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Héctor H. León Santiesteban [1], Juan Aguirre Aguilar [1], Deyanira Ángeles Beltrán [2], José Luis Contreras Larios [1], Ricardo Reyes Chilpa [3,*], Julio C. García Martínez [4] and Margarita M. González Brambila [1,*]

[1] Energy Department, Universidad Autónoma Metropolitana-Azcapotzalco. Av. San Pablo # 420, C.P. 02128, México City, México

[2] Basic Sciences Department, Universidad Autónoma Metropolitana-Azcapotzalco. Av. San Pablo # 420, C.P. 02128, México City, México

[3] Chemistry Institute, Universidad Nacional Autónoma de México, Circuito Exterior s/n, Ciudad Universitaria, 04510, Coyoacán, México City, México

[4] Biophysics Department, Instituto Politécnico Nacional. Prolongación de Carpio y Plan de Ayala, s/n. Col. Santo Tomás, Miguel Hidalgo. C. P. 11340. México City, México

* Correspondence: chilpa@unam.mx (R.R.C.); margarita.gonzlezbrambila@gmail.com (M.M.G.B.); Tel.: +52 5526531825 (M.M.G.B.)

Abstract

From 1950 to the present, plastic production and use have increased mainly because plastics possess qualities like stability, light weight, versatility, and decreasing production costs. However, most plastics are not biodegradable, and only a small portion is recycled worldwide. Bioplastics serve as an alternative if they are biodegradable and derived from residual materials, promoting a circular economy. PHB is a polymer with characteristics similar to some commercial plastics. It was discovered in the 1920s and has been examined by researchers and engineers since then due to its potential as a biodegradable bioplastic. Some microorganisms can produce PHB under controlled conditions. In this work, PHB production was analyzed using two strains: *Bacillus subtilis* and *Bacillus megaterium*. Using two byproducts—whey and glycerol—as substrates and varying the culture media compositions. Both byproducts and both strains are suitable for PHB production; the absence of nitrogen and trace element sources enhances PHB yield. Additionally, bacterial growth, substrate uptake, and PHB production were modeled using logistic growth and the Luedeking-Piret models.

Keywords: *Bacillus megaterium*; *Bacillus subtilis*; polyhydroxyalkanoates; polyhydroxybutyrate; plastics; bioplastics; environmental impact; circular economy

1. Introduction

The annual production of plastics has doubled from 234 million tons (Mt) in 2000 to 460 Mt in 2019. Moreover, in the same period, plastic waste has more than doubled from 156 Mt in 2000 to 353 Mt in 2019 (1).

Some basic actions are needed to reduce the impact of plastic pollution. Some of the most important are:

1) To promote the circular economy, including the design of reusable and recycled products.

2) To increase bioplastics production from renewable feedstocks by utilizing residual byproducts as raw materials (2).

This work examines the use of whey and glycerol as substrates for bioplastic production. Whey is a byproduct of the dairy industry, and glycerol is the main byproduct of biodiesel manufacturing.

Biopolymers produced by microorganisms are easily biodegraded by them and within the digestive systems of higher animals, including humans. The PHAs, a type of biopolymer, are natural

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polyesters made up of various D-hydroxyalkanoic acids, starting with polyhydroxybutyrate (PHB). PHAs are generated by a range of prokaryotes and some eukaryotic organisms, such as plants, animals, and fungi.

Figure 1 displays the chemical structure diagram of PHAs. Depending on the bacterial strain, carbon source, and growth conditions, molecular weights can vary from tens of thousands to hundreds of thousands (2). All PHAs originate from renewable materials, are genuinely biodegradable, and exhibit high biocompatibility (3). They hold great promise for food packaging and compare favorably to plastics like polyethylene and polypropylene.

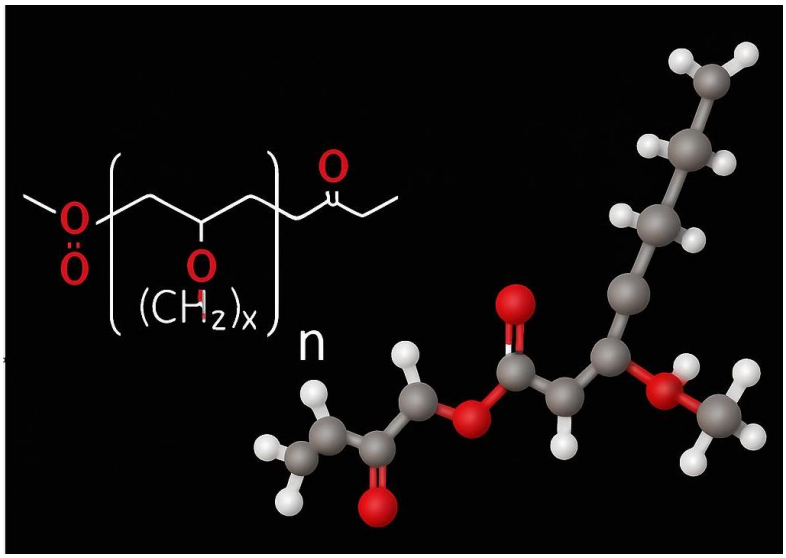

Figure 1. Chemical structure of PHAs in 2 and 3D, drawn with BIOMODEL (2).

Some bacterial and fungal species store PHA as a carbon and energy source to survive during starvation conditions. PHA accumulates in cells when there is an excess of a carbon source in the culture medium, but bacterial growth is limited by another nutrient; these limiting nutrients can include nitrogen, sulfur, phosphate, iron, magnesium, potassium, or oxygen (4).

One of the most promising bioplastics is polyhydroxybutyrate (PHB), which belongs to the PHA family of bioplastics, where the molecular weight coefficient ***x*** (shown in Figure 1) equals 3. It is a non-toxic polymer produced and stored by certain bacterial and fungal genera, such as *Alcaligenes, Azotobacter, Bacillus, Beijerinckia, Pseudomonas, Rhizobium, and Rhodospirillum* (5). The PHB molecules in the cytoplasm are crystalline, forming granules about 0.5 μm in diameter. These granules can be isolated or extracted using solvents. Some researchers believe that PHB has significant potential to replace high-density polyethylene (PE) and polypropylene (PP) due to its chemical and mechanical properties (see Table 1) and with proper disposal practices to establish a fully circular plastic lifecycle (6). However, its current use is limited by high production costs, low yields, manufacturing technology, and downstream purification challenges. Therefore, it is necessary to study and optimize conditions that enhance its accumulation within cells, develop methods for cell disruption without damaging the PHB molecules, and improve purification from the culture media.

Table 1. Comparison of properties of PHB and PP.

Property	PHB	PP
Crystalline melting point (°C)	**175**	176
Crystallinity (%)	**80**	70
Molecular weight (Da)	**5 x 10^5**	2 x 10^5
Glass transition temperature (°C)	**4**	-10
Density [g/cm^3]	1.25	0.905
Flexural modulus (GPa)	4	1.7

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Tensile strength (MPa)	40	38
Extension to break (%)	6	400
Ultraviolet resistance	good	poor
Solvent resistance	poor	good

PHB biodegradation occurs within a reasonable timeframe when it contacts degrading microorganisms in biologically active environments, such as soils, freshwater, and aerobic and anaerobic composting (7).

Metabolic Pathways

The production of PHB using bacteria has been extensively documented in the literature. A variety of raw materials have been reported, including simple sugars like glucose and sucrose, vegetable oils, agro-industrial waste (molasses, whey, fruit peels), crude glycerol, and even methane and methanol.

Whey as Substrate

The main compound of whey (excluding water) is lactose. Lactose catabolism begins with the transport of lactose into the cell through the cell's membrane via a lactose-specific phosphotransferase system (8,9). Figure 2 illustrates the steps of the lactose metabolic pathway leading to PHB production, including the enzymes that catalyze each reaction.

Glycerol as Substrate

This study examines the production process using crude glycerol, a byproduct of biodiesel manufacturing. The metabolic pathway that converts glycerol to PHB is illustrated in Figure 3 (10).

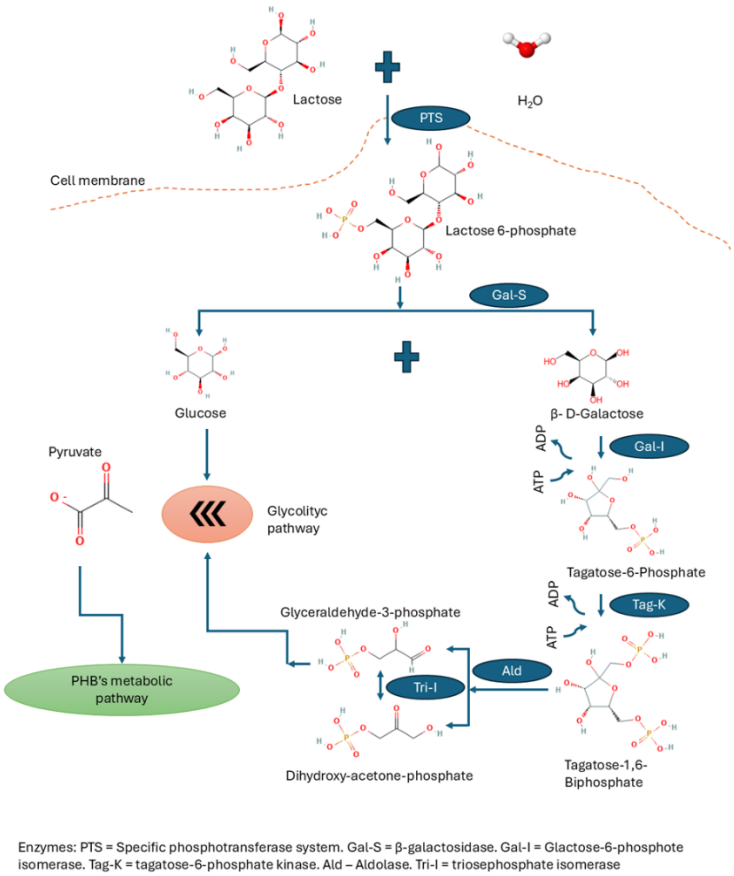

Figure 2. Lactose to PHB's metabolic pathway.

