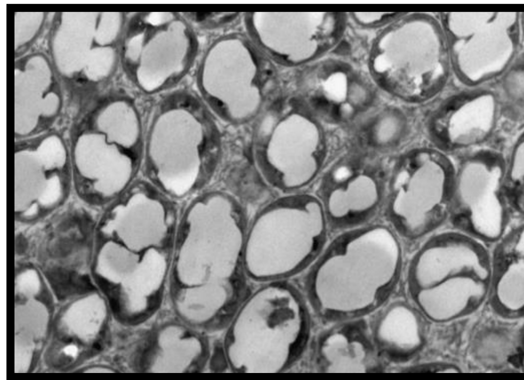
UNIVERSITAT ROVIRA i VIRGILI CATÀLISI HETEROGÈNIA Interfibio

TRANSFORMING PRETREATED SPENT COFFEE GROUNDS INTO POLYHYDROXYALKANOATE BIOPLASTIC THROUGH FERMENTATION PROCESSES

Lídia García Sánchez

BACHELOR'S DEGREE FINAL PROJECT IN BIOTECHNOLOGY



Academic tutor: Magdalena Constantí Garriga, Chemical Engineering Department, magdalena.constanti@urv.cat

In cooperation with: Interfibio and Catheter research groups from URV

Supervisor: Francesc Medina, Chemical Engineering Department, francesc.medina@urv.cat and Vaibhav Vilas Andhalkar, Chemical Engineering Department, vaibhavvilas.andhalkar@fundacio.urv.cat

Data de convocatòria: setembre 2024

Jo, **Lídia García Sánchez**, amb DNI 39951825C, soc coneixedora de la guia de prevenció del plagi a la URV *Prevenció, detecció i tractament del plagi en la docència: guia per a estudiants* (aprovada el juliol 2017) (http://www.urv.cat/ca/vidacampus/serveis/crai/que-us-oferim/formaciocompetencies-nuclears/plagi/) i afirmo que aquest TFG no constitueix cap de les conductes considerades com a plagi per la URV.

Tarragona, 2 de setembre de 2024

ACKNOWLEDGMENTS

First, I would like to sincerely thank to my main supervisor, Ph.D. candidate Vaibhav Andhalkar, who has been an outstanding mentor through my stay. His guidance, supervision, and support have ensured that I gained valuable experience in this field of research. This work is as much his as it is mine.

I also want to express my gratitude to all the members of Catheter and Interfibio research groups, from the students to the lab technicians, for welcoming me and making every day in the laboratory an enjoyable experience during my internship. Ikram, Regan, Dominic, Yürani, Margarita, Susana and Sergi, I am very grateful to you for bringing a smile to my face when I needed it the most.

Special thanks to Magdalena Constantí for her tutoring and assistance with this work, as well as to Francesc Medina for his support with the project.

Finally, I am deeply grateful to my family and friends for their unwavering support. Even though they may not realize it, they have helped me maintain the balance between lab work and everyday life.

INDEX

1. ABOUT CATHETER AND INTERFIBIO **6**

2. ABSTRACT **8**

3. INTRODUCTION **9**

- **3.1** **ENVIRONMENTAL IMPACT OF CONVENTIONAL PLASTICS** 9
- **3.2** **POLYHYDROXYALKANOATES AS AN ALTERNATIVE TO FOSSIL-BASED PLASTICS** 9
- **3.3** **LIGNOCELLULOSIC BIOMASS AS A CARBON SOURCE** 11
- **3.4** **GREEN BIOMASS PRETREATMENT FOR VALORIZATION OF LIGNOCELLULOSIC BIOMASS** 11
- **3.5** ***BURKHOLDERIA SACCHARI* IN PHB BIOSYNTHESIS** 12

4. OBJECTIVE AND HYPOTHESIS **14**

5. MATERIALS AND METHODS **15**

- **5.1** **MATERIALS** 15
  - *5.1.1* *Raw biomass* *15*
  - *5.1.2* *Culture mediums composition* *15*
  - *5.1.3* *Microorganism* *16*
- **5.2** **METHODOLOGY** 16
  - *5.2.1* *Oxalic acid pretreatment of spent coffee grounds* *16*
  - *5.2.2* *High cell density fermentation* *17*
  - *5.2.3* *Analytical methods* *18*

6. RESULTS AND DISCUSSION **24**

- **6.1** **INITIAL COMPOSITION OF SCG AND EFFECT OF OXALIC ACID PRETREATMENT** 24
- **6.2** **OXALIC ACID PRETREATED SCGH HYDROLYSATE COMPOSITION** 26
- **6.3** **PHB PRODUCTION EMPLOYING *BURKHOLDERIA SACCHARI*** 27
  - *6.3.1* *From normal concentration SCGH* *27*
  - *6.3.2* *From diluted concentration SCGH* *28*
- **6.4** **PHB DETERMINATION** 30
  - *6.4.1* *Fourier Transform Infrared Spectometry* *30*
  - *6.4.2* *Gas Chromatography – Mass Spectrometry* *31*
  - *6.4.3* *Gas Chromatography* *32*
  - *6.4.4* *^{1}H Nuclear Magnetic Resonance* *33*
  - *6.4.5* *Transmission Electron Microscopy* *34*

7. CONCLUSION **36**

8. BIBLIOGRAPHY **37**

9. SELF-EVALUATION **39**

10. APPENDIX **40**

1. ABOUT CATHETER AND INTERFIBIO

Catheter and Interfibio are two research groups located in Chemical Engineering Department at Universitat Rovira i Virgili (URV). Both groups have direct access to advanced analytical and characterization equipment at the URV's Scientific and Technical Resources Service (SRCiT). Throughout my internship, I had the privilege of being part of both groups, as their collaboration was essential in efficiently advancing both the chemical engineering and microbiology aspects of the project.

Catheter

The name "Catheter" originates from the combination of "Catalysis" and "Heterogeneous," reflecting the group's core focus on the application of new materials in environmental and industrial fields. The group is led by Dr. Francesc Medina Cabello and is funded through european, national, and regional grants. The research team currently consists of 12 members, not including laboratory technicians, doctoral students, and interns. They work in three fully equipped research laboratories of 200 m^2 and they also have access to the URV's pilot plant, where they can scale and demonstrate the feasibility of the processes they develop.

Catheter group, with a strong foundation in heterogeneous catalysis, include over 15 research lines. During my time with the group, I had the opportunity to take part in the project named "*catalytic processes for the transformation of non-food biomass and the production of liquid fuel and chemical products from biomass*". Other notable areas of study include the synthesis of new catalysts based on metallic nanoparticles, the separation of nitrogen and oxygen from air, the removal of nitrates/nitrites/ammonia from aqueous phases, and the application of supported ionic liquids in fine chemical synthesis. Over the past five years, the group has published more than 60 articles, establishing Catheter as an active and innovative research group.

Interfibio

As the name suggests, Interfibio group merges engineering and biology, focusing on research at the interface between biological systems and materials. Their primary work involves creating intelligent, non-invasive, and low-cost diagnostic systems applicable to clinical, environmental, and food safety fields, leveraging advances in biotechnology, molecular biology, microtechnology, and nanotechnology. A prominent example is the

development of ultrasensitive immunosensors and genosensors, as well as new sensors for pathogen detection in microarray applications.

The group comprises 8 senior members, including my thesis advisor, Magdalena Constantí. The team also includes 4 researchers, 7 doctoral students, and 3 technicians. Interfibio operates in four specialized laboratories: electrochemistry, microbiology (class 2), molecular biology, chemical synthesis, and bioanalytics, each equipped with the necessary technology for their research.

Interfibio's research is funded by international, european, national, regional, and local projects. Among the national projects, funded by the Ministerio de Ciencia, Innovación y Universidades, is the research I participated in during my internship: "Valorization of hydrolysates from lignocellulosic biomass via a combined catalytic and biological approach" (code RTI2018-098310-B-I00, 2019-2021), with a funding of €176,660.

An innovative aspect of Interfibio is its commitment to scientific outreach and activities aimed at fostering curiosity about science among diverse audiences. The group's members actively participate in science education programs, workshops, talks, fairs, and mentorship of research projects for high school students, as well as laboratory internships for university students.

2. ABSTRACT

Lignocellulosic biomass is a sustainable and economically viable feedstock for polyhydroxyalkanoate (PHA) production. However, a harsh pretreatment step is often required to break its recalcitrant structure that leads to formation of inhibitors, which hamper the growth microorganisms and PHA production. To mitigate inhibition, this work thoroughly investigates effectiveness of the oxalic acid pretreatment for improve fermentable sugars (glucose, galactose, and mannose) from spent coffee ground waste (SCG). Subsequently, the hydrolysed obtained from SCG was combined with bacterial fermentation using *Burkholderia sacchari* IPT 101. It was found that the mild oxalic acid pretreatment reduced the formation of inhibitors and produced a total sugar concentration of 23.53 g/L, leading to a PHA production with a polyhydroxybutyrate (PHB) concentration of 1.187 g/L and a PHB content of 55.37%. This research demonstrates that oxalic acid pretreatment is effective in enhancing biomass conversion and significantly increasing PHB production from spent coffee grounds.

Keywords: Polyhydroxyalkanoates, Spent Coffee Grounds, Oxalic Acid Pretreatment, *Burkholderia sacchari*.

3. INTRODUCTION

3.1 Environmental impact of conventional plastics

Traditional plastics have become ubiquitous in our daily lives because of their affordability, resistance, durability, and malleability. These properties make them an almost perfect material for use in multiple industries such as construction, packaging, and healthcare. However, their widespread use entails significant environmental drawbacks.

One of the most problematic issues is that fossil-based plastics are neither biodegradable nor biocompatible. This means they persist in the environment for centuries, as they are not metabolized by living organisms, leading to a global waste crisis. Additionally, the entire lifecycle of conventional plastics is associated with greenhouse gas emissions, which aggravate climate change.

