-trans-enoyl-CoA.

4.3.4 Comparison of metabolic patterns of the iCN1361 and iCNH2025A models

The GEM of iCNH2025A was used to evaluate the metabolic capabilities of *C. necator* to grow on dextrose from bitter cassava starch as a sole carbon source. A quantitative evaluation of the initial model was carried out to measure the ability of the model to predict growth rates and intracellular fluxes of the bacterium growing on fructose. The experimental data published by Park et al. [10] was used in the first stage. For this simulation, the fructose uptake rate was fixed according to experimentally determined values. Uptake rates of the main inorganic nutrients in the minimal medium were constrained to predefined bounds representing minimal medium conditions, while trace elements were supplied in non-limiting amounts by assigning large lower bounds to their respective exchange reactions.

Here, fluxes from all three models were compared to determine whether the optimal FBA solution included the correct metabolic pathways. A comparison was made with the metabolic flux results obtained by Percy et al. [11] and Alagesan et al. [193] using fructose as a carbon source. In this case, the authors quantify the intracellular fluxes of central carbon metabolism by implementing ¹³C metabolic flux analysis (¹³C-MFA). For this purpose, 33 reactions of the ¹³C-MFA model were aligned with our model corresponding to the pathways ED, PPP, EMP, CBB, and citric acid cycle. A parsimonious FBA (pFBA) was applied to maximize the growth rate by minimizing the total sum of fluxes. It is important to mention that a good correlation between flux and directionalities was found for 27 of the 33 reactions. It can be highlighted that

our model shows a similar flux distribution to that of the ^{13}C MFA model, with a high correlation between the predictions of both models ($R^2 = 0.76$, calculated from the Pearson correlation coefficient). Likewise, when comparing the flux distribution published by Pearcy et al. [11] with our model, the correlation between the predictions of both models was $R^2 = 0.78$.

Furthermore, the fluxes predicted by the *i*CNH2025A model were compared with the experimental fluxes of *C. necator* in chemostat mode reported by Park et al. [10] for fructose as a carbon source. The simulations were carried out using a two-step optimization approach. The experimental growth rate was compared with the value obtained from the model. The exchange flux of fructose (v_{ex}) was constrained according to the experimental data. The other medium components and oxygen limits were set to allow free import. The values for the experimental (μ_{exp}) and *in silico* (μ_{ins}) growth rates are shown in Table 4.2. The complete results of the simulations can be found in the supplementary material S7 (<https://github.com/mascencio27/GSM-iCNH2025A/tree/main>).

Table 4.2 Summary of experimental constraints and *in silico* growth rate on fructose, using the *i*CNH2025A model.

Substrate	Experimental constraints		Results
	v_{ex}	μ_{exp}	μ_{ins}
Fructose	-2.6	0.25 ± 0.08	0.22

v in [mmol/(g dry cell weight (DCW)*h)], and μ in [1/h].

As shown in Figure 4.6, the intracellular fluxes were quantified, using *i*CNH2025A to simulate growth on fructose. Notably, both glycolytic pathways, EMP and ED, are activated; however, the carbon flux directed through the EMP pathway to produce pyruvate is limited because the key enzyme phosphofructokinase, essential for fructose synthesis, is not encoded in the genome of *C. necator* [194,195]. Nevertheless, Figure 4.6 shows that the rest of the EMP pathway is indeed activated. The fructose uptake by the cell is converted into glucose-6-phosphate, entering the ED pathway. The results show the formation rates of acetyl-CoA and pyruvate, which are required for PHB synthesis. These simulation results align with those published by [11]. A correlation of $R^2 = 0.78$ (calculated from Pearson's correlation coefficient) between the two flux distributions highlights that our model predicts similar metabolic patterns to those reported by other models and is consistent with *in vivo* data.

(S2), representing exponential growth in the fed-batch phase of Strategy 1 (described in the chapter 3) (36–48 h); and Scenario 3 (S3), referring to exponential growth during the fed-batch phase of Strategy 3 (described in the chapter 3) (84–96 h). The lower and upper bounds of the exchange fluxes for the components of the defined medium are constrained between -1 and 1, respectively. The uptake of nutrients, specifically glucose, was established as an equality constraint based on the experimentally obtained flux, representing the network’s main exchange fluxes.