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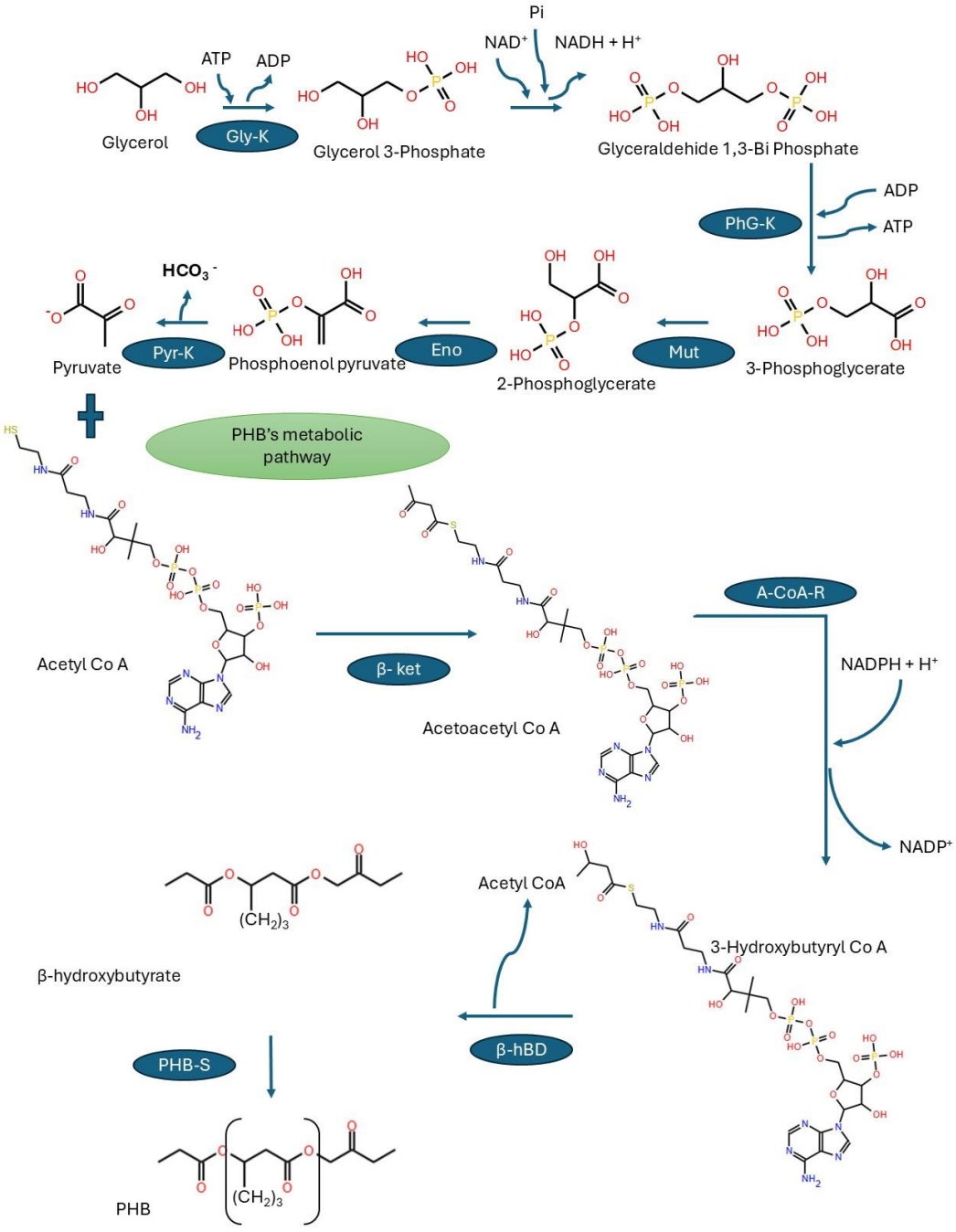

Figure 3. Metabolic pathway of Glycerol to PHB.

2. Results and Discussion

2.1. Experiments with Whey

The experimental tests were conducted in triplicate; the results shown in Figure 4 are the averages of the three measurements for each experiment. At a pH level of 8, the production of PHB was 2.35 times higher than at pH 3, 1.35 times higher than at pH 6, and 1.24 times higher than at pH 7. Therefore, a basic pH promotes PHB production because it stresses the cells of Bacillus megaterium.

That makes sense because *B. megaterium* is a neutrophilic bacterium, and its optimal growth pH ranges from 6.5 to 7.5. However, at acidic pH levels, these bacteria do not accumulate PHB in their

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cytoplasm. This occurs because low pH levels denature certain enzymes involved in the PHB production pathway, such as β-ketothiolase [β-ket], Acetoacetyl-CoA reductase [A-CoA-R], and β-PHB synthase [PHB-S].

Table 2 presents results from other studies reported in the literature on *Bacillus megaterium* and various substrates for PHB production. This table shows that one of the key issues researchers aim to address is finding low-cost raw materials or, ideally, residues from other industries such as biodiesel production (glycerol), cacao liquid wastes, and oil palm empty fruit bunches (waste from oil palm cultivation). The PHB concentration observed in this work falls within the same range as reported in other similar studies. The PHB concentration at each hour of the bioreaction is the most comparable variable across different studies.

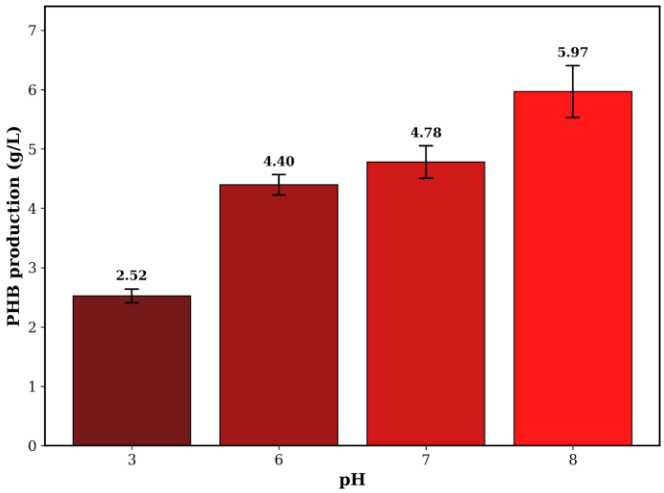

Figure 4. PHB concentration obtained experimentally, with Whey at different pH levels, and the standard deviation of each one.

Table 2. PHB production comparison with *B. megaterium* and different substrates reported.

Microorganism	Substrate	PHB [gL^{-1}]	PHB [g L^{-1} h^{-1}]	Reference
Bacillus megaterium B2	Glycerol	1.2	0.11	(11)
Bacillus megaterium B2	Glycerol	1.59	0.044	(12)
Bacillus megaterium B2	Cacao liquid wastes	11.6	0.16	(13)
Bacillus megaterium DSM32T	Saccharose		0.162	(13)
Bacillus megaterium S29	Glucose	5.4	0.45	(14)
Bacillus megaterium R11	OPEFB*	12.48	0.26	[(15)
Bacillus megaterium BBST4	Glucose	3.3	0.103	(16)
Bacillus megaterium BBST4	Glycerol	4.8	0.114	(17)
Bacillus megaterium BA-019	Molasses	4.16	0.35	(18)
Bacillus megaterium	Whey	5.97	0.124	This work

*** OPEFB – Oil palm empty fruit bunch.**

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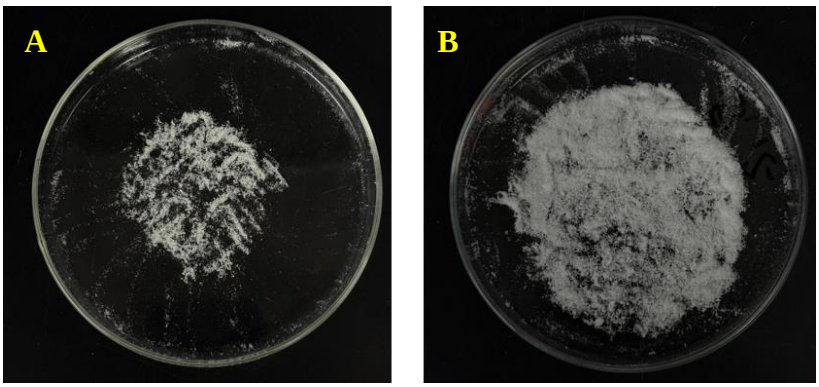

Figure 5. PHB obtained with A] Bacillus subtilis, B] Bacillus megaterium.