Globaly, around 400 million tonnes of plastic waste are produced per annum, with projections suggesting this could rise to 1,100 million tonnes by 2050 (*Visual Feature | Beat Plastic Pollution, 2022b*). This massive accumulation of plastic waste threatens ecosystems and poses health risks to both humans and wildlife. To address these challenges, there is an urgent need to develop green alternatives like polyhydroxyalkanoates (PHAs) that enhance environmental sustainability and reduce the environmental footprint of fossil-based plastics.

3.2 Polyhydroxyalkanoates as an alternative to fossil-based plastics

Polyhydroxyalkanoates (PHAs) are linear polyesters synthetised and accumulated intercellularly by various prokaryotic microorganisms. These organisms produce PHAs as energy and carbon storage granules under stress conditions such as nutrient limitation (nitrogen, phosphorous, iron, sulfur, magnesium, potassium, or oxygen) combined with an excess of carbon source.

PHAs are considered promising substitutes for petroleum-based plastics due to their similar mechanical properties as synthetic polymers, coupled with their biodegradability and biocompatibility. Moreover, PHAs lifecycle promotes sustainability since they can be synthetized from renewable resources through bacterial fermentation, and their

biodegradation process leaves a carbon zero footprint, releasing harmless by-products like water and carbon dioxide (Zhang et al., 2022).

PHAs are classified based on the number of carbon atoms in their monomer units, which significantly influence their properties and applications. Among them, polyhydroxybutyrate (PHB) is a short chain length-PHA (SCL-PHA) that has been extensively studied and well-characterized. PHB is known for its high crystallinity, thermoplastic properties, and low oxygen permeability, making it comparable to conventional plastics like polypropylene (PP).

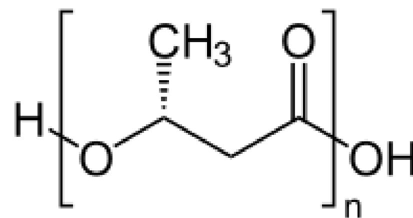


Fig. 1: Poly-(R)-3-hydroxybutyrate structure.

As illustrated in *Figure 2*, SCL-PHA polyhydroxybutyrate (PHB) is synthesized by the condensation of two molecules of acetyl-CoA by 3-ketothiolase PhaA into acetoacetyl-CoA, with a subsequent reduction by NADPH dependent acetoacetyl-CoA reductase PhaB into (R)-3-hydroxybutyryl-CoA (R-3HB-CoA). Finally, the PHA synthase PhaC polymerizes R-3HB-CoA into PHB. (Alvarez-Santullano et al., 2021)

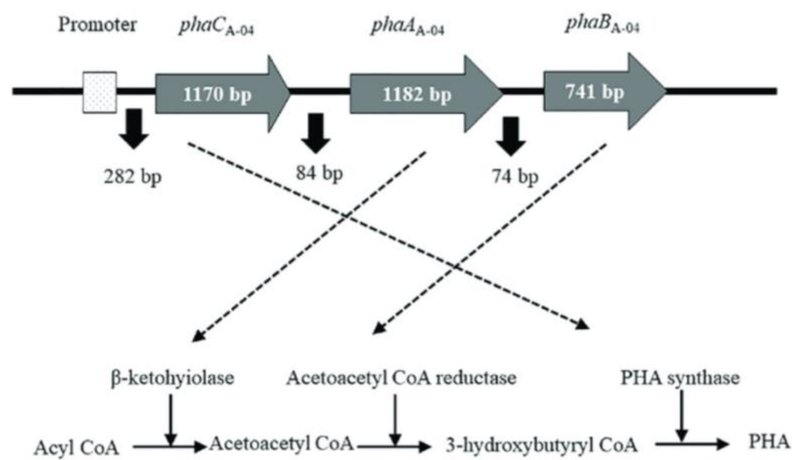


Fig. 2: Genes and enzymes involved in PHB biosynthesis.

3.3 Lignocellulosic biomass as a carbon source

Traditionally, PHA is produced from refined sugars, oils, or fatty acids. However, the cost of the substrate accounts for about 50% of the total production cost, creating an economic barrier for the widespread adoption of bioplastics. Consequently, there is an urgent demand to search for new, cheap, locally available, renewable, and sustainable sources for the industrialization and commercialization of bioplastics if they want to compete against conventional plastics prices (Zhang et al., 2022).

Lignocellulosic biomass waste may be promising feedstock for PHA production (Al-Battashi et al., 2019). Among various lignocellulosic sources, spent coffee grounds (SCGs) have garnered interest due to their abundance and the fact that they are a major by-product of one of the world's most popular beverages. Currently, SCGs are often discarded as waste, contributing to environmental issues. Their limited applications as fertilizers, livestock feed, or compost materials further underscore the need for innovative valorization strategies (Obruca et al., 2014).

The valorization of SCGs in a circular bioeconomy perspective has become highly relevant in the last years, due to their high organic content, composed of (w/w) 30-40% hemicellulose, 9-13% cellulose, 25-33% lignin, 3% polyphenols, 7-14% proteins, and 10-20% lipids (Corrado et al., 2024). Waste stream of coffee industry has been considered as a raw substrate for extraction and transformation into various added-value products such as polyphenols, bioethanol, biodiesel, manooligosaccharides or polyhydroxyalkanoates (Obruca et al., 2014).

3.4 Green biomass pretreatment for valorization of lignocellulosic biomass

Lignocellulosic biomass is a complex molecule surrounded by lignin, cellulose and hemicellulose interconnected in a complex manner providing recalcitrance to the plant cell. The conversion of biomass into oligomers and monomers requires pretreatment processes including physical, chemical, physicochemical, and biological approaches (Panyarachun et al., 2023).

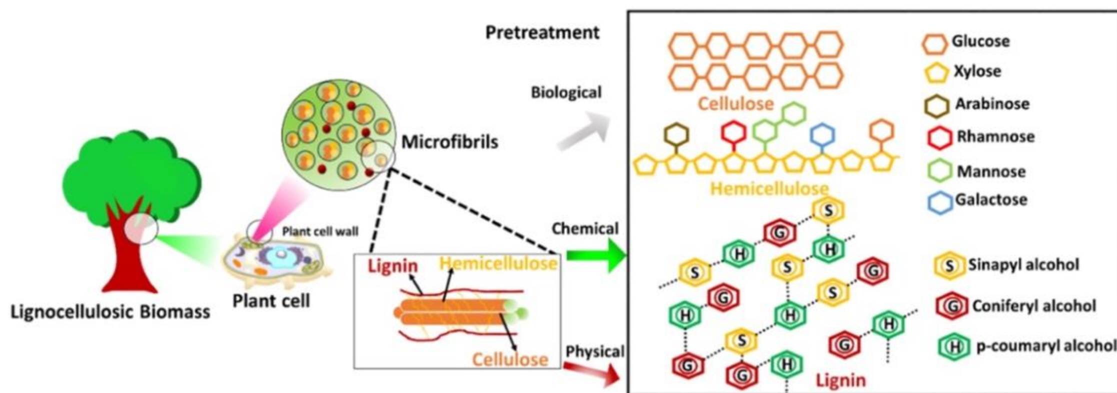


Fig. 3: Schematic representation of lignocellulose and its main components. Adapted from Andhalkar et al. (2024).

Among the various types of pretreatments, chemical, including acid and alkaline pretreatments are the most employed for biomass utilization. These conventional methods normally demand high operational costs, generate toxic by-products for fermentation processes, and causes corrosion of the equipment (Andhalkar et al., 2022). Nevertheless, in the dilute acid pretreatment process, previous studies demonstrated the potential of organic acids as compared to inorganic such as sulfuric acid or hydrochloric acid (Kundu et al., 2015; Panyarachun et al., 2023)

Oxalic acid pretreatment is a soft organic acid that produces low concentration of fermentation inhibitors (generally, acetic acid, furfural, and 5-hydroxymethylfurfural coming from pentose and hexose sugars), reduces cellulose crystallinity, and provides more catalytic efficiency of hydrolysis producing more monosaccharides than oligosaccharides from hemicelluloses and celluloses. Therefore, this pretreatment improves the efficiency of sugar production, which can be fermented by bacteria to produce bioplastics such as polyhydroxybutyrate (PHB).

3.5 *Burkholderia sacchari* in PHA biosynthesis

Burkholderia sacchari is a gram-negative, rod-shaped bacteria belonging to the genus *Burkholderia*. This genus is known for its metabolic versatility and ability to degrade a wide range of organic compounds, making some of its species valuable in biotechnological applications. Strain *IPT101* stands out for its efficient use of sucrose, glucose, xylose, arabinose, fructose, mannose, and galactose to synthesize PHA under nitrogen limitation (Alvarez-Santullano et al., 2021; Oliveira-Filho et al., 2021). Sugars are metabolized via the Entner–Doudoroff (ED), pentose-phosphate (PP), and lower

Embden–Meyerhoff–Parnas (EMP) pathways, which produce reducing power through NAD(P)H synthesis and pyruvate, the PHA precursor that will be transformed to acetyl-CoA through the pyruvate dehydrogenase enzymatic complex, used in PHB biosynthesis pathway described above (Alvarez-Santullano et al., 2021).