The experimental growth rate was compared with that obtained using the model. Based on the experimental substrate consumption data, glucose exchange fluxes ($v_{ex,Gluc}$) were calculated by normalizing the glucose consumption rate by the corresponding dry cell weight and cultivation time, yielding specific uptake rates expressed in $\text{mmol} \cdot \text{gDCW}^{-1} \cdot \text{h}^{-1}$. These values were implemented as constraints on the glucose exchange reaction, following the standard FBA convention in which negative flux values represent substrate uptake. Similarly, the limits for other medium components and oxygen allowed free import. The values of the experimental growth rates (μ_{exp}) and simulation results for specific growth rate (μ_{ins}) and PHB flux values are summarized in Table 4.3. The complete results of FBA simulations are available in supplementary material S8 (<https://github.com/mascencio27/GSM-iCNH2025A/tree/main>).

According to the data presented in Table 4.3, the performance of the model varied across the evaluated scenarios, with statistical errors ranging from 5.1% to 66%, calculated as the relative percentage difference between experimental and simulated specific growth rates. The lowest error (5.1%) was obtained in scenario S3, showing a close agreement between model predictions and experimental results, whereas higher deviations were observed in batch and early fed-batch conditions. Overall, the results indicate that the model is able to capture the main metabolic trends of *C. necator*, including growth behavior, PHB synthesis, and intracellular flux distributions under the tested experimental conditions. The carbon flux distributions for the simulated metabolic scenarios under the constraints described in Table 4.3 are summarized in Figure 4.7.

Table 4.3 Summary of experimental constraints and *in silico* growth rate using *iCNH2025A* in batch and fed-batch cultures of *C. necator*.

Scenario	Operation mode	Experimental constraints	Results	Statistical error
----------	----------------	--------------------------	---------	-------------------

		v_{ex}	μ_{exp}	μ_{ms}	v_{ex} PHB	%
S1	Batch	-0.20	0.018	0.012	0.006	44.0
S2	Fed batch culture strategy 1	-0.079	0.0060	0.0036	0.002	66.7
S3	Fed batch culture strategy 3	-0.12	0.0075	0.0079	0.095	5.1

v in [mmol/(g dry cell weight (DCW)*h)], and μ in [1/h].

Figure 4.7 reveals that the carbon flux is directed toward glycolysis to produce pyruvate via the ED pathway under PHB production conditions. This pathway metabolizes sugars into pyruvate and glyceraldehyde-3-phosphate (GAP) and is encoded by two enzymes: 6-phosphogluconate dehydratase and 2-keto-3-deoxy-6-phosphogluconate aldolase, enabling the formation of NADPH, which is essential for PHB synthesis.