2.2. Experiments with Glycerol

Figure 5 shows solid PHB obtained after the recovery process with *B. subtilis* [A] and *B. megaterium* [B]. It can be observed with the naked eye that the amount of PHB obtained with *B. megaterium* is greater than that obtained with *B. subtilis.*

Figures 6 and 7 show the experimental results of glucose consumption, biomass growth, and PHB production with *B. subtilis*. Figure 6 shows the results of experiments carried out without trace elements, and Figure 7 shows the results with trace elements. As can be seen without trace elements (Figure 6), there are three phases of biomass growth. The first one **[I]** is the exponential phase from time 0 to 12 hours; the stationary phase begins at 12 hours and ends at 60 hours; then the death phase begins. Glucose is not totally consumed in these experiments; the percentage of glucose consumed at the end of the exponential growth phase [$\boldsymbol{C_S{}^E}$] was 16.4% [Eq. 1], and at the end of the experiment [$\boldsymbol{C_S{}^F}$] was 49.6%, calculated with Eq. 2, where $\boldsymbol{C_{Si}}$ is the glucose initial concentration, and $\boldsymbol{C_{Sf}}$ is the glucose concentration at the end of the exponential growth phase.

The experimental yield coefficient biomass/substrate, calculated at the end of the exponential growth phase, was 0.38 grams of cell produced per liter per gram of glucose in the phase [$\boldsymbol{Y_{X/S}{}^E}$], see Eq. 3. Where $\boldsymbol{C_{Sfe}}$ is the glucose concentration at the end of the exponential growth phase.

Part of the substrate consumed during the stationary growth phase is used to produce PHB, while the rest supports cell maintenance. That is because, even though biomass stopped increasing at hour 12 of the experiment, the PHB concentration continued to increase, quickly until the end of the stationary growth phase and slowly thereafter. The yield coefficient product / substrate calculated at the maximum PHB concentration [$\boldsymbol{Y_{P/S}{}^M}$] obtained was: 0.45, see Eq. 5. Where $\boldsymbol{C_{Pfm}}$ is the maximum PHB concentration, and $\boldsymbol{C_{Sfm}}$ is the glucose concentration at time equal to maximum PHB concentration.

$$\%\, C_S{}^E = \frac{C_{Si} - C_{Se}}{C_{Si}} 100 \quad (1)$$

$$\%\, C_S{}^F = \frac{C_{Si} - C_{Sf}}{C_{Si}} 100 \quad (2)$$

$$Y_{X/S}{}^E = \frac{C_{Sfe}}{(C_{Si} - C_{Sfe})} \quad (3)$$

$$Y_{P/S}{}^E = \frac{C_{Pfe}}{(C_{Si} - C_{Sfe})} \quad (4)$$

$$Y_{P/S}{}^M = \frac{C_{Pfm}}{(C_{Si} - C_{Sfm})} \quad (5)$$

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Figure 7 displays the experimental results for *B. subtilis* in a culture medium with trace elements. The same three growth phases are observed, but the durations of each phase differ from those in the previous case. The exponential growth phase lasts until hour 33 of the experiment. Then, a stationary phase occurs until hour 48, followed by the death phase.

Glucose is not completely consumed in these experiments, but the final concentration is lower than in the previous set. The percentage of glucose used up at the end of the exponential growth phase was 42.8%, and at the end of the experiment, it was 64.8%, as calculated using Equations 1 and 2, respectively.

The yield coefficient of biomass substrate during the exponential growth phase was 0.34, dropping to 0.14 by the end of the experiment. Conversely, the yield coefficient of product substrate was 0.07 at the end of the exponential growth phase and increased to 0.45 by the experiment's conclusion. This is because a major portion of PHB is produced after the exponential growth phase has ended. The yield coefficient of PHB/substrate was 0.22 at the end of the exponential growth phase and rose to 0.61 by the end of the experiment, calculated using the same equations as before.

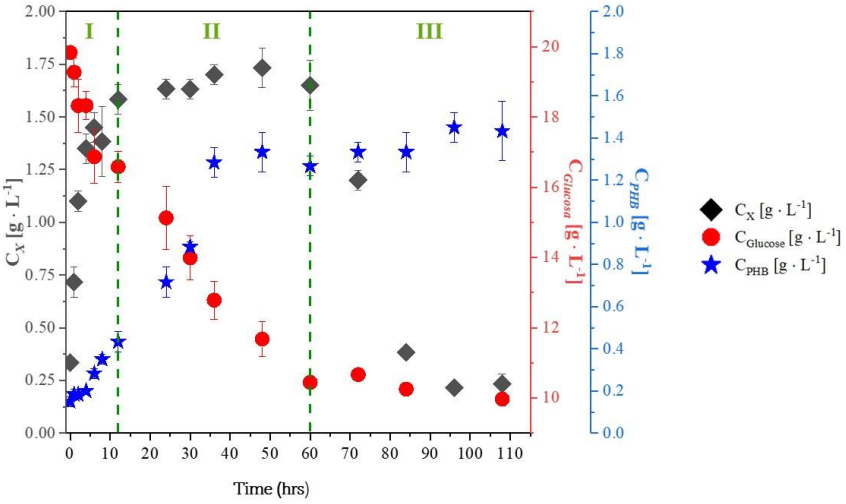

Figure 6. Experimental results of glucose consumption, biomass growth, and PHB production [g L^{-1}], with B. subtilis without trace elements.

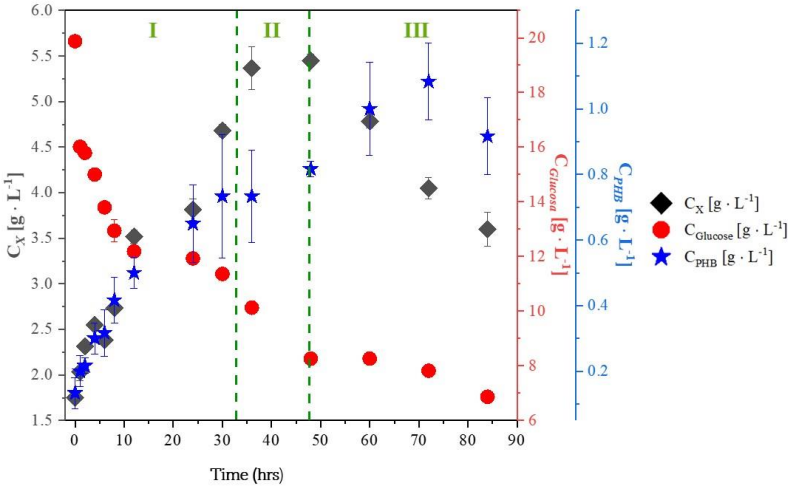

Figure 7. Experimental results of glucose consumption, biomass growth, and PHB production [gL^{-1}], with *B. subtilis* with trace elements.

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Figure 8 shows the experimental results for *B. megaterium* in a broth without trace elements. The same three growth phases were observed, with different durations for each. The exponential growth phase ended at hour 18, the stationary phase concluded at hour 40, and the death phase persisted until the end of the experiments.

The maximum biomass concentration was 1.9 g L^{-1}, and the maximum PHB concentration reached was 3.1 g L^{-1}. *B. megaterium* did not consume all the glucose in the broth; the glucose concentration at the end of the experiment was 11 g L^{-1}. The yield coefficient of biomass/substrate, at the end of the exponential growth phase, was 0.3, and at the end of the experiment was 0.026. The yield coefficient product/substrate at the end of the exponential growth phase was 0.2633, and the yield coefficient at the end of the experiment was 0.8.

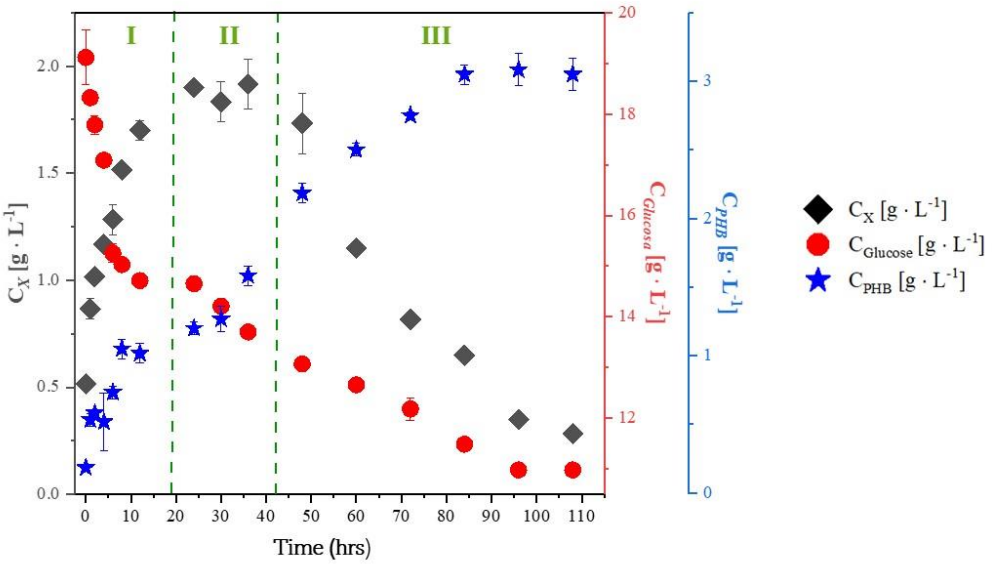

Figure 8. Experimental results of glucose consumption, biomass growth, and PHB production [g L^{-1}], with B. megaterium without trace elements.