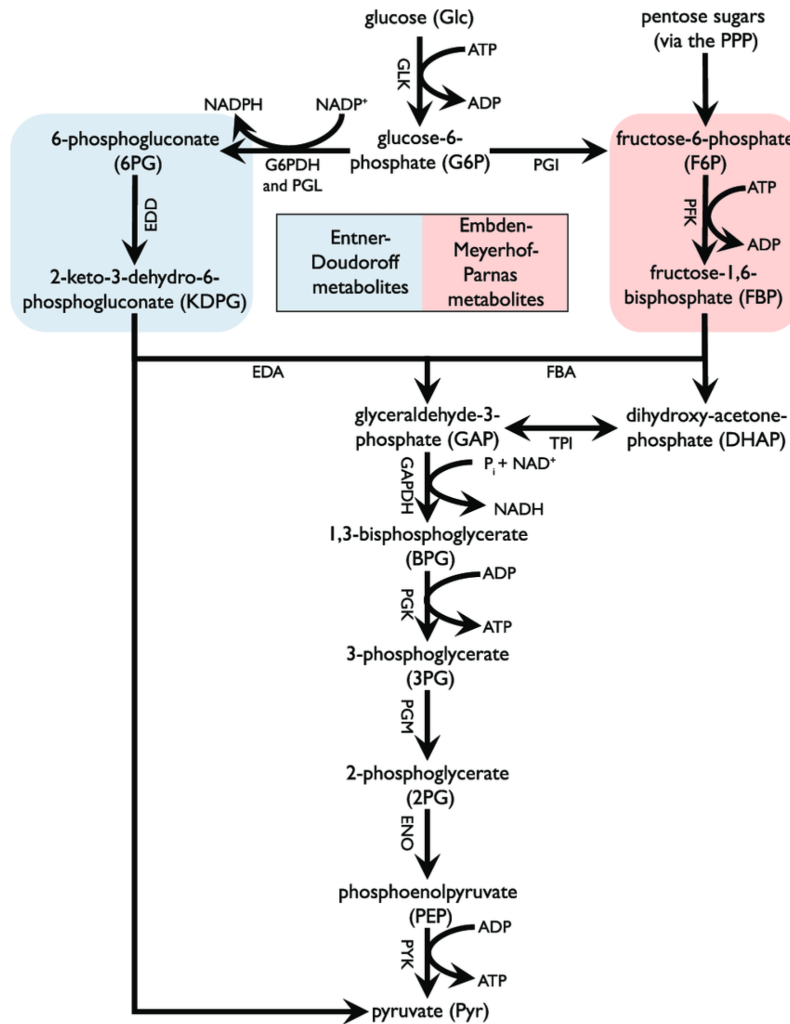


Fig. 4: Schematic diagram of Entner–Doudoroff (ED), pentose-phosphate (PP), and Embden–Meyerhoff–Parnas (EMP) methabolic pathways until pyruvate synthesis.

In this study, the *Burkholderia sacchari* IPT101 strain was selected due to its broad carbon source utilization capabilities, particularly relevant for the hydrolysate derived from spent coffee grounds. Additionally, this strain is considered safe and well-suited for industrial-scale applications, as it is non-pathogenic and demonstrates resilience under diverse environmental conditions.

4. OBJECTIVE AND HYPOTHESIS

The objective of this thesis is to investigate the effectiveness of oxalic acid pretreatment and microbial fermentation in enhancing the conversion of spent coffee ground waste into PHA. This approach aims to develop a sustainable industrial process for bioplastic production by utilizing a widely available biomass waste.

It is hypothesized that oxalic acid pretreatment of spent coffee ground waste will enhance the availability of fermentable substrates, leading to improved microbial fermentation efficiency and higher yields of PHA compared to conventional pretreatment.

5. MATERIALS AND METHODS

5.1 Materials

5.1.1 Raw biomass

In this research, spent coffee grounds (SCG) were used as a representative lignocellulosic carbon substrate. They were obtained from the coffee automat machine at the cafeteria of Universitat Rovira i Virgili, Tarragona, Spain. The waste material was firstly oven-dried at 105ºC to achieve a moisture content below 5%.

5.1.2 Culture mediums composition

5.1.2.1 Reasoner's 2A medium (R2A)

R2A culture medium is used for resuscitation of heterotrophic bacteria present in water and soil that does not need high levels of nutrients. It was chosen by recommendation of DSMZ, because its main purposes are:

- Promoting growth of better adapted bacteria to suboptimal conditions.
- Minimizing contamination and competition in cultures.
- Maintaining bacteria in a controlled growth phase, which can be favourable in large-scale production and management.

Its composition is shown in Table 1.

Compound	Concentration (g/L)
Yeast extract	0.5
Proteose peptone (Difco no. 3)	0.5
Casamino acids	0.5
Glucose	0.5
Starch (soluble)	0.5
Na-pyruvate	0.30
K_2HPO_4	0.30
$MgSO_4 \cdot 7H_2O$	0.05
Agar	15

Table 1: Composition of Reasoner's 2A medium.

Final pH is adjusted at 7.2 with crystalline K_2HPO_4 or KH_2PO_4 before adding agar.

5.1.2.2 Mineral medium

The seeding medium composition employed in this work was adapted from Cesário et al., as it was designed for nitrogen to be the first limiting nutrient (Table 2). The preparation of this medium should be done by autoclaving separately the basic compounds from the rest, and then mixing them by filtration in sterile conditions when needed. Not doing it in this way could drive to precipitation of some reagent, leading to turbidity, which would inhibit bacterial growth.

Compound	**Concentration (g/L)**	**Concentration (mL/L)**
Na_2HPO_4	4.5	
KH_2PO_4	1.5	
Yeast extract	1.0	
$(NH_4)_2SO_4$	1.0	
$MgSO_4 \cdot 7H_2O$	0.2	
Trace element solution (TES)*		1.0

*Table 2: Composition of the adapted mineral medium. The compounds related to the basic medium are shown in green, and those that must be added separately are shown in orange. *The composition of the trace element solution can be found in Appendix A.*

Final pH is adjusted at 6.8 by adding 2M HCl.

5.1.3 Microorganism

In this research, *Burkholderia sacchari IPT 101* strain (DSM 17165) was used. It was obtained from Leibniz Institute DSMZ, Germany. It was used for PHA production with sugars enriched SCG hydrolysate as the sole carbon source and nitrogen as the limiting nutrient to induce stressful conditions.

The bacterial culture was resuscitated by plating the contents stored in the glycerol stocks onto an R2A agar plate at 35ºC in the incubator. The grown plates were stored for one month in the fridge at 4 ºC.

5.2 Methodology

5.2.1 Oxalic acid pretreatment of spent coffee grounds

To hydrolyse the celluloses and hemicelluloses, 3 g of oven-dried SCG were weighted, combined with 0.4 g of oxalic acid, and mixed with 30 mL of deionized water.

The mixture was then autoclaved for 1 h at 121 ºC. The solid residue remaining after hydrolysis was separated from the liquid fraction by vacuum filtration with Filter-LAB® filter papers. The recovered liquid was neutralized with 3M of potassium hydroxide obtaining the final spent coffee grounds hydrolysate (SCGH).

The solid sample was stored for subsequent compositional analysis, while the hydrolysate was kept in a bottle at 4 ºC until further use. Both the initial composition of spent coffee grounds and the compositional changes after pretreating the biomass were determined by using the NREL protocol as described below.

5.2.2 High cell density fermentation

For seed culture, a single colony from an agar plate was aseptically transferred into 100 mL of R2A medium and grown in a shaker incubator at 35 ºC, 150 rpm for 12-15h. After bacterial growth, cells were centrifuged in a centrifuge (Thermo Fisher Scientific) at 10,000 rpm for 10 minutes and 5 ºC. The pellet was then collected and resuspended in 5 mL of basic mineral medium.

The cultivations were performed in 250 mL Erlenmeyer flasks containing 100 mL of culture broth. The fermentation broth was prepared by aseptically reconstituting 100 μL $MgSO_4 \cdot 7H_2O$ 10X, 100 μL TES, and variable volumes of SCGH and autoclaved milli-Q water depending on the experiment, ensuring the pH was adjusted to 7, if necessary, using 2M HCl. The resuspended pellet (5% inoculum) was then added and appropriately mixed to initiate fermentation under the same incubation conditions as the initial growth phase.

For the positive controls, SCGH in the fermentation flask was replaced with the same volume of a synthetic hydrolysate made from commercial sugars and inhibitors dissolved in Mili-Q water. The sugar concentrations for the synthetic hydrolysate were determined based on the HPLC analysis of the SCGH. Meanwhile, negative controls were performed with the same conditions as the positive controls, but without bacterial inoculation. This helped to verify sterility conditions and discard external factors such as the natural decomposition of the substrates.

During the fermentation process, samples were withdrawn to analyse the evolution of the biomass, PHB yields, and the composition of the culture medium as described below.

5.2.3 Analytical methods

5.2.3.1 Quantification of sugars and inhibitors by HPLC

On one hand, sugar analysis performed in this project were carried out on an Agilent 1100 Series HPLC System equipped with an Aminex HPX-87P carbohydrate analysis column (Bio-Rad Laboratories, CA) and a refractive index detector (RID). HPLC milli-Q water was used as the mobile phase at a flow rate of 0.6 mL min^{-1} at a column temperature of 80 ºC. Before using this equipment, each sample had to be neutralized, if necessary, with $BaCO_3$.

On the other hand, acetic acid, furfural and 5-hydroxymetylfurfural (HMF) content was analysed by a HPLC equipped with an Aminex HPX-87H column. The eluent was an aqueous solution of sulfuric acid (5 mM H_2SO_4) and the flow rate was 0.6 mL min^{-1} at a column temperature of 80 ºC.

Sample peaks were identified and quantified by comparing the retention times of the calibration curves plotted previously with analytical standards of glucose, xylose, arabinose, galactose, mannose, acetic acid, furfural, and HMF in both columns.

Every sample analysed in both HPLC were previously filtered through a 0.22 µm filter and transferred into an HPLC vial to avoid unwanted interactions with the columns.

5.2.3.2 Cell dry weight (CDW)

Cellular growth was measured gravimetrically by determining the cell dry weight (CDW) during the fermentation process of the hydrolysate. This method was chosen because the darkness and opacity of the culture broth derived from SCGH hindered the use of UV spectrophotometry for determining cellular growth via OD_{600}. However, this issue did not affect the control cultures, as they were slightly white, making them easily to dilute and allowing their absorbance values to fall within the range from 0-1.