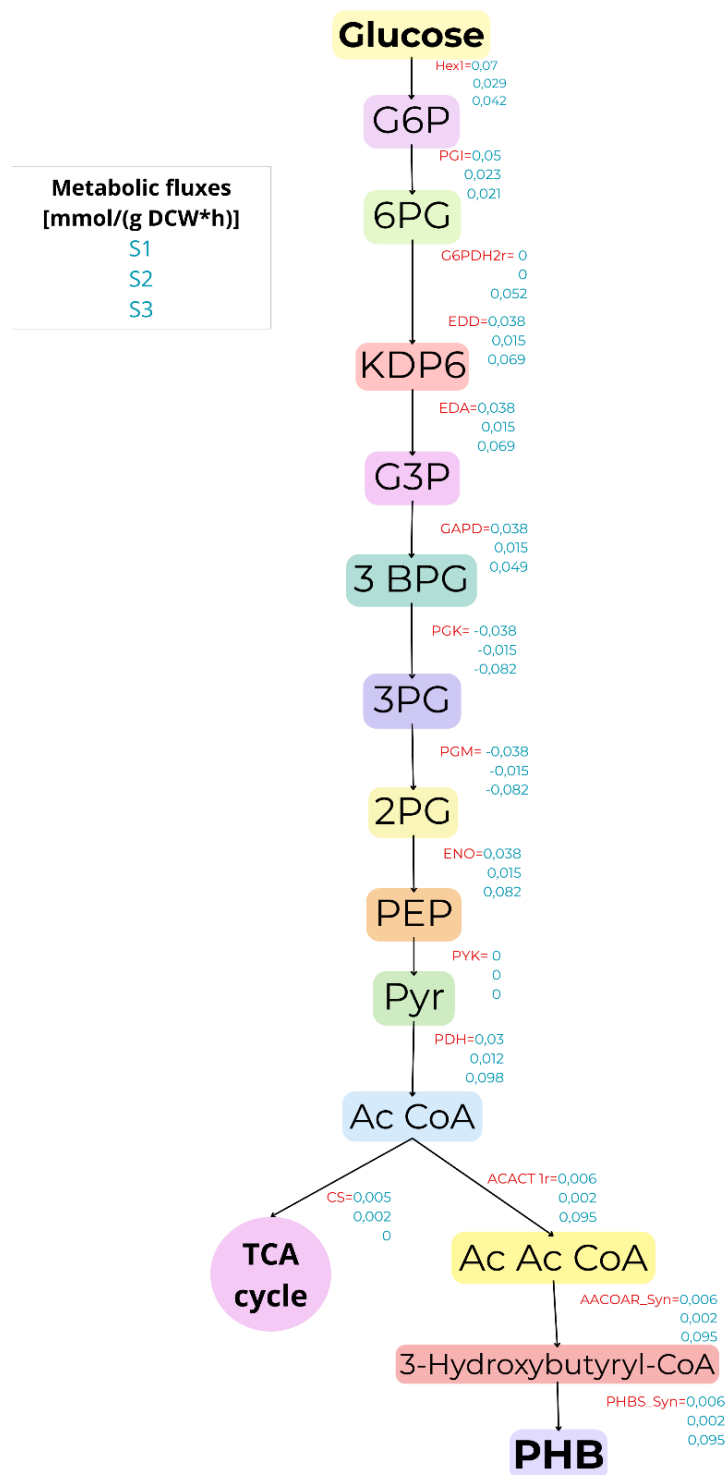


Figure 4.7 Summary of *in silico* carbon flux distribution along the central metabolism predicted by *i*CNH2025A for growth on dextrose derived from bitter cassava starch and PHB biosynthesis for *C. necator* for the metabolic scenarios (S1-S3).

Comparing the fed-batch scenarios S2 and S3, the highest reaction flux toward the PHB pathway (0.095 mmol/(g DCW*h)) was observed in S3, coinciding with the maximum experimental PHB production recorded (Figure 4.7). However, this scenario macroscopically indicated a low growth rate but a value very close to the experimentally obtained result, with an error of 5.1%. This aligns with the experimental results, demonstrating that our model can effectively represent these metabolic scenarios.

The GEM of *C. necator* published by Park et al. [10] and Percy et al. [11] have been utilized to simulate bacterial growth with fructose. The metabolic model published by Lopar et al. [196] for *C. necator* DSM 545 simulated PHB production from glucose, revealing a high degree of similarity between experimental and simulated results. Moreover, it emphasized that the biomass flux was up 102% higher than the PHB flux. This contrasts with the behavior observed in simulations using our model, where the biomass flux was 180% lower than the PHB flux.

As highlighted in the experimental results, these findings suggest that nitrogen source depletion likely occurred in the cultures, promoting a higher carbon source (dextrose from bitter cassava starch) conversion into PHB. Our model accurately represented this metabolic behavior.

PhPP Analysis

In this analysis, ammonium and glucose uptake rates (see Figure 4.8a) and ammonium uptake and PHB production rates (see Figure 4.8b) were chosen as the independent variables, with each of them varying from 0–3 mmol/(g DCW*h). These figures show how cell growth rate is affected by different compound uptake rates, where the different colored regions represent different growth behaviors. Figure 4.8a shows a pyramid-shaped response surface, where a maximization of cell growth is observed when both sources (glucose and ammonium) are available at appropriate concentrations.