Figure 9 displays the experimental results for *B. megaterium* in a culture medium with trace elements. In this case, glucose was also not fully consumed. The biomass/substrate yield coefficients at the end of the exponential growth phase and at the end of the experiment were 0.2 and 0.04, respectively. The yield coefficients of PHB substrate at the end of the exponential growth phase and at the end of the experiment were 0.18 and 0.42, respectively.

Figures 10 and 11 display the comparison of yield coefficients for biomass/substrate and PHB/substrate, respectively, across the four experiments conducted. In Figure 10, the biomass substrate yield coefficients are depicted in red for the culture medium without trace elements and in blue for the medium with trace elements. As demonstrated, *B. subtilis* produced more cells per gram of substrate consumed in the medium lacking trace elements than in the one containing them, and it achieved the highest biomass-substrate yield coefficient among all the experiments.

The same trend was observed with *B. megaterium*: the biomass/substrate yield coefficient was higher in media without trace elements compared to those with trace elements. It is important to note that the C:N ratio used in all experiments was significantly higher than the optimal C:N ratio for growth. The optimal C:N ratio for *B. subtilis* ranges from 8:1 to 11.5:1 (19), (20), and for *B. megaterium*, it ranges from 10:1 to 20:1 (21). In these experiments, the C:N ratio exceeded 200.

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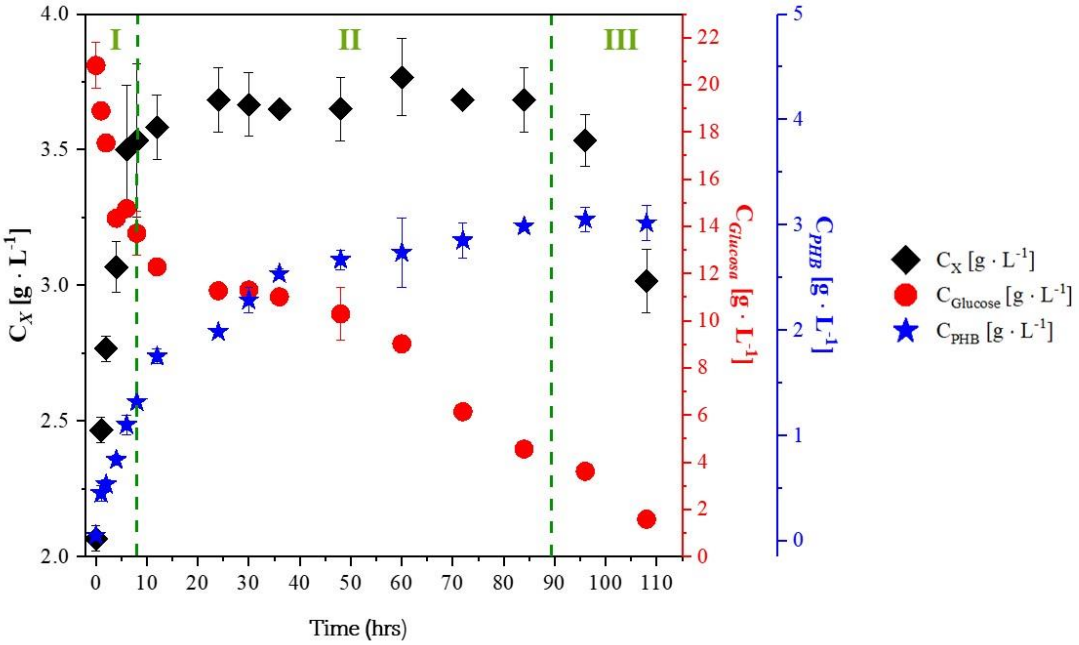

Figure 9. Experimental results of glucose consumption, biomass growth, and PHB production [g L-1], with B. megaterium with trace elements.

Regarding PHB substrate yield coefficients, *B. megaterium* growing in a culture medium lacking trace elements produced the highest amount, as shown in Figure 11. In this experiment, the PHB production was 1.8 times greater than the production obtained in second place (*B. subtilis* without trace elements), 1.9 times greater than the production of *B. megaterium* with trace elements, and more than 6 times greater than the production with *B. subtilis* with trace elements.

In three of the experiments (1, 3, and 4), the amount of substrate used by cells to produce PHB was greater than the amount used for growth. Experiments conducted without TE increased PHB production in both strains. As previously reported by Gómez Cardozo et al. (22), B. megaterium is more efficient at producing PHB than B. subtilis.

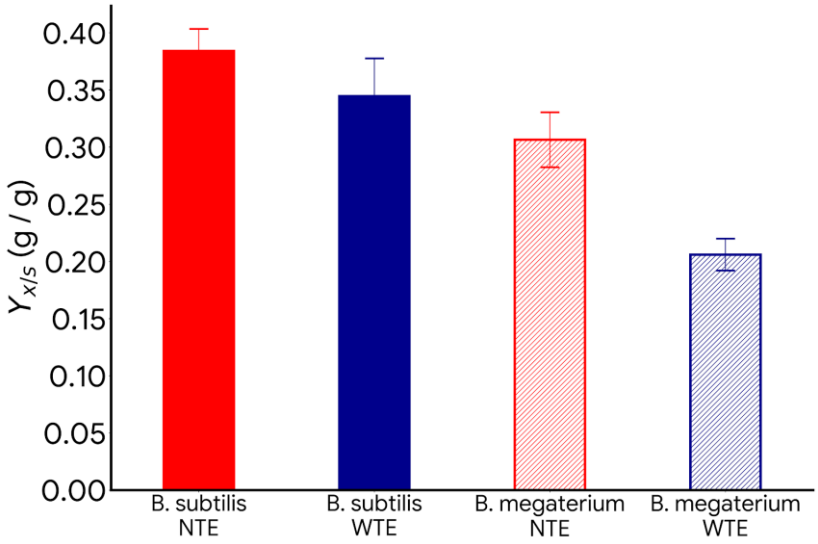

Figure 10. Yield coefficients biomass substrate [Y x/s], at the end of each experiment. Solid bars show coefficients for *B. subtilis*, and not solid bars *for B. megaterium*. Red bars are for the no trace elements experiment, and blue bars are for experiments with trace elements in culture media.

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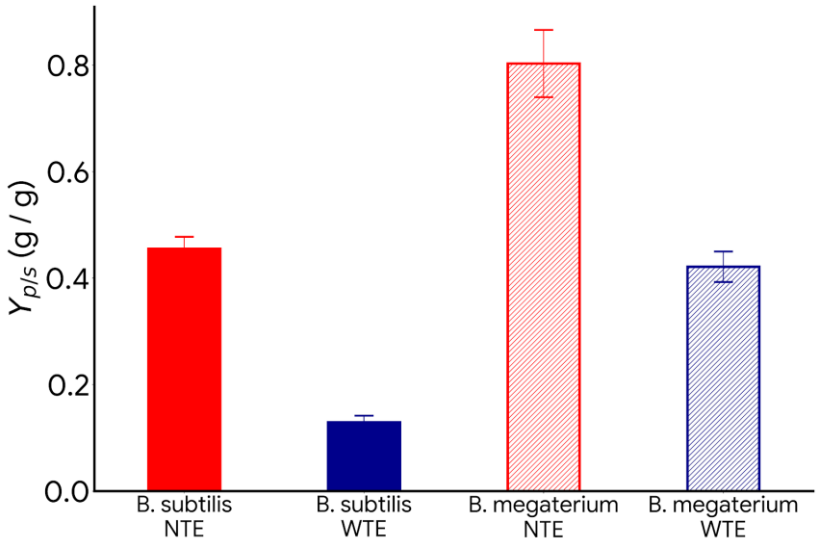

Figure 11. Yield coefficients product substrate [Y p/s], at the maximum PHB concentration obtained in each experiment. Solid bars show coefficients for *B. subtilis*, and not solid bars *for B. megaterium*. Red bars are for the no trace elements experiment, and blue bars are for experiments with trace elements in culture media.

To achieve short exponential growth phases and extended stationary phases, optimize PHB production. Trace element concentration has a greater impact on PHB production than the C:N ratio when the C:N ratio is too high.

The characterization of PHB was performed by Fourier-transform infrared spectroscopy (FTIR). The characteristic signals of PHB were identified in both *Bacillus* strains. One of the most important signals is the signal observed in the 1700–1750 cm^{-1} region, characteristic of the carbonyl group (>C=O) present in PHB, as shown in Figure 12. This signal is very intense in the PHB spectrum produced by *B. subtilis*. Another characteristic signal was observed in the 1250-1150 cm^{-1} range, corresponding to esters ($C_2H_7C_6$). In the 3000-2850 cm^{-1} range, the characteristic signals of the CH_3 and CH_2CH_2 groups are visible in Figure 12 (23).