For CDW determination (*Figure 5*), 1 mL of culture broth was centrifuged at 10,000 rpm for 10 minutes at 5 ºC, using pre-dried and pre-weighed 1.5 mL Eppendorfs. The supernatant was removed and stored for substrate consumption analysis via HPLC, while the pellet was dried all night and weighted again to calculate the difference and subsequently the CDW in grams of biomass per litre of culture broth, as shown in equation (1).

$$CDW\ (\%) = \frac{final\ weight\ (g) - initial\ weight\ (g)}{sample\ volume\ (L)} \cdot 100 \qquad \text{Eq. (1)}$$

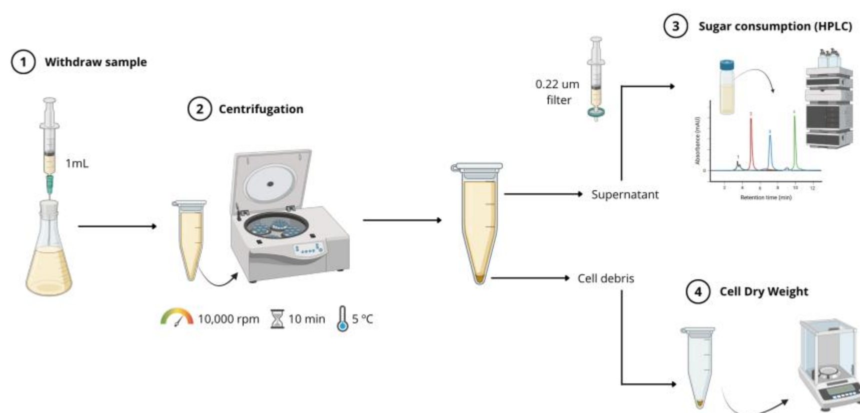


Fig. 5: Schematic diagram of the monitorization of fermentation process.

5.2.3.3 Biomass compositional analysis

The compositional analysis of spent coffee ground waste was performed as shown in National Renewable Energy Laboratory (NREL) protocol described by Sluiter et al. (2008).

Firstly, for oil (extractives) content determination, a Soxhlet extraction was carried out using α-hexane as a polar solvent according to the NREL protocol.

In brief, 0.3 g of oven-dried SCG were treated with 3 mL of 72 % w/w of sulfuric acid in glass test tubes. This mixture was then placed in a water bath at 30 ◦C ± 2 ◦C for 60 minutes, with stirring every 10 minutes. After the 60-minute hydrolysis reaction, the tubes were removed from the water bath. The mixture was then transferred to autoclave bottles, and the sulfuric acid concentration was diluted to 4 % by adding 84 mL of deionized water. The autoclave bottles were placed in an autoclave rack and autoclaved

at 121 ◦C ± 2 ◦C for 60 minutes. Once the autoclave cycle was completed, the mixture was allowed to cool to room temperature. The solid residue and liquid hydrolysates were then separated by vacuum filtration. The solid residue was recovered and used to determine the acid-insoluble lignin using the gravimetric method. Meanwhile, the liquid hydrolysate samples were neutralized with $BaCO_3$ and analysed using HPLC equipped with the Aminex HPX-87H column.

5.2.3.4 PHB quantification by gas chromatography

To determine the PHB content from the bacterial pellets, the biopolymer is extracted into an organic phase in an acidic medium (*Figure 7*), followed by its transformation into the structural monomers that constitute it. These monomers are then converted into methyl esters, which can subsequently be detected by gas chromatography and mass spectrometry (GC/MS). The reaction that takes place is known as methanolysis and was carried out according to Braunegg et al.

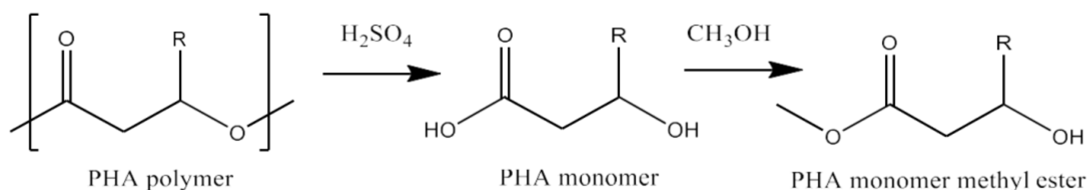


Fig. 6: Schematic representation of the methanolysis procedure.

First, a sample was withdrawn from the culture broth and centrifuged at 10,000 for 10 minutes at 5 ºC. The resulting pellet was washed with deionized water. This process was repeated twice.

Next, 0.01 g of the pellet was weighted and placed in a test tube containing a mixture of 5% w sulfuric acid and 30% w methanol in chloroform (total volume 3 mL), with 0.5 g/L of benzoic acid added as an internal standard. The mixture was incubated in an oil bath at 105 ºC for 180 minutes in an extraction hood. After methanolysis, 1 mL of water was added, and the mixture was vortexed.

Fig. 7: Visualization of the two phases formed after water addition. Aqueous phase is in the upper part, while the organic phase is in the lower part.

The chloroform layer at the bottom, containing the PHB, was transferred into a clean vial with approximately 30 mg of sodium sulfate to remove any remaining water. The sample was filtered through a 0.22 µm nylon filter and transferred to a previously labeled chromatography vial. Finally, PHB was identified using a gas chromatography-mass detector (GC–MS) from URV's SRCiT and quantified using a gas-chromatography-flame ionization detector (GC-FID) from Catheter's laboratories.

Polymer content in GC was determined by constructing a calibration curve (*Appendix B*) with standard methyl(R)-3-hydroxybutyrate (3HB) (Sigma Aldrich, purity-99 %). The GC analysis was performed using an Agilent 6890 equipped with a capillary column, 30 m in lenght x 0.25 mm of internal diameter. GC conditions were as follows: 10 ºC/min ramp from 40 to 80 ºC and temperature increased in 25 ºC steps from 80 to 250 ◦C, and was held at 250 ◦C for 3 min. Nitrogen was the carrier gas with a flow rate of 30 mL/min. Analytes were identified and quantified by GC–MS and GC-FID at a temperature of 275 ◦C with a hydrogen flow of 30 mL/min and an airflow rate of 350 mL/min (Andhalkar et al., 2024).

5.2.3.5 PHB extraction

PHB extraction was performed following the method of Andhalkar et al., (2024), with modifications to ensure complete extraction. In brief, chloroform was used to extract the PHB polymer from the cell pellets. Specifically, 0.5 g of oven-dried cell pellet was mixed with 20 mL of chloroform, and the mixture was incubated at 37 ºC for three days with continuous stirring at 320 rpm. After incubation, the suspension was filtered through a 0.22 µm nylon filter to remove any remaining cell debris. The filtrate was poured into ice-cold methanol to precipitate the biopolyester. Finally, the precipitated PHB was redissolved in chloroform, evaporated using a rotary evaporator, and dried to obtain the purified PHB biopolyester (*Figure 8*).

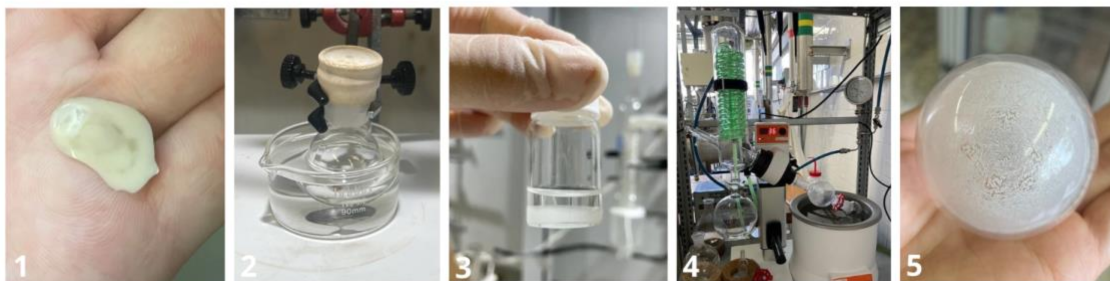


Fig. 8: Sequenced steps of extraction of PHB. 1: oven-dried cell pellet; 2: round flask where the extraction takes place in a bath oil; 3: precipitation of PHB after ice-cold methanol addition; 4: evaporation of chloroform in the rotary evaporator; 5: dried and purified PHB.

5.2.3.6 ATR-FTIR

Attenuated Total internal Reflectance - Fourier Transform Infrared Spectometry (ATR-FTIR) analysis is a useful technique to identify and characterize the presence of different functional groups in the cell pellet. For PHB identification, ATR-FTIR can detect the distinctive band related to the carbonyl group (C=O) in the region of 1720 cm^{-1} – 1740 cm^{-1}.

The samples were measured by averaging 32 scans from 400 to 4000 cm^{-1} at a spectral resolution of 1 cm^{-1} using Jasco FT/IR-6700 from URV's SRCiT.

5.2.3.7 Proton Nuclear Magnetic Resonance (^1H NMR)

^1H NMR provide detailed information about structural information of PHB. The technique detects the hydrogen atoms in the PHB polymer chain to confirm the presence of its characteristic protons. These are, methyl ($-CH_3$), methylene ($-CH_2$), and methine (-CH) groups. ^1H NMR technique, also shows the presence of impurities in the sample and the degree of polymerization, which means how long are the PHB polymer chains by the relative intensities of the signals in the spectrum.

Sample preparation was performed by dissolving 5 mg of PHB extracted and purified sample in 1 mL of $CDCl_3$ solvent to avoid interferences during analysis. The reference standard used was TMS (tetramethylsilane) as it chemical shift is universally set at 0 ppm in ^1H NMR. The technique was conducted using a Bruker Avance Neo 400 MHz ^1H NMR model from URV's SRCiT, and the spectra was processed with MestReNOVAx64 software.