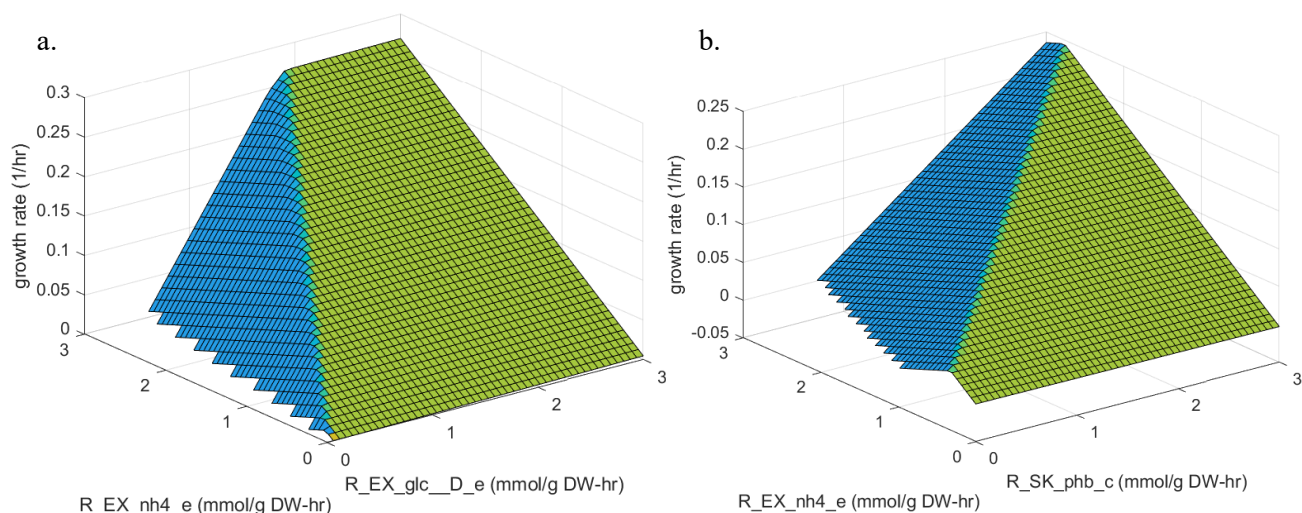


Figure 4.8 Phenotype phase plane analysis of the growth rate of *iCNH2025A*. All fluxes have the unit of mmol/(g DCW*h).

According to the results, the growth rate shows a progressive increase, reaching a rate of 0.32 h^{-1} as glucose (X-axis) and ammonium (Y-axis) uptake increases under conditions of high nutrient availability. However, when one of the two substrates is at low levels (near the axes), the growth rate becomes limited. This indicates that both carbon and nitrogen are essential and co-limiting for biomass production. This behavior is consistent with the central metabolism of *C. necator*, in which nitrogen assimilation is crucial for the biosynthesis of amino acids and other nitrogen-containing molecules, while glucose serves as the main source of energy and carbon skeletons [197]. The flat region at the top of the pyramid represents a saturation zone, where growth does not significantly increase despite higher nutrient levels. This may be related to internal metabolic limitations or enzymatic constraints.

Figure 4.8b evaluates the relationship between ammonium uptake rate and PHB synthesis on the growth rate of *C. necator*. A pyramid-shaped surface is also observed in this case, where the growth rate reaches approximately 0.27 h^{-1} under conditions of high ammonium uptake and high flux toward PHB. However, as ammonium flux decreases, a significant reduction in the growth rate becomes evident, even in the presence of a high flux toward PHB. Furthermore, at very low ammonium uptake rates ($\leq 0.5 \text{ mmol/(g DCW*h)}$), the predicted growth rate approaches zero, indicating an unsustainable condition for sustained bacterial growth under these constraints. Nevertheless, such conditions favors the redirection of cellular metabolism toward PHB synthesis. This phenotypic phase plane highlights how nitrogen availability directly influences the balance between cell growth and PHB accumulation. This behavior is consistent with previous studies, which have shown that *C. necator* accumulates PHB as a metabolic response to nitrogen-limiting and carbon-excess conditions. [198]. The region of the plane with positive PHB flux and low growth represents a metabolically favorable zone for PHB production, as the carbon not used for growth is redirected toward this anabolic pathway.

These results are important for optimizing PHB production bioprocesses in bioreactors and can also serve as a guide for designing engineered strains that maximize polymer accumulation while reducing the growth rate.

4.3.6 Identification of potential metabolic targets susceptible to genetic modification.

Based on the methodology described in the Materials and Methods section, the OptKnock algorithm (the script used can be found on our GitHub repository: <https://github.com/mascencio27/GSM-iCNH2025A/tree/main>) was applied to our *iCNH2025A* model to identify potential metabolic targets susceptible to genetic modification. The simulated conditions were those defined for scenario 3 (S3) as described in Table 4.3, using dextrose derived from bitter cassava starch as the carbon source (glucose). As a result, OptKnock identified two knockouts composed of objective reactions associated with genes whose deletion would favor the accumulation of key precursors for PHB biosynthesis:

- knockout 1: deletion of the genes *galU* (R_GALUi) and *pgi* (R_PGI).
- knockout 2: deletion of the genes *H16_B2567* (R_EDD), *pgi* (R_PGI), and *H16_B2555* (R_PGM).