Figure 13 shows the SEM micrographs of the PHB produced, recovered, and purified from both strains, where homogeneous and amorphous masses with smooth textures are observed (23).

Image A displays PHB from *B. megaterium* observed at 1000x magnification. Amorphous structures with irregular surfaces and some aggregation are visible. The scale indicates a reference of 100 µm, suggesting the PHB aggregates are quite large.

Figure 13B displays the PHB produced by *B. subtilis*, visualized at a magnification over 2000X, showing a morphology with denser, rougher structures. The 20 µm scale indicates that the fragments observed are smaller than those in Figure 13A.

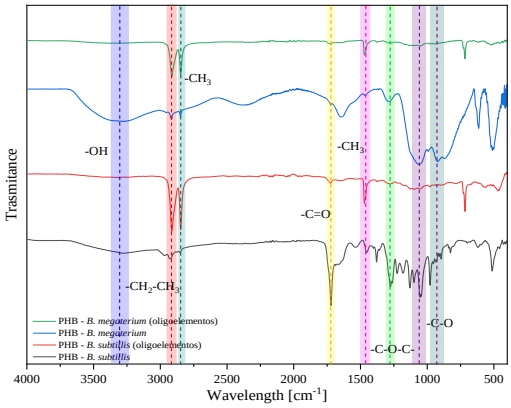

Figure 12. FTIR spectra for different PHB samples produced experimentally.

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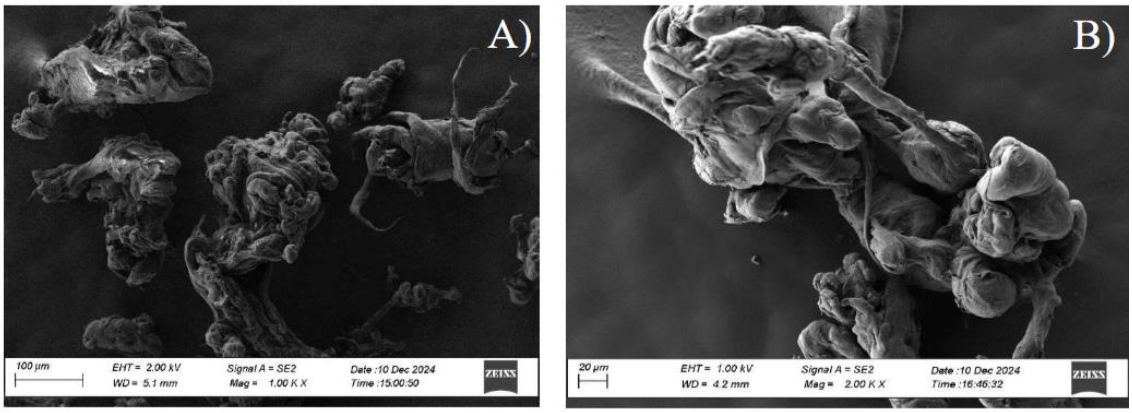

Figure 13. PHB micrography produced by A] *B. megaterium* [100 µm], and B] produced by *B. subtilis* [20 µm].

2.3. Material Balance

A carbon mass balance was conducted to determine the carbon dioxide produced by cell respiration and to investigate why glycerol was not consumed in the experiments. The initial carbon dioxide concentration in the culture media was set to 0.001 g L-1. This value represents the average CO2 concentration in water at 2,440 meters altitude, with an atmospheric pressure of 0.77 atm and a temperature of 25 °C, which are the conditions in Mexico City. Figure 14 illustrates the time-dependent carbon dioxide production for each experiment.

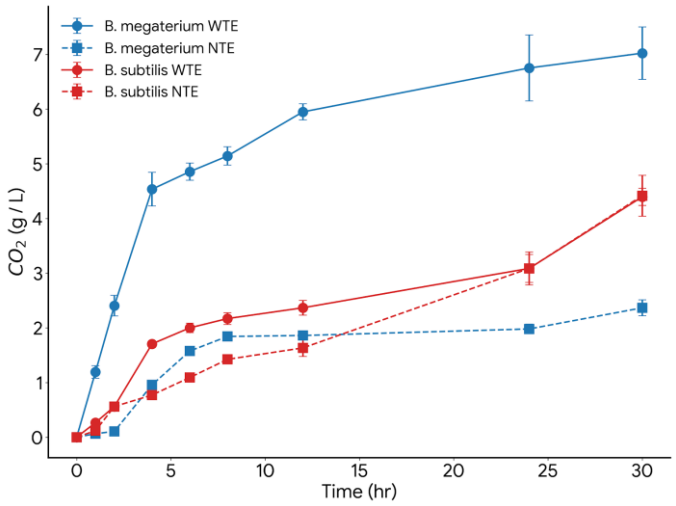

Figure 14. Evolution of carbon dioxide calculated by mass balance in time for each experiment.

The yield coefficients of carbon dioxide to glucose obtained are shown in Table 3 for each experiment, compared with the coefficients reported in literature (18,24). All of these values are within the range reported in the literature. Therefore, glycerol was not consumed in the experiments.

Table 3. Yield coefficients CO2 / glucose calculated by carbon mass balance, for each experiment set.

Experiment set	Strein and conditions	Yield coefficient [C-mol CO_2/C-mol Glucose]	Values reported [C-mol CO_2/C-mol Glucose]
1	*B. subtilis* NTE	0.54	0.3 - 0.6
2	*B. subtilis* WTE	0.49	0.3 - 0.6
3	*B. megaterium* NTE	0.39	0.4 – 0.7
4	*B. megaterium* WTE	0.41	0.3 - 0.6

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2.4. Model

The growth kinetics of B. megaterium and B. subtilis were modeled with the logistic model. The production of PHB for both strains was simulated using the Luedeking-Piret model, with an initial product concentration [PHB] set to zero. Using the experimental results from all batch experiments in the exponential growth phase, kinetic parameters were estimated with the Marquardt non-linear estimation method (25).

Comparisons of model and experimental results for all cases are shown in Figure 14. Graph A shows the results for *B. subtilis* grown in culture media without trace elements. Graph B shows the results for *B. megaterium* with trace elements in the culture media. Symbols represent experimental data, and lines represent model results. As shown in Figure 14, the model fits the experimental data reasonably well, with parameters obtained using the Marquardt algorithm.

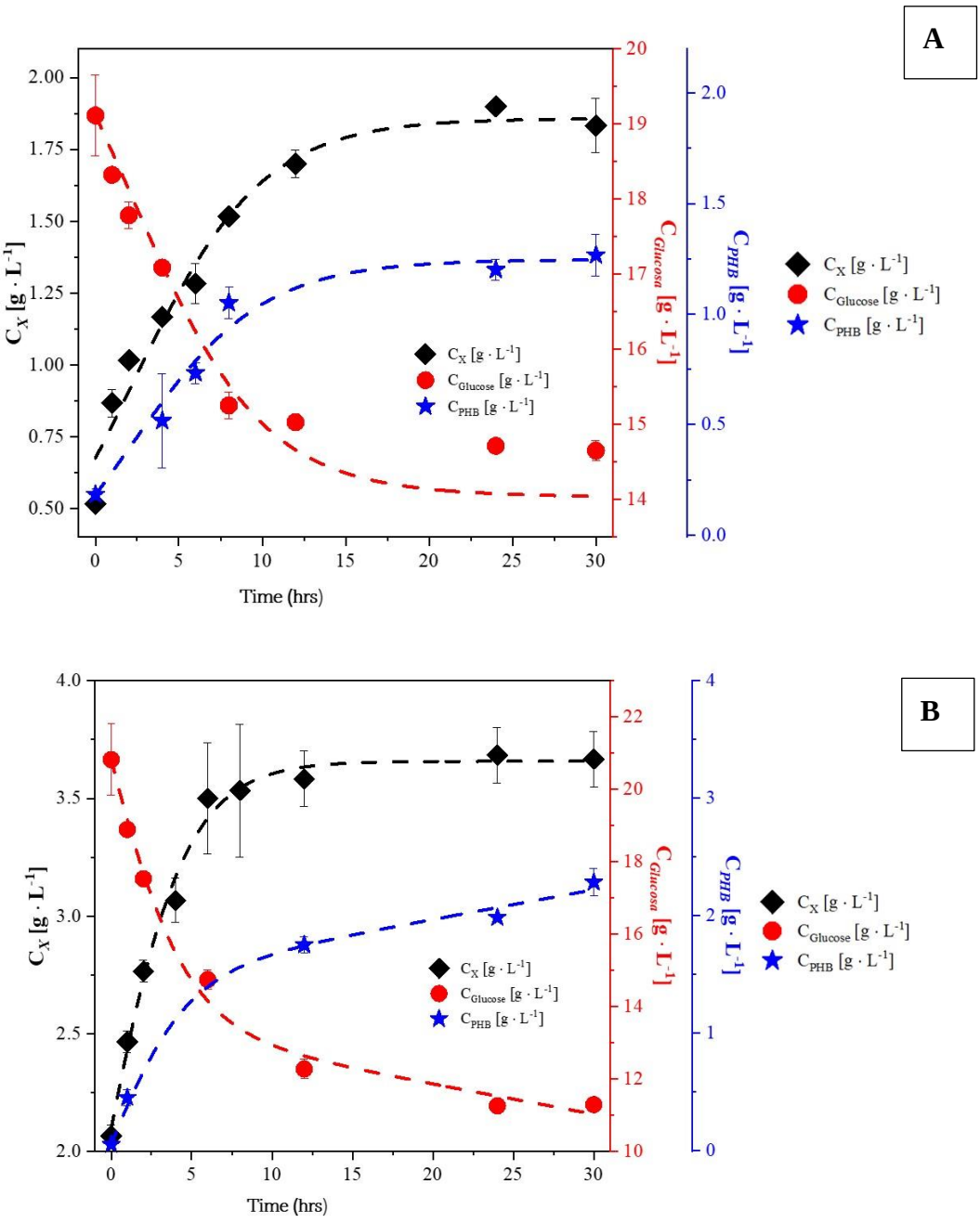

Figure 14. Comparison between experimental and model results, during the exponential phase. A for *B. subtilis* NTE, and B for *B. megaterium* WTE.