5.2.3.8 Transmission Electron Microscopyy (TEM)

TEM provides high-resolution images of the intracellular structures where PHB granules are stored. It allows for the visualization of the size, distribution, and morphology of PHB granules within the cells. A positive image for the PHB bacterial production will show clearly the cell containing white granules contrasting with the dark background.

Sample preparation for TEM observation requires six previous steps that are fixation, post-fixation, dehydration, embedding, sectioning, and staining. These steps require special equipment and reagents, so they are carried out directly in Microscopy Unit from URV's SRCiT by the technicians. This microscopy technique was performed with a JEOL 1011 TEM model with magnifications ranging between 200x and 1,000,000x.

Fig. 9: TEM equipment used.

6. RESULTS AND DISCUSSION

This section addresses the initial hypothesis: *"oxalic acid pretreatment of spent coffee ground waste will enhance the availability of fermentable substrates, leading to improved microbial fermentation efficiency and higher yields of PHB compared to conventional pretreatment".* The results obtained during the experimentation are discussed hereunder.

Before data interpretation, it is important to note that some issues occurred with both HPLC columns. The Aminex HPX-87P column was not effectively splitting the mannose and arabinose peaks, likely due to an elution problem. Simultaneously, the Aminex HPX-87H column experienced a double peak phenomenon, which suggested a blockage. To resolve this, the column was washed with a solvent in reverse direction. Consequently, the HPLC remained unavailable for an extended period, so the analysis of inhibitors could not be performed.

6.1 Initial composition of SCG and effect of oxalic acid pretreatment

The effect of oxalic acid treatment on the composition of spent coffee grounds (SCG) compared to its initial composition is presented in Table 3. The compositional content by weight (%) of each component was calculated using equations (2) and (3).

$$Content\ (w\ \%) = \left(\frac{[sugar]_{HPLC} \left(\frac{g}{L}\right) * V_{sample}\ (L)}{m_{sample}(g)} \right) \cdot \%\ solid\ recovery \qquad \text{Eq. (2)}$$

$$\%\ solid\ recovery = \frac{m_{after\ pretreatment}}{m_{before\ pretreatment}} \cdot 100 \qquad \text{Eq. (3)}$$

Several important observations regarding the impact of this pretreatment on the residual solid are as follows:

a) The glucan content increased significantly from 7.00% in raw SCG to 12.34% in oxalic acid pretreated SCG. This increase suggests that the pretreatment may have exposed glucans in the solid fraction by liberating other sugars from the hemicellulosic fraction, consistent with the findings of Panyarachun et al. (2023)
b) Mannan content remained relatively constant, with a slight decrease from 16.62% to 16.05%. This indicates that the pretreatment had a limited impact on mannan degradation.

c) Galactan experienced a significant reduction, decreasing from 32.61% in raw SCG to just 1.40% in pretreated SCG. This suggests that oxalic acid was highly effective in degrading galactan, releasing galactose into the liquid medium.
d) Lignin content increased from 26.99% to 31.71% in the solid fraction after treatment. This result aligns with the literature (Kundu et al., 2015), where both glucan and lignin contents increased compared to raw biomass. Additionally, the absence of complete delignification may have prevented the formation of lignin-derived phenolic compounds, which could be toxic during bacterial fermentation at concentrations up to 2 g/L, as observed in other *Burkholderia* species (Cesário et al., 2014).

In summary, oxalic acid treatment has effectively modified the composition of spent coffee grounds, liberating soluble components, particularly galactan, and increasing the relative concentration of glucan and lignin in the remaining solid fraction. This type of pretreatment could be beneficial for releasing fermentable sugars, although the increase in lignin content may pose challenges in subsequent biomass processing stages.

	Content (w%)	
Compound	**Raw SCG**	**Oxalic acid pretreated SCG**
Glucan	7.00 ± 0.17	12.34 ± 0.03
Mannan	16.62 ± 1.92	16.05 ± 0.01
Galactan	32.61 ± 0.26	1.40 ± 0.00
Arabinan	2.12 ± 0.83	N/A
Lignin	26.99 ± 0.49	31.71 ± 0.09
Ash	0.51 ± 0.51	N/A
Extractives (oil)	16.06 ± 1.06	N/A
Solid recovery	100	68

Table 3: Composition of the solid fraction of SCG biomass before and after oxalic acid pretreatment. Results are in form average (duplicates) ± standard deviation. N/A – not available.

6.2 Oxalic acid pretreated SCGH hydrolysate composition

Main sugars identified in SCGH after oxalic acid pretreatment are mannose (13.21 ± 0.52 g/L) and galactose (9.13 ± 0.15 g/L), followed by glucose (1.19 ± 0.68 g/L) as shown in Table 4. These sugars are hexoses, which are likely a key factor positively influencing the PHB production, as *B. sacchari* prefers hexoses over pentoses. This preference is due to the greater production of ATP and NADPH during hexose metabolism, which supports higher PHB yields (Obruca et al., 2014).

		Pretreatment		
		Oxalic acid	**Conventional: Alkaline pretreatment (4.8% NaOH) + Acidic hydrolysis (5% H_2SO_4) + detoxification**	**Acidic hydrolysis (1% H_2SO_4) + cellulose enzymatic digestion + detoxification**
Sugars	**Glucose**	1.19 ± 0.68	0.51	3.9 ± 0.2
	Galactose	9.13 ± 0.15	3.60	17.3 ± 0.3
	Mannose	13.21 ± 0.52	12.79	23.6 ± 0.6
	Total	23.53 ± 1.21	16.90	50.3 ± 2.2
Inhibitors	**Acetic acid**	N/A	0.00	N/A
	HMF	N/A	2.45	0.15 ± 0.03
	Furfural	N/A	0.45	n.d.
Reference		This project	Experiment performed during my internship (data not shown)	Obruca et al. (2014)

Table 4: Chemical composition of SCGH hydrolysate obtained through different pretreatment approaches. N/A – Not available. n.d. – not detected.

When comparing the total concentration of sugars obtained in the hydrolysate using oxalic acid as a catalyst to a hydrolysate produced through acidic hydrolysis followed by enzymatic digestion of cellulose as reported by Obruca et al. (2014), it is observed that the oxalic acid method yields 23.53 g/L of total sugars, while the other method yields 50.3 g/L. Although the sugar concentration in the first case is 53.2% lower than on the second, the oxalic acid has the advantage of significantly reducing the necessary steps of hydrolysate obtention, which simplifies the process and may provide benefits in terms of efficiency and operational costs. Additionally, although inhibitors concentrations could not be analysed, as reported in literature, they should be lower compared to other types of pretreatments due its mild conditions (Panyarachun et al., 2023).

Compared to both methods, the conventional pretreatment approach is the less effective. It results in high levels of fermentation inhibitors, lower sugar yields, and longer, more expensive, and corrosive process. Therefore, oxalic acid is a better alternative for biomass pretreatment.

6.3 PHB production employing *Burkholderia sacchari*

This section presents the most significant results obtained in this study. The main variables discussed are the percentage of PHB accumulated in *Burkholderia sacchari* during fermentation, as defined in equation (2), and the PHB yield from the substrate (Y_{PS}), calculated using equation (3).

$$PHB\ content\ in\ \%\ w = \frac{[PHB]_{GC}}{CDW} \cdot 100 \qquad \text{Eq. (2)}$$

$$Y_{PS} = \frac{[PHB]_{GC}\ (t)}{[total\ sugars]_{HPLC}\ (t_0) - [total\ sugars]_{HPLC}\ (t)} \qquad \text{Eq. (3)}$$

6.3.1 From normal concentration SCGH

A fermentation experiment was conducted in duplicate (SCGH OX 1 and 2) as described in the methodology section, where 95 mL of SCGH obtained with oxalic acid was added to the fermentation flask. The initial sugar concentrations in these cultures were 0.5 g/L of glucose, 9.12 g/L of galactose, and 11.76 g/L of mannose. After fermenting for 114 hours, the culture media composition was analysed.

While the positive control showed biomass growth and a total sugar consumption of 14.53 g/L, the first experimental flask (SCGH OX 1) exhibited no sugar consumption or biomass growth. In its duplicate (SCGH OX 2), 10.51 g/L of sugars were consumed, but no PHB was detected by either FT-IR analysis or GC.

The conclusions drawn from these results are:

a) The initial pH adjustment plays a crucial role in bacterial growth. The pH meter we had was not well-calibrated, so we had to rely on pH strips, which reduced accuracy. This could explain the phenomenon observed in SCGH OX 1, where the pH at inoculation may have been slightly above 7.

b) For the SCGH OX 2 experiment, there are two possible explanations: First, the volume of mineral medium used to resuspend the cell pellet may have been too large, disrupting the optimal C/N ratio necessary for stress induction and thus PHB production. Second, the bacteria might have degraded previously synthesized PHB granules to obtain endogenous carbon source, extending their growth and survival under both carbon and nitrogen limitation.

c) Although the inhibitor content was not analyzed, it did not seem to affect bacterial growth, as observed in SCGH OX 2. This means, a detoxification step of SCGH obtained after oxalic acid pretreatment is not required but could improve fermentation.

In light of the findings, a new hypothesis was formulated: diluting SCGH by half could stabilize the pH of the culture medium, reduce potential inhibitor concentrations, and enhance nutrient bioavailability.

6.3.2 From diluted concentration SCGH

A new experiment named SCGH OX 2X dil. was designed by replacing the 95 mL of SCGH with 47.5 mL of SCGH and 47.5 mL of Milli-Q water. This experiment was allowed to ferment for 160 hours, resulting in a maximum intracellular PHB accumulation of 55.37%, a CDW of 2.14 g/L, a PHB concentration of 1.187 g/L, and a PHB production yield (Y_{PS}) of 0.17 g/g.