These identified metabolic targets are part of competing pathways in central metabolism, whose deletion can block conventional glycolysis and pathways associated with polysaccharide synthesis, thereby redirecting the carbon flux toward the generation of acetyl-CoA and NADPH production, both of which are essential for PHB biosynthesis.

OptKnock predicted PHB fluxes 62.4 and 1.2% higher for the two identified gene combinations, respectively, compared to the base PHB flux without deletions (see Table 4.4 and Figures 4.9 and 4.10). Cell growth remained viable with specific growth rates of 0.0024 and 0.0079 h⁻¹, respectively.

Table 4.4 Effect of gene deletion on PHB flux and its relative increase

Gene Deletion Strategy	PHB Flux (mmol/(g DCW*h))	Relative PHB Flux Increase (%)
Without deletion	0.0952	--
<i>galU</i> + <i>pgi</i>	0.1544	+62.4%
<i>H16_B2567</i> + <i>pgi</i> + <i>H16_B2555</i>	0.0958	+1.2%

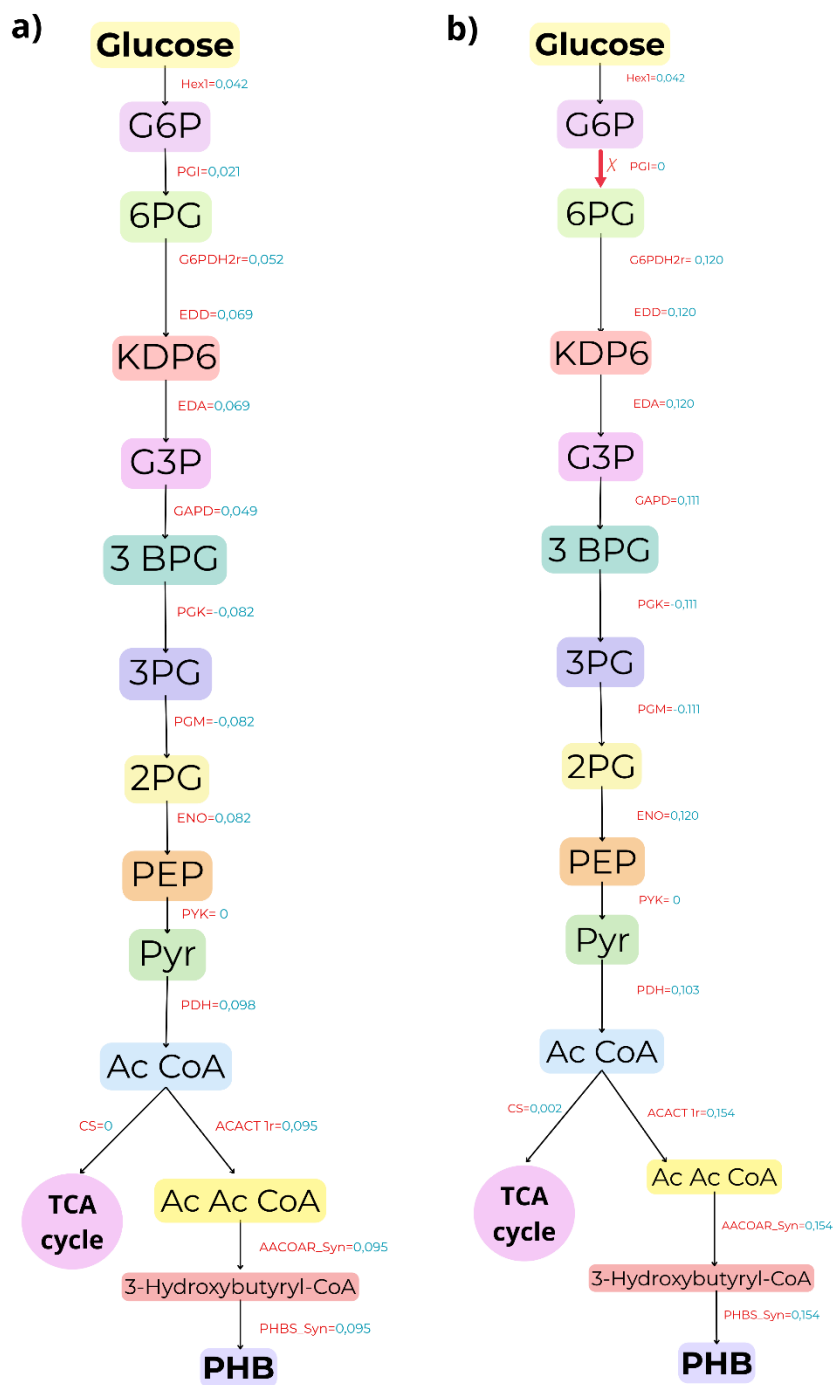


Figure 4.9 Comparison of the *in silico* carbon flux distribution along the central metabolism for growth on dextrose derived from bitter cassava starch and PHB biosynthesis in *C. necator*: a) *i*CNH2025A b) knockout 1 suggested by OptKnock.

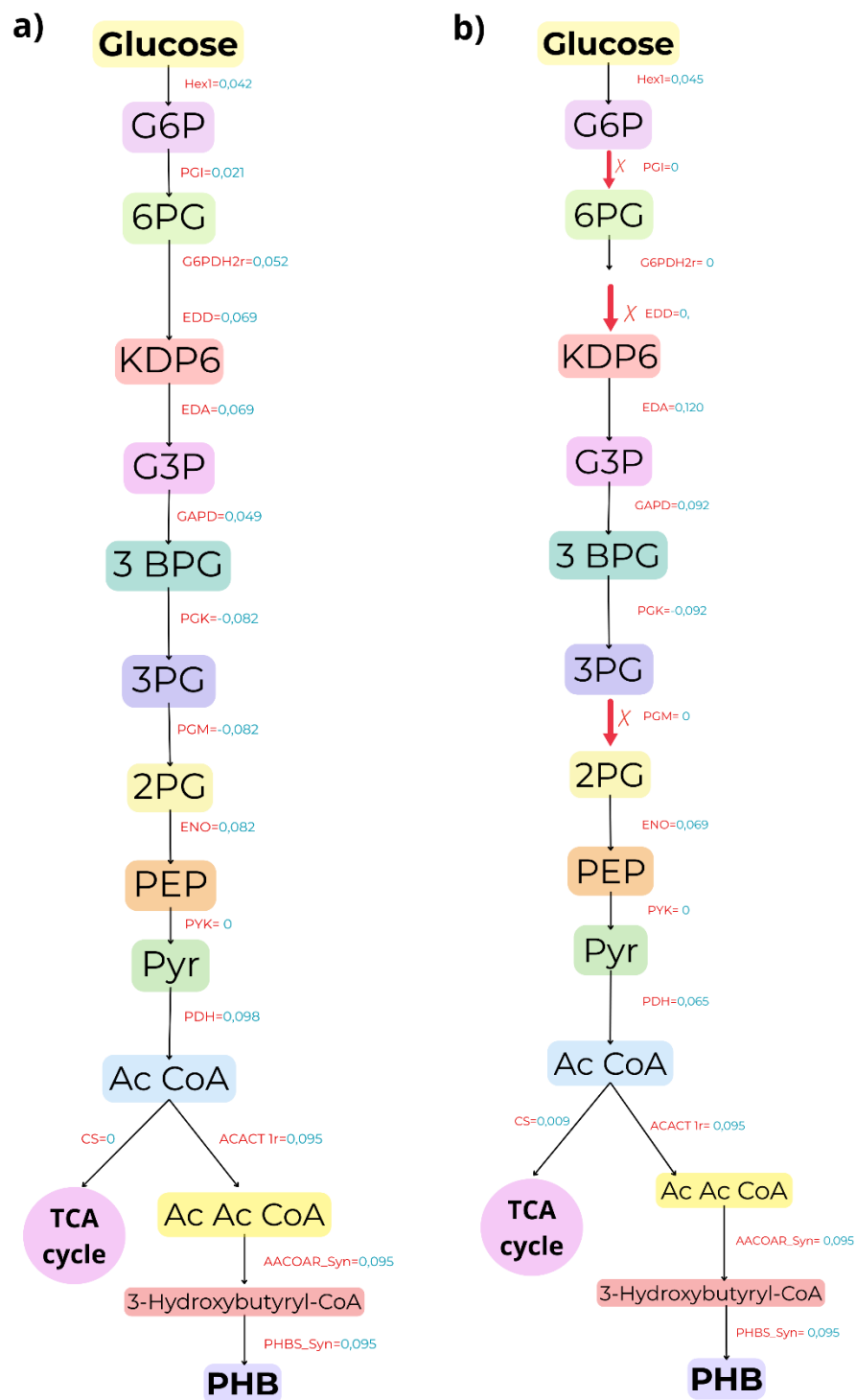


Figure 4.10 Comparison of the *in silico* carbon flux distribution along the central metabolism for growth on dextrose derived from bitter cassava starch and PHB biosynthesis in *C. necator*: a) iCNH2025A b) knockout 2 suggested by OptKnock.