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Table 4 shows the model results for parameters related to growth kinetics, PHB production, and substrate consumption using the models outlined in the Model section. As seen, among the maximum biomass concentration parameters, *B. subtilis* in a culture medium with trace elements had the highest x_{max}, followed by *B. megaterium* with TE, B. megaterium without TE, and B. subtilis without TE. Likewise, the yield coefficients for biomass production per gram of substrate were higher in experiments without TE, probably due to excess carbon relative to nitrogen.

Respect the comparison between the parameters α and β of the Ludeking-Piret model: in all cases, α is greater than β, because PHB is produced in the metabolic pathways of glycolysis and the Krebs cycle, which are part of the growth metabolic pathway.

The highest product yield coefficients were achieved without TE in the culture media, and B. megaterium produced more PHB per gram of substrate than B. subtilis.

Table 4. Model results for kinetic growth. PHB production and substrate consumption of B. subtilis and B. megaterium, with and without trace elements.

	B. subtilis				*B. megaterium*			
	No Trace Elements		With Trace Elements		No Trace Elements		With Trace Elements	
Kinetics growth parameters								
x_0, $[g_x \cdot L^{-1}]$	0.39716	± 0.0656	1.800076	± 0.05831	0.67537	± 0.05963	2.09683	± 0.05106
$x_{máx}$, $[g_x \cdot L^{-1}]$	1.57173	± 0.04205	3.75857	± 0.05852	1.85731	± 0.06434	3.65795	± 0.03589
$\mu_{máx}$,$[h^{-1}]$	0.86199	± 0.15153	0.22845	± 0.02728	0.25732	± 0.04036	0.39183	± 0.39183
χ^2	0.00677		0.00659		0.00861		0.00396	
R^2	0.97034		0.99084		0.96063		0.98871	
Kinetics of PHB production								
P_0, $[g_{PHB} \cdot L^{-1}]$	0.15	± 0.024	0.13333333	± 0.0471	0.18333333	± 0.02357	0.05	± 0.02357
α $[g_{PHB} \cdot g_x^{-1}]$	0.0168	± 0.00921	0.23024	± 0.001858	0.89208	± 0.08946	0.92823	± 0.07818
β $[g_{PHB} \cdot (g_x \cdot h)^{-1}]$	0.0168	± 0.000441	0.0014	± 0.000491	0.00018554	± 0.00293	0.0069	± 0.00153
χ^2	0.00027		0.00046		0.00621		0.00549	
R^2	0.99598		0.98919		0.96558		0.9944	
Kinetics of substrate consumption								
S_0, $[g_S \cdot L^{-1}]$	19.83544304	± 0.143	19.8734177	± 0.0895	19.1139241	± 0.9846	20.8227848	± 0.9846
$Y_{X/S}$, $[g_S \cdot g_S^{-1}]$	0.52739		0.386		0.38179		0.41147	
$Y_{PHB/S}$, $[g_{PHB} \cdot g_S^{-1}]$	0.215383		0.13576		0.55		0.39995	
m_S, $[g_S \cdot L^{-1}]$	0.00153		0.00001		0.001		0.00543	
χ^2	0.05979		1.12524		0.28086		0.18093	
R^2	0.987		0.91621		0.9186		0.98795	

The absence of trace elements in the culture media, specifically Fe, Mo, and Ni, inhibits the activity of nitrogenase and hydrogenase enzymes, redirecting reductive equivalents to the β-cetocetiolase → acetoacetyl CoA reductase → PHA polymerase pathway, which promotes PHB accumulation. The lack of Mg in the culture media has been shown to increase PHB production by up to 80%. Additionally, omitting trace elements from the culture medium reduces the cost of PHB production.

A light, basic pH level, between 7.5 and 8, enhances the activity of the PHA-polymerase enzyme because it influences the solubility of certain trace elements, decreasing the activity of some enzymes and increasing the activity of others involved in the PHB production pathway, as demonstrated by the experimental results with Whey.

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3. Conclusion

This study shows the technical feasibility of using industrial by-products, especially residual glycerol and cheese whey, in circular economy pathways to produce polyhydroxybutyrate (PHB) through biochemical methods. Based on the experimental results, the following conclusions can be made:

1. Effect of pH on Biosynthesis: The pH level of the culture medium was identified as a key factor directly affecting bioproduction yield. PHB production showed a positive correlation with increasing pH within the evaluated range, rising from 2.52 g/L at pH 3 to a maximum at pH 8.
2. Effect of nitrogen and carbon sources: The lack of nitrogen and the excess of carbon sources promote PHB production with *B. megaterium* and *B. subtilis*.
3. Effect of trace element deficiency: The absence of trace elements in the culture media increases PHB production and the product-to-substrate yield coefficient.
4. PHB production after the exponential growth phase: In all experiments with glycerol, PHB production continued during the stationary and death phases. This is because trace elements like magnesium and calcium, which are involved in enzyme activity, return more slowly to the metabolic pathways responsible for growth and energy generation.
5. Waste Valorization and Sustainability: Using glycerol and whey as sources of carbon and nitrogen not only cuts production costs related to pure substrates but also offers a sustainable method for managing agro-industrial waste. This strategy advances the shift toward a circular bioeconomy by converting polluting waste into high-value, biodegradable bioplastics.
6. This work shows that using residual material, suppressing certain trace elements, maintaining a high C:N ratio, and controlling pH can create cost-effective and environmentally friendly processes to produce bioplastics like PHB.

4. Materials and Methods

In this part of the study, *Bacillus megaterium* was used to produce PHB in the laboratory with whey as the substrate, and both *B. megaterium* and *B. subtilis* were employed to produce PHB using glucose and glycerol as carbon sources. Glycerol is a primary byproduct of biodiesel production, while whey is a byproduct of the dairy industry. Some aspects considered were:

1. Pyruvate produces ethanol or lactate under anaerobic conditions in a nutrient-balanced culture medium.
2. Under aerobic conditions, pyruvate is converted into oxaloacetate and enters the Krebs cycle to produce energy and support cell growth, using a nutrient-balanced culture media.
3. PHB production is increased in culture media with excess carbohydrates and a deficiency of nutrients such as nitrogen, phosphorus, oxygen, sulfur, or trace elements. When cells are cultured in media containing excess glucose, sucrose, lipids, glycerol, and other carbon sources, Acetyl CoA accumulates. The lack of nitrogen, phosphorus, oxygen, and sulfur halts the synthesis of nucleic acids and proteins, leading to a decrease in cell growth.
4. The excess of NADPH and Acetyl Co-A increased the synthesis of 3-hydroxybutyryl-CoA, the monomer of PHB, because high levels of NADPH and NADH inhibit citrate synthase in the TCA cycle, which ensures the availability of acetyl CoA to connect with 3-Phosphoglycerate.
5. Cells form PHB's conglomerate in the cytoplasm as a future source of carbon and energy.

All experiments took place in the Process Analysis Laboratory at the Metropolitan Autonomous University campus Azcapotzalco. Two raw materials were tested: whey from a cheese factory and glycerol from biodiesel production.

Both *Bacillus* species were grown in flasks containing culture medium containing 15 g L^{-1} of meat peptone, 1.5 g L^{-1} of yeast extract, and 5 g L^{-1} of NaCl at 30 °C and 150 rpm for 24 hours to obtain the inoculum.

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Experiments with Whey

In the initial set of experiments, whey was used as a source of carbon and energy. Estimates show that for every kilogram of cheese, this industry produces between 9 and 10 liters of whey; therefore, Mexico generates about 4,000 million liters of whey annually (26).

Whey contains over 90% water, 5% lactose, less than 1% protein, and less than 9.5% fat, along with minerals like calcium, phosphorus, sodium, potassium, and magnesium, plus traces of vitamins, especially B-complex. The overall composition of whey is shown in Table 5 (27).

Table 5. Whey general composition (27).