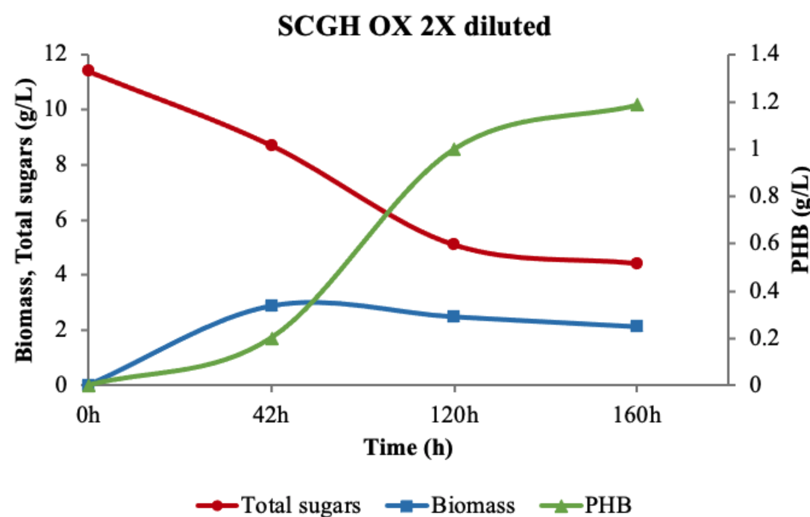


Fig. 10: Time course of biomass, PHA, and total sugar concentrations during cultivation of B. sacchari in SCGH OX 2X dil. experiment.

As observed in *Figure 5*, at 42 h of fermentation *B. sacchari* began synthetizing PHB. At this point, biomass concentration reached its peak, with a maximum CDW of 2.9 g/L. According to *Figure 6*, this point also coincides with the complete depletion of glucose and a significant reduction in mannose levels.

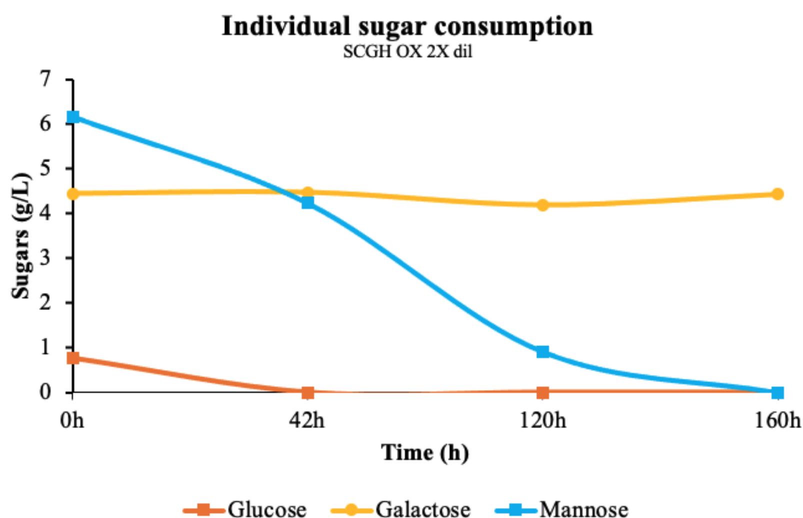


Fig. 11: Individual sugar consumption profile during cultivation of B. sacchari in SCGH OX 2X dil. experiment.

Based on the experimental results, it can be confirmed that the synthesis of polyhydroxybutyrate (PHB) by *Burkholderia sacchari* through the fermentation of spent coffee grounds hydrolysate, obtained after oxalic acid pretreatment, is indeed viable. However, some important aspects should be discussed:

a) The time required to reach the highest PHB production (160 hours) is significantly longer compared to other studies using the same bacterium with different substrates. For instance, Lopes et al. (2009) reported yields ranging from 0.22 to 0.29 g PHB/g substrate within a maximum of 48 hours using glucose, xylose, and arabinose, as well as mixtures of these sugars, for fermentation. In contrast, in this study, the sugars used as substrates are not pure but are part of a mixture containing unknown potential inhibitors that may have slowed bacterial growth and thus reduced PHB production. Other possible explanations for the metabolic slowdown could be related to the quality and quantity of the inoculum, pH (as previously discussed), the lack of essential nutrients in the mineral medium, or the bioavailability of the substrate.

b) Regarding substrate bioavailability, during the fermentation process, both glucose and mannose were gradually consumed, while galactose remained unutilized over the 160-h period (*Figure 6*). This phenomenon can be explained by carbon catabolite repression (CCR), a regulatory mechanism where the metabolism of certain sugars is inhibited in the presence of a preferred carbon source.

Glucose and mannose can be metabolized through the Embden-Meyerhof-Parnas (EMP) or Entner-Doudoroff (ED) pathways after an initial isomerization step. In contrast, galactose metabolism is more complex, as it is processed via the Leloir pathway. This pathway requires multiple enzymatic conversions, including the formation of UDP-galactose before it can be converted to glucose-6-phosphate and subsequently enter the EMP or ED pathways to form pyruvate. The complexity of galactose metabolism relative to glucose and mannose justifies the occurrence of CCR, leading to the preferential consumption of the latter two sugars when they are present. Because of catabolite repression, sugar mixtures cannot be metabolized in a rapid and efficient way implicating in lower poductivity in bioprocesses using lignocellulosic hydrolysates (Lopes et al., 2011).

c) In response to the new hypothesis, the half-dilution of the hydrolysate show effectivity in improving the fermentability of sugars, as well as pH stabilization and reducing potential inhibitor concentration.

6.4 PHB determination

The structural characterization of intracellular polymer was investigated combining the following analytical procedures. In exception of ^{1}H NMR, all of them were executed for both positive control and SCGH OX 2X dil. samples.

6.4.1 Fourier Transform Infrared Spectometry

The functional groups present in the polymer extracted from *B. sacchari* were detected in both the control sample and SCGH sample using FT-IR analysis. The FT-IR data revealed several characteristic bands: a band at 3296 cm^{-1} corresponding to the

stretching of a terminal OH group; bands at 2968 cm^{-1} and 1926 cm^{-1} indicating C-H stretching in methyl and methylene groups; a band at 1718 cm^{-1} indicative of C=O stretching of a carboxyl group; and bands between 1020 cm^{-1} and 1248 cm^{-1} related to the stretching vibrations of the ester bond C-O.

Among these, the band associated with the carboxyl group is crucial for confirming the presence of PHB in the sample, as it is directly related to polymer's ester bonds. Although, this could be confused with the C=O amide bond of the amino acids, if it were an amide, we would expect to see a corresponding N-H stretching vibration band around 3500 cm^{-1}, which was not detected.

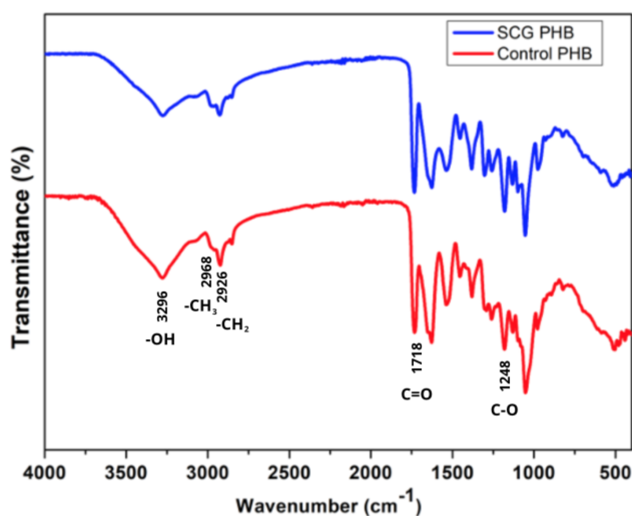


Fig. 12:ATR-FTIR analysis of the oven-dried cellular pellet of positive control and SCGH OX 2X dil. samples.

6.4.2 Gas Chromatography – Mass Spectrometry

The GC/MS spectra of methanolysed PHB extracted from *B. sacchari* and are shown in *Figures 13* and *14*. Methylated 3HB was the only monomeric structure detected in the sample (*m/z*-118.06) besides the internal standard, methyl benzoate (*m/z*-116.05). This result was the expected considering the PHAs constituents accumulated by *B. sacchari* when consuming the sugars present in our SCGH, as reported in literature by Oliveira-Filho et al. (2021).

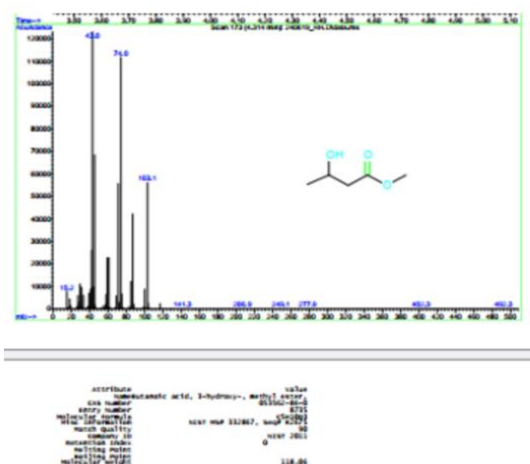


Fig. 13: 3-polyhydroxybutirate detected by GC-MS after methanolysis of cellular pellet of SCGH OX 2X dil.