These results suggest that the knockout proposed by the algorithm does represent significant improvements in the biosynthetic capacity of PHB, while maintaining viable growth conditions, highlighting the potential of metabolic engineering to optimize the production of this type of biopolymer.

With the joint deletion of the *galU* and *pgi* genes, which encode UTP-glucose-1-phosphate uridylyltransferase and phosphoglucose isomerase, respectively, a significant improvement in PHB synthesis is observed. This may be related to the disruption of competitive pathways by key intermediate metabolites such as glucose-1-phosphate and fructose-6-phosphate. The knockout of *pgi* would force the flux of glucose-6-phosphate toward the pentose phosphate pathway, increasing the availability of NADPH, an essential cofactor in the reduction step of acetoacetyl-CoA to 3-hydroxybutyryl-CoA in PHB biosynthesis. This approach has been used to increase PHB production in recombinant *Escherichia coli* strains [199,200]. While the knockout of *galU* is likely to prevent the consumption of glucose-1-phosphate in non-essential anabolic pathways associated with UDP-glucose and exopolysaccharide synthesis [201], releasing carbon that can be redirected toward the generation of PHB precursors such as acetyl-CoA, as shown in Figure 4.9. On the other hand, the knockout of the genes *H16_B2567*, *pgi*, and *H16_B2555*, which encode 6-phosphogluconate dehydratase, phosphoglucose isomerase, and phosphoglucomutase, respectively, involved in the pentose phosphate pathway and in the interconversion of sugars, shows a marginal increase (1.2%). In this case, metabolic disruption is likely affecting the energy balance or the ability to regenerate intermediates, limiting the net benefit for PHB synthesis.

Both knockouts show an increase in the flux toward PHB synthesis, with minimal effect on growth, making them viable metabolic targets for future genetic interventions aimed at increasing bioplastic production in *C. necator*.

4.4 Conclusions

The *i*CNH2025A model represents a significant advancement in the metabolic reconstruction of *C. necator*, offering a highly curated and refined GEM that outperforms previous models in annotation quality, consistency, and predictive accuracy. Through extensive validation, including MEMOTE scoring, FBA, and correlation with experimental data, *i*CNH2025A has demonstrated its ability to accurately simulate metabolic fluxes and growth rates under various conditions.

A key strength of the model lies in its comprehensive inclusion of metabolic pathways, particularly those involved in PHB and PHBV biosynthesis, as well as its refined thermodynamic properties, which improve the reliability of metabolic predictions. Comparative analyses highlight the model's superior annotation and consistency scores compared to earlier GEMs, ensuring its alignment with community standards in metabolic modeling.

Furthermore, the model effectively predicts metabolic responses under different carbon sources, such as fructose and dextrose, and aligns closely with experimental observations. Notably, *iCNH2025A* successfully captures the redirection of carbon fluxes toward PHB synthesis, particularly under nitrogen-limited conditions, demonstrating its robustness in simulating industrially relevant fermentation processes.

Similarly, the identified metabolic targets susceptible to genetic modification showed an increase in PHB flux of over 60%, indicating that identifying and modifying key metabolic pathways is a viable alternative for optimizing PHB production in *C. necator*.

Overall, *iCNH2025A* serves as a powerful tool for metabolic engineering and synthetic biology applications, paving the way for optimized biotechnological strategies in *C. necator* for sustainable biopolymer production.

Publication: This chapter was submitted as Martha L. Ascencio-Galván, Víctor A. López-Agudelo, Andreas Dräger, Julio C. Caicedo, David Gómez-Ríos, Rodrigo Andler, and Howard Ramírez-Malule. Integrating fed-batch pulse feeding and flux balance analysis for efficient PHB production by *Cupriavidus necator* from cassava-based dextrose.