Component	Concentration [% mass]	Notes
Water	≈ 93	Main component
Lactose	≈ 5.1	Primary carbohydrate
Proteins	≈ 0.8	Includes β-lactoglobulin, α-lactalbumin, glycomacropeptide, serum albumin
Fats	0.1 – 0.4	Lower in acid whey
Mineral (ash)	≈ 0.5 – 0.7	Calcium, phosphorus, sodium, potassium, magnesium
Vitamins	Traces	B-complex, especially riboflavin

Equipment: The equipment used in this set of experiments was an incubator (Yamato Scientific American IC403CW), an autoclave (TOMY SX-500 high-pressure steam sterilizer), a pH meter (ST20 Series Pen Meers), an FTIR (Alpha II Bruker), a centrifuge (Eppendorf 5910 R), and a spectrophotometer (Jenway 7305).

Reagents and Materials: The materials used were whey, NaOH solution 12 M, meat peptone (Bioxon), urea (Sigma), Bacteriological agar (Bioxon), NaClO solution 5% w/v, chloroform (Meyer) solution 1% v/v, and 4 µm filter paper. The Whey used was collected from a farm named "La Estancia", in San Juan del Río, Querétaro.

Bioreaction

The whey was sterilized twice at 120 °C for 20 minutes, then filtered into a Kjeldahl flask to precipitate and remove all proteins.

Culture media was prepared with 0.8 g of meat peptone in 100 ml of deionized water, along with 0.8 g/l of urea solution. This mixture was combined with 30% whey. The solution was sterilized for 20 minutes at 120 °C. The pH was adjusted using a 1% NaOH solution. Three pH levels (6, 7, and 8) were tested; an additional experiment was performed without pH control.

Bioreactions were performed in flasks inoculated with B. megaterium at 200 rpm stirring, 35 °C, and a sterile airflow of 5 L/min for 48 hours at the three tested pH levels.

Recovery and Washing of PHB

At the end of the bioreaction period, the mixture was centrifuged at 3,000 rpm for 20 minutes. Then, 30 mL of 30% v/v chloroform and 30 mL of 30% v/v NaCl solution were added. This new mixture was placed on an orbital shaker at 150 rpm and 30 °C overnight. Finally, the mixture was centrifuged for 20 minutes at 3,000 rpm. Three phases were observed: the top contained the NaClO solution, the middle contained the cell material, and the bottom contained PHB dissolved in chloroform.

The hypochlorite solution was separated using a pipette, and the other two phases were separated by vacuum filtration. Two volumes of methanol were added to the PHB/chloroform phase, and the mixture was heated at 60 °C for 20 minutes with gentle stirring. Methanol and chloroform were then evaporated. The resulting solid was dried and weighed (see Figure 15).

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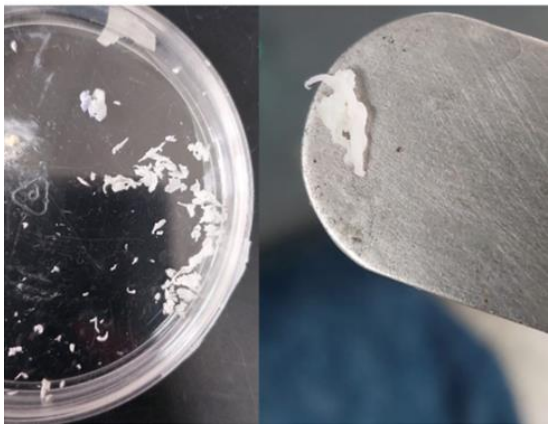

Figure 15. Solid PHB obtained experimentally.

The solid samples obtained were analyzed using a UV-Vis spectrophotometer. Afterwards, they were dissolved in 10 mL of concentrated sulfuric acid in test tubes and placed in a water bath for 10 minutes. The solution was compared with a PHB blank solution in H_2SO_4 using a spectrophotometer (Jenway 7305) at 235 nm.

Experiments with Glycerol

In this set of experiments, glycerol and glucose were used to produce PHB. Experiments with *B. megaterium* and *B. subtilis* were carried out. Equipment and reactive materials were the same as in the experiments described in the previous section.

In previous experiments, biodiesel was produced from residual oil collected from the University's cafeteria. Glycerol generated during biodiesel production was filtered and washed with water, and the water was then evaporated to remove all unwanted compounds. Glycerol was sterilized at 120 °C for 20 minutes to kill any remaining microorganisms and residual particles (see Figure 16).

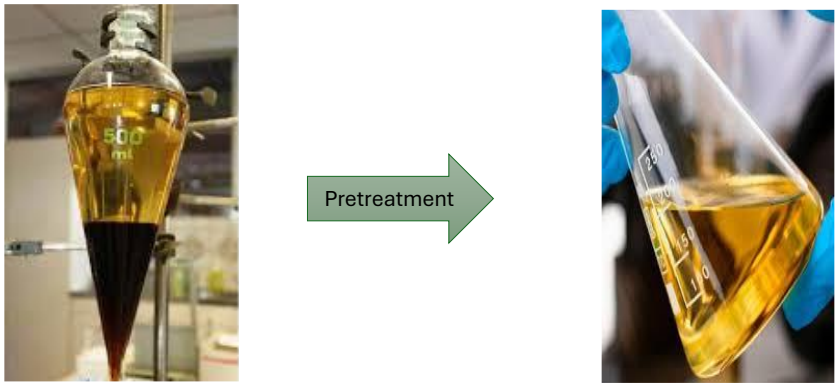

Figure 16. Glycerol before and after the pretreatment process.

Bioreaction

The bioreactions with glycerol were conducted using the two *Bacillus* species. According to the literature, both species can produce PHB. *B. megaterium* has higher efficiency, but *B. subtilis* is more sensitive to the composition of the culture media (22).

PHB was produced in a Lambda Minifor bioreactor in fed-batch mode with mixing vibration and a culture media volume of 1.7 L, as shown in Figure 17. The culture media composition and bioreaction conditions are detailed in Table 6. As indicated, the C: N ratio was 210:1.

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Two sets of experiments were conducted for each strain: one with trace elements (WTE) in the culture media and one without (NTE). The purpose of the experiments without trace elements was to determine whether the absence of those elements increases cellular stress, resulting in decreased biomass growth and higher PHB production.

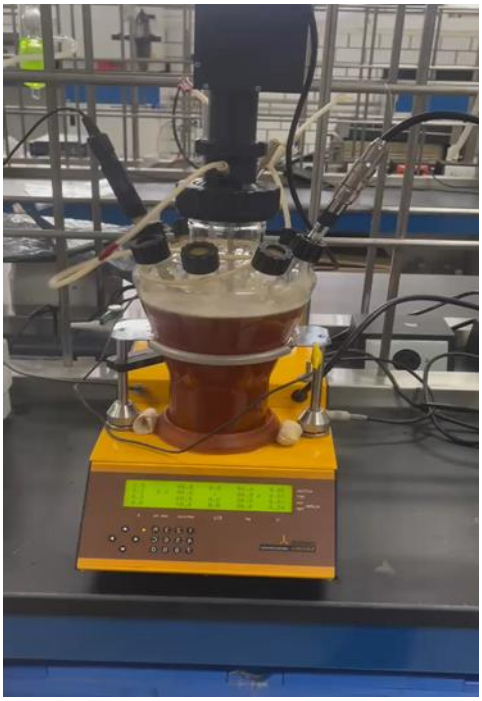

Figure 17. Bioreactor for PHB production.

Recovery of PHB and Glucose

Extraction of PHB was performed using chloroform, sodium hypochlorite, and sonication. Initially, biomass was treated with a 5% v/v sodium hypochlorite solution at 30 °C under gentle stirring to break the cell membranes without affecting PHB. Then, the solution was sonicated twice for 30 minutes at 80% power, with a 10-minute pause in between.

The remaining cells were removed by centrifugation at 3500 rpm and 10 °C for 10 minutes, followed by vacuum filtration. Afterwards, PHB was dissolved in chloroform, and the organic phase was separated by stirring previously. Finally, PHB was precipitated with cold ethanol, recovered by vacuum filtration, and dried at 60 °C until reaching a constant weight.

Table 6. Culture media composition.

Compound	Concentration [g L^{-1}]	Concentration [mol L^{-1}]
Glycerol $C_3H_8O_3$	60.0	0.6516
Glucose $C_6H_{12}O_6$	20.0	0.1111
Ammonium sulfate $[NH_4]_2SO_4$	0.8	0.00605
Magnesium sulfate $MgSO_4 \bullet 7\ H_2O$	0.2	0.00081
Trace elements (NulanZa brand) [mL L^{-1}]	1	
Total carbon		2.4546
Total nitrogen		0.0121

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C/N relation		220:1
Initial pH level		7.0
Temperature	[°C]	30
Agitation	3.5 Hz	210 rpm
Total volume	[L]	1.7
Time	[hrs.]	180
Inoculum volume	[L]	0.3

Analysis

Glucose was obtained by centrifugation of the supernatant. Afterwards, the colorimetric reaction of reducing sugars with the DNS reagent was performed. The glucose concentration was determined by spectrophotometric analysis using the Dinitrosalicylic Acid method (28), measuring absorbance at 540 nm. Biomass and PHB were separated and weighed using the dry-weight method at 60 °C.

The PHB obtained was analyzed using Fourier-transform infrared spectroscopy. Spectra were averaged over 8 scans in the wavenumber range 4000-4000 cm^{-1} at a resolution of 4 cm^{-1}. Additionally, scanning electron microscopy [SEM] was used to evaluate the morphology of PHB.