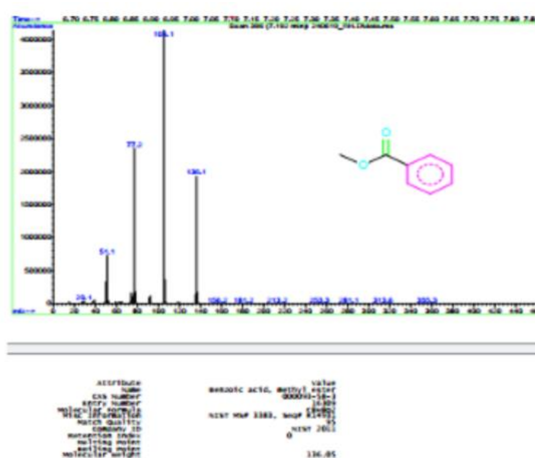


Fig. 14: benzoic acid detected by GC-MS after methanolysis of cellular pellet of SCGH OX 2X dil.

6.4.3 Gas Chromatography

GC-FID analysis was conducted to quantify the monomeric composition of PHB extracted from *B. sacchari* cellular pellets, specifically 3-hydroxybutyrate (3HB), as previously demonstrated by GC-MS. The GC-FID chromatogram (*Figure 15*) showed a peak related to 3HB monomer at a retention time of 3.659 minutes and another peak for benzoic acid at 6.902 minutes. The PHB concentration was determined by substituting the GC area into the calibration curve equation, detailed in the *Appendix B*. The control experiment showed a PHB concentration of 2.879 g/L, while the SCGH OX dil. experiment yielded 1.187 g/L.

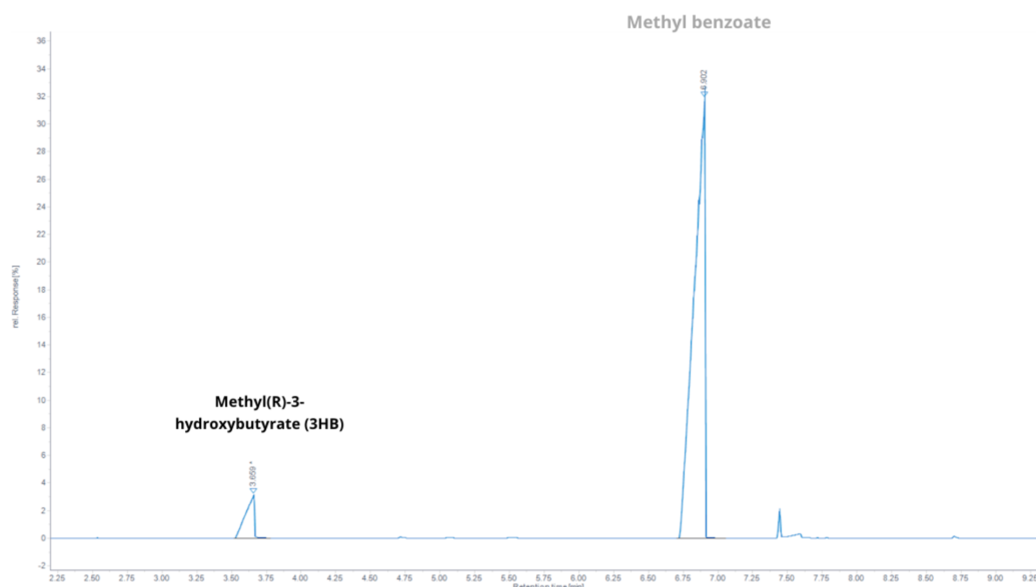


Fig. 15: Gas chromatogram from the methanolized cellular pellet corresponding to SCGH OX 2X dil. fermentation, where both 3HB and benzoic acid methyl esters appear.

6.4.4 ^{1}H Nuclear Magnetic Resonance

The ^{1}H NMR spectrum of the extracted PHB (*Figure 16*) revealed signals of a methyl group ($-CH_3$) at a chemical shift of δ = 1.26 ppm. The multiplet proton splitting pattern of a methylene group joint to a carbonyl group ($-CH_2$) was observed at δ = 2.46-2.61 ppm. Multiple signals appearing at δ = 5.20 ppm corresponded to a methine group (-CH). All these peaks are characteristic of the functional groups present in the 3HB monomer of PHB molecule.

Peaks associated with solvent impurities were also detected. The internal standard, tetramethylsilane (TMS), produced a peak at 0 ppm. Methanol, used during the extraction, appeared at δ = 3.49 ppm. Acetone, which was employed for cleaning the NMR test tube, also showed a signal at δ = 2.17 ppm. Finally, deuterated chloroform ($CDCl_3$) also appeared as a residual peak at δ = 7.26 ppm.

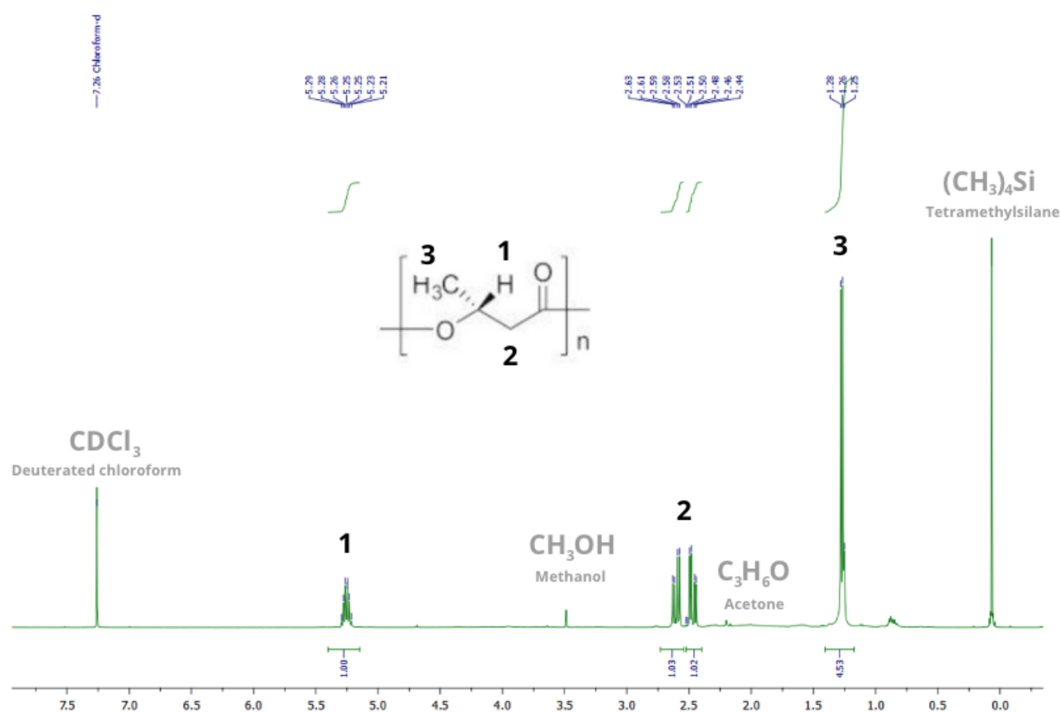


Fig. 16: ^{1}H NMR spectrum of PHB extracted from Burkholderia sacchari.

6.4.5 Transmission Electron Microscopy

The TEM images revealed PHB granules with a diameter between 0.2-0.5µm accumulating inside *Burkholderia sacchari* during fermentation process of the SCGH OX 2X dil. (*Figure 18*). The observation differs slightly from the control sample results (*Figure 17*).

As shown in the control sample, both the bacterial cells and the PHB granules are more widely spaced, whereas in the SCGH OX 2X dil. sample, the cells appear more closely packed with a seemingly higher intracellular PHB content, despite showing 42.30% less PHB than the control. This discrepancy can be attributed to the different states of the samples when submitted for TEM analysis. The control sample was delivered in a wet state, while the SCGH OX 2X dil. pellet had been previously dried in an oven.

The drying process results in dehydration, leading to shrinkage, collapse, or distortion of PHB granules due to water loss. Consequently, the granules may lose their original shape, appearing more irregular or flattened in TEM images. Additionally, cell shrinkage can compress internal structures, further distorting the appearance of the granules, as observed in the SCGH OX 2X dil. sample.

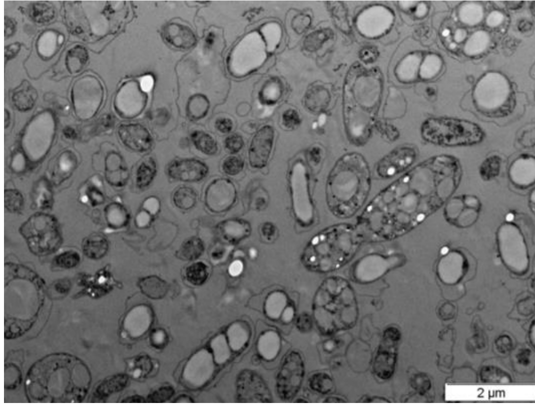


Fig. 17: PHB granules from the control sample observed in TEM at 10,000X magnification.

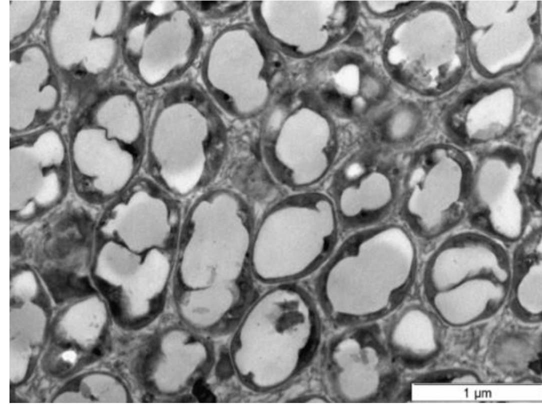


Fig. 18: PHB granules from the SCGH OX 2X dil. sample observed in TEM at 30,000X magnification.

7. CONCLUSION

In summary, this research explored a combined oxalic acid pretreatment and microbial fermentation approach to produce bio-based biodegradable and biocompatible PHB bioplastic using industrial waste spent coffee grounds as a feedstock. The oxalic acid pretreatment efficiently curbs the formation of inhibitors, improves biomass fermentability, and boosts the yield of PHB. Specifically, the half-diluted hydrolysate generated the maximum PHB concentration, accumulation, and yield (1.187 g/L, 55.37% and 0.17 g/g, respectively).