Chapter 5 Conclusions and future research

Over the past decade, increasing scientific interest has focused on the use of low-cost and renewable feedstocks for the sustainable production of PHA. In this context, the present thesis evaluated the potential of dextrose derived from bitter cassava starch as an alternative carbon source for PHB production by *C. necator*, integrating experimental cultivation strategies with genome-scale metabolic modeling to analyze and optimize PHB biosynthesis.

From an experimental perspective, this study clearly demonstrates that the feeding strategy, and particularly the timing of carbon pulse addition, plays a decisive role in PHB accumulation. Batch cultures using dextrose as the sole carbon source achieved a PHB concentration of 2.01 g/L with an accumulation of 47.10%, confirming that this substrate supports both bacterial growth and polymer synthesis under simple cultivation conditions.

In fed-batch cultures employing pulse-feeding strategies, marked differences were observed depending on the moment of carbon addition. Early pulse applications favored biomass formation and extended the exponential growth phase but resulted in lower PHB accumulation. In contrast, carbon supplementation during the advanced stationary phase led to reduced growth rates and significantly higher PHB accumulation. These results experimentally demonstrate that late carbon feeding promotes the redirection of metabolic fluxes toward PHB synthesis, outperforming both batch cultures and early pulse-feeding strategies in terms of polymer accumulation.

The enhanced PHB accumulation observed under late feeding conditions is consistent with the hypothesis of nitrogen limitation, a well-known trigger for PHB biosynthesis in *C. necator*. In this work, this interpretation is supported by indirect evidence, including reduced biomass growth, increased PHB content, and agreement with previously reported studies. However, direct measurements of nitrogen depletion or intracellular nitrogen status were not performed, and therefore this mechanism should be regarded as a supported hypothesis rather than a conclusively demonstrated result.

The structural and thermal characterization of PHB produced under batch and fed-batch conditions confirmed its semicrystalline nature with an orthorhombic phase and characteristic functional groups. Thermal analyses revealed melting temperatures, glass transition temperatures, and degradation profiles comparable to values reported in the literature. These findings indicate that the PHB obtained exhibits suitable structural and thermal properties from a potential application standpoint; however, claims regarding processability or industrial performance must be considered preliminary and remain contingent upon further verification through mechanical, rheological, and processing studies, which were beyond the scope of this thesis.

To complement the experimental work, a genome-scale metabolic model of *C. necator*, named *iCNH2025A*, was reconstructed and extensively curated. The model comprises 2,737 reactions, 1,803 metabolites, and 1,059 genes, and represents a substantial improvement over previously published models in terms of annotation quality, mass and charge balance, and metabolic consistency. Validation using MEMOTE and additional tools yielded high scores, confirming

that *i*CNH2025A meets current community standards for genome-scale metabolic reconstructions.

Simulations performed with *i*CNH2025A showed strong agreement with experimental observations, accurately predicting growth rates and intracellular flux distributions under different cultivation conditions and carbon sources. In PHB-producing scenarios, the model successfully captured the redirection of carbon flux toward polymer biosynthesis, providing a mechanistic explanation for the experimental trends observed. Additionally, *in silico* optimization analyses identified potential genetic targets whose deletion could enhance PHB accumulation while maintaining viable growth, offering a rational basis for future metabolic engineering strategies that will require experimental validation.

Limitations and Future Research

This study was conducted under pseudo-steady-state assumptions and did not include direct measurements of key physiological variables such as residual nitrogen concentration, intracellular redox state, or time-resolved flux dynamics, which limits the ability to conclusively confirm some of the proposed metabolic mechanisms. Moreover, polymer characterization was restricted to structural and thermal analyses, without addressing mechanical, rheological, or processing properties relevant to industrial implementation.

Based on the results obtained, future research should prioritize:

1. Direct quantification of nitrogen consumption and limitation, to validate its role in triggering PHB accumulation.
2. Systematic optimization of pulse-feeding strategies, including timing and substrate concentration effects.
3. Integration of omics data into the *i*CNH2025A model, enabling improved resolution of metabolic regulation and flux control.
4. Expanded polymer characterization, incorporating mechanical, rheological, and processing assessments.
5. Pilot-scale evaluation and sustainability analyses, including life-cycle assessment, to assess the feasibility of industrial implementation.

Overall, the findings of this thesis confirm the potential of *C. necator* and bitter cassava starch-derived dextrose as a promising platform for PHB production and establish a solid experimental and computational foundation for the development of more efficient and sustainable biopolymer production processes.

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