Material Balance

The material balance of the bioreactions was developed to determine the relationship between glucose and glycerol used by cells as sources of carbon and energy. To develop this balance, a general chemical formula of biomass was employed.

Biomass's chemical formula was developed by some authors, based on 1 mole of carbon (29–31), considering the average elemental composition of many microorganisms as: $x = CH_{1.62}O_{0.52}N_{0.155}S_{0.0017}P_{0.012}$, with a molecular weight of 24.6 g/C-mol. In a culture medium with a lack of trace nutrients, as in the PHB production, the biomass's formula is like the PHB's: $CH_{1.5}O_{0.5}$, in these cases, the molecular weight of biomass is equal to 21.5 (32).

The following reaction equation depicts the biochemical process for producing PHB in both strains. It encompasses all biochemical reactions within the metabolic pathways shown in Figures 2 and 3, based on 1 mol of carbon [C-mol]. Clearly, this is a significant simplification, as all reactions responsible for converting substrates into biomass and the PHB product are combined into a single reaction, treated as a black box model (31).

As in our experiments, the culture media contained two substrates (glucose and glycerol), both of which were considered.

$$CH_2O + CH_{2.67}O + CH_{1.5}O_{0.5} + Y_{SO_2}\,O_2 + Y_{NX}\,(NH_4)_2SO_4$$
$$Y_{SX}\,CH_{1.5}O_{0.5} + Y_{SN}NH_3 + Y_{SC}CO_2 + Y_{SW}H_2O + Y_{SPHB}CH_{1.5}O_{0.5} +$$
$$Y_{GX}\,CH_{1.79}O_{0.5}N_{0.2} + Y_{GN}NH_3 + Y_{GC}CO_2 + Y_{GW}H_2O + Y_{GPHB}CH_{1.5}O_{0.5}$$

Where: $\boldsymbol{CH_2O}$ represents the glucose chemical formula on the basis of 1 carbon mol; $\boldsymbol{CH_{2.67}O}$ is the formula of glycerol in the same base of 1 C-mol; $\boldsymbol{CH_{1.5}O_{0.5}}$ is the formula of biomass in the same base, in a culture media with lack of nitrogen and trace elements; and $\boldsymbol{CH_{1.5}O_{0.5}}$ is the chemical formula of PHB in the same base.

$\boldsymbol{Y_{SO_2}}$ is the yield coefficient that represents the oxygen consumed by 1 mol of carbon of glucose consumed by the cells; $\boldsymbol{Y_{SN}}$ is the yield coefficient of ammonium consumed by 1 C-mol of glucose consumed; $\boldsymbol{Y_{SC}}$ is the yield coefficient of carbon dioxide produced by 1 C-mol of glucose consumed; $\boldsymbol{Y_{SW}}$ is the yield coefficient of water produced by 1 C-mol of glucose consumed; and $\boldsymbol{Y_{SPHB}}$ is the yield coefficient of PHB produced by 1 C-mol of glucose consumed.

$\boldsymbol{Y_{GO_2}}$ is the yield coefficient that represents the oxygen consumed by 1 C-mol of glycerol consumed by the cells; $\boldsymbol{Y_{GN}}$ is the yield coefficient of ammonium consumed by 1 C-mol of glycerol;

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Y_{GC} is the yield coefficient of carbon dioxide produced by 1 C-mol of carbon of glycerol consumed; Y_{GW} is the yield coefficient of water produced by 1 C-mol of carbon of glycerol consumed; and Y_{GPHB} is the yield coefficient of PHB produced by 1 C-mol of glycerol consumed.

Considering the mass balance equation, the carbon mass balance is calculated as follows: it is the sum of glucose consumed, glycerol consumed, and initial biomass concentrations, minus the PHB produced from glucose and glycerol, minus the biomass generated, and minus the carbon dioxide produced, as shown in the next reaction equation.

$$CH_2O + CH_{2.67}O + CH_{1.5}O_{0.5} - Y_{SX}\,CH_{1.5}O_{0.5} - Y_{SC}CO_2 - Y_{SPHB}CH_{1.5}O_{0.5} - Y_{GX}\,CH_{1.5}O_{0.5} - Y_{GC}CO_2 - Y_{GPHB}CH_{1.5}O_{0.5} = 0$$

Experimental results for glucose, glycerol, PHB, and biomass were obtained, and the carbon dioxide concentrations were calculated by mass balance. To assess whether the results were sufficient, the yield coefficients for both strains were compared with those reported in the literature for PHB production. For B. subtilis, the reported yield coefficient CO_2/substrate ranges from 0.35 to 0.55 C-mol of CO_2 per C-mol of glucose. For *B. megaterium*, the same yield coefficient ranges from 0.4 to 0.7 C-mol of CO_2 per C-mol of glucose (24,33).

Model

The growth kinetics of *B. megaterium* and *B. subtilis* were modeled using the logistic equation, based on the experimental results for each strain.

The logistic growth model assumes that biomass growth depends on cell biomass; however, this growth is limited by factors that prevent cells from surpassing a maximum population size (see equation 6). In these cases, the limiting factors are the lack of nitrogen, and sometimes, trace elements. The initial condition at time = 0 was the biomass initial concentration for each experiment.

$$\frac{dx}{dt} = \mu_{max}\left(1 - \frac{x}{x_{max}}\right)x; \qquad x(0) = x_i \tag{6}$$

The production of PHB for both strains was simulated using the Luedeking-Piret model, with an initial product concentration [PHB] set to zero. This is an empirical model used to describe the specific production rate of metabolites. It considers the production rate to be the sum of primary and secondary metabolites; primary metabolites are produced through pathways related to growth, while secondary metabolites are produced through pathways unrelated to growth. Refer to equation 7, where α is a factor related to the products within growth's metabolic pathways, and β is a term that represents the rate of product formation independent of cell growth. The initial condition for this equation is that the P (PHB) concentration is zero.

$$\frac{dP}{dt} = \alpha\,\frac{dx}{dt} + \beta x; \qquad P[0] = 0 \tag{7}$$

The substrate used for maintenance was modeled with the Pirt model to describe how microorganisms consume energy for growth and maintenance, including cell material turnover and maintaining concentration gradients across cell membranes (see equation 8). The initial condition for this equation is that the substrate concentration equals the concentration at time zero.

$$\frac{1}{x}\frac{dS}{dt} = \frac{\mu}{Y_G} + m; \qquad S(0) = S_i \tag{8}$$

Using the three models, the change in substrate concentration over time results from growth, PHB production, and cell maintenance; see equation 9.

$$\frac{dS}{dt} = \frac{1}{Y_{x/S}}\frac{dx}{dt} + \frac{1}{Y_{P/S}}\frac{dP}{dt} + m_S x \tag{9}$$

Author Contributions: Conceptualization: MMGB and HHLS; methodology: HHLS, JAA, DAB, JLCL; validation: RRC, JCGM; formal analysis: MMGB; investigation: JAA, HHLS, MMGB; resources: RRC; writing—

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original draft preparation: MMGB; writing—review and editing: JCGM, RRC, MMGB; All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare no conflicts of interest.

Abbreviations

The following abbreviations are used in this manuscript:

A-CoA-R	Acetoacetyl coenzyme A reductase
ADP	Adenosine diphosphate
Ald	Aldolase enzyme
ATP	Adenosine triphosphate
Bio-PE	Bio-based-polyethylene
Bio-PET	Bio-based polyethylene terephthalate
Bio-PP	Bio-based-polypropylene
BM	Biomass
$\%\ C_S^E$	Percentage of glucose consumed at the end of the exponential growth phase
$\%\ C_S^F$	Percentage of glucose consumed at the end of the cell death phase
C_{Si}	Substrate concentration at time = 0
C_{Sf}	Substrate concentration at the end of the experiment
C_{Pfe}	PHB concentration at the end of the exponential growth phase
C_{Pfm}	Maximum PHB concentration obtained
C_{Se}	Substrate concentration at the end of the exponential growth phase
$\frac{dP}{dt}$	Specific PHB production rate [$g_P\ L^{-1}\ h^{-1}$]
$\frac{dS}{dt}$	Specific substrate consumption rate [$g_S\ L^{-1}\ h^{-1}$]
$\frac{dx}{dt}$	Specific growth rate [$g_x\ L^{-1}\ h^{-1}$]
Eno	Enolase enzyme
g	Gram
GAL-I	Galactose-6-phosphate isomerase enzyme
Gal-S	B-galactosidase enzyme
Glu-K	Glycerol kinase enzyme
Gly-K	Glycerol kinase enzyme
3HB	3-hydroxybutyrate
4HB	4-hydroxybutyrate
HD	3-Hydroxydecanoate
HHc	3-Hydroxyhexanoate
3HV	3-Hydroxyvalerate
L	Liter

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MT	Million tons
Mut	Mutase enzyme
NAD	Nicotinamide adenine dinucleotide
NTE	Without trace elements
P	Product [PHB] concentration [g L^{-1}]
PE	Polyethylene
PET	Polyethylene terephthalate
PP	Polypropylene
PHAs	Polyhydroxyalkanoates
PHB	Polyhydroxybutyrate