This approach not only contributes to improving PHB production but also emphasized the importance of maximizing resource utilization in bioplastic production. However, the next step of the investigation should address optimizing the fermentation process to reduce the time required for PHB accumulation, possibly by exploring different bacterial strains or enhancing the nutrient composition of the fermentation medium. Additionally, scaling up the process and evaluating the economic feasibility and environmental impact will be crucial for transitioning from laboratory-scale production to industrial applications. Further studies could also explore the integration of this process into a circular economy framework, where other waste streams could be utilized, and the by-products of fermentation could be repurposed to achieve a more sustainable and cost-effective bioplastic production system.

8. BIBLIOGRAPHY

Al-Battashi, H., Annamalai, N., Al-Kindi, S., Nair, A. S., Al-Bahry, S., Verma, J. P., & Sivakumar, N. (2019). Production of bioplastic (poly-3-hydroxybutyrate) using waste paper as a feedstock: Optimization of enzymatic hydrolysis and fermentation employing Burkholderia sacchari. *Journal of Cleaner Production*, *214*, 236–247. https://doi.org/10.1016/j.jclepro.2018.12.239

Alvarez-Santullano, N., Villegas, P., Mardones, M. S., Durán, R. E., Donoso, R., González, A., Sanhueza, C., Navia, R., Acevedo, F., Pérez-Pantoja, D., & Seeger, M. (2021). Genome-wide metabolic reconstruction of the synthesis of polyhydroxyalkanoates from sugars and fatty acids by burkholderia sensu lato species. *Microorganisms*, *9*(6). https://doi.org/10.3390/microorganisms9061290

Andhalkar, V. V., Ahorsu, R., Domínguez De María, P., Winterburn, J., Medina, F., & Constantí, M. (2022). Valorization of Lignocellulose by Producing Polyhydroxyalkanoates under Circular Bioeconomy Premises: Facts and Challenges. In *ACS Sustainable Chemistry and Engineering* (Vol. 10, Issue 50, pp. 16459–16475). American Chemical Society. https://doi.org/10.1021/acssuschemeng.2c04925

Andhalkar, V. V., Montané, D., Medina, F., & Constantí, M. (2024). Biorefinery design with combined deacetylation and microwave pretreatment for enhanced production of polyhydroxyalkanoates and efficient carbon utilization from lignocellulose. *Chemical Engineering Journal*, 149754. https://doi.org/10.1016/j.cej.2024.149754

Braunegg, G., Sonnleimer, B., & Lafferty, R. M. (1978). A Rapid Gas Chromatographic Method for the Determination of Poly-~-hydroxybutyric Acid in Microbial Biomass. In *European J. Appl. Microbiol. Biotechnol* (Vol. 6).

Cesário, M. T., Raposo, R. S., de Almeida, M. C. M. D., van Keulen, F., Ferreira, B. S., & da Fonseca, M. M. R. (2014). Enhanced bioproduction of poly-3-hydroxybutyrate from wheat straw lignocellulosic hydrolysates. *New Biotechnology*, *31*(1), 104–113. https://doi.org/10.1016/j.nbt.2013.10.004

Corrado, I., Argenziano, R., Borselleca, E., Moccia, F., Panzella, L., & Pezzella, C. (2024). Cascade disassembling of spent coffee grounds into phenols, lignin and fermentable sugars en route to a green active packaging. *Separation and Purification Technology*, *334*. https://doi.org/10.1016/j.seppur.2023.125998

Kundu, C., Lee, H. J., & Lee, J. W. (2015). Enhanced bioethanol production from yellow poplar by deacetylation and oxalic acid pretreatment without detoxification. *Bioresource Technology*, *178*, 28–35. https://doi.org/10.1016/j.biortech.2014.08.082

Lopes, M. S. G., Gosset, G., Rocha, R. C. S., Gomez, J. G. C., & Ferreira Da Silva, L. (2011). PHB biosynthesis in catabolite repression mutant of Burkholderia sacchari. *Current Microbiology*, *63*(4), 319–326. https://doi.org/10.1007/s00284-011-9981-6

Obruca, S., Benesova, P., Petrik, S., Oborna, J., Prikryl, R., & Marova, I. (2014). Production of polyhydroxyalkanoates using hydrolysate of spent coffee grounds. *Process Biochemistry*, *49*(9), 1409–1414. https://doi.org/10.1016/j.procbio.2014.05.013

Oliveira-Filho, E. R., Gomez, J. G. C., Taciro, M. K., & Silva, L. F. (2021). Burkholderia sacchari (synonym Paraburkholderia sacchari): An industrial and versatile bacterial chassis for sustainable biosynthesis of polyhydroxyalkanoates and other bioproducts. In *Bioresource Technology* (Vol. 337). Elsevier Ltd. https://doi.org/10.1016/j.biortech.2021.125472

Panyarachun, D., Panakkal, E. J., Tawai, A., Rodiahwati, W., Paramasivam, B., Asavasanti, S., & Cheenkachorn, K. (2023). Oxalic Acid Pretreatment on Enhancement of Enzymatic Saccharification from Napier Grass for Biofuel Production. *E3S Web of Conferences*, *428*. https://doi.org/10.1051/e3sconf/202342801008

Sluiter, A., Hames, B., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., & Crocker, D. (2008). *Determination of Structural Carbohydrates and Lignin in Biomass: Laboratory Analytical Procedure (LAP) (Revised July 2011)*. http://www.nrel.gov/biomass/analytical_procedures.html

Visual Feature | Beat Plastic Pollution. (2022b, marzo 1). https://www.unep.org/interactives/beat-plastic-pollution/?gad_source=1&gclid=Cj0KCQjwzva1BhD3ARIsADQuPnWV2Fq0vwwRN_wvmkJE8Au1V3wULNkyCYH0wyzFPXZBcIiquJGlG0aArWWEALw_wcB

Zhang, L., Jiang, Z., Tsui, T. H., Loh, K. C., Dai, Y., & Tong, Y. W. (2022). A Review on Enhancing Cupriavidus necator Fermentation for Poly(3-hydroxybutyrate) (PHB) Production From Low-Cost Carbon Sources. In *Frontiers in Bioengineering and Biotechnology* (Vol. 10). Frontiers Media S.A. https://doi.org/10.3389/fbioe.2022.946085

9.SELF-EVALUATION

Working on my bachelor's final degree project has been a meaningful and educational experience. During my internship with the Catheter and Interfibio groups, I was pushed to explore new areas, particularly in the physicochemical treatment of biomass, which was initially unfamiliar to me. This project also gave the opportunity to apply some theoretical concepts I learned throughout my degree in a practical setting, deepening my understanding of both the subject matter and the dynamics of research groups, especially the importance of knowledge-sharing.

On a personal level, I am proud of myself for persevering, even when things became difficult, both in the lab and my personal life. There were many failed experiments and frustrating moments, but pushing through those challenges made the final successes even more satisfying. I have learned that failure is just part of the process in science, and it is what drives you to keep improving.

This project has also helped me build confidence in my abilities as a researcher. Now, I feel more comfortable in the laboratory, having handled sophisticated equipment such as HPLCs and gas chromatograph, and worked in a laminar flow cabinet during microbiological procedures. As I look forward to my future career, I am confident that the knowledge and experience I gained during this project will be invaluable, and I am excited to contribute to the development of sustainable solutions through biotechnology.

10. APPENDIX

A) Trace Element Solution composition

Compound	Concentration (g/L)
H_3BO_3	0.3
$CoCl_2 \cdot 6H2O$	0.2
$ZnSO_4 \cdot 7H_2O$	0.1
$MnCl_2 \cdot 4H_2O$	0.03
$(NH_4)_6Mo_7O_{24}$	0.03
$NiCl_2 \cdot 6H_2O$	0.02
$CuSO_4 \cdot 5H_2O$	0.01

Table 5: Trace Element Solution composition.

B) GC calibration curve

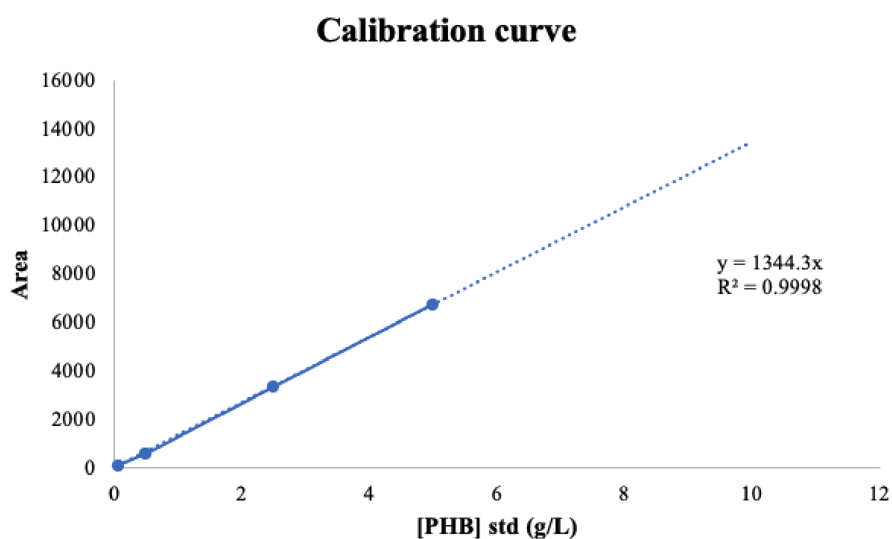


Fig. 19: Calibration curve obtained with concentrations ranging from 0.0625 g/L to 5 g/L obtained using standard methyl(R)-3-hydroxybutyrate (3HB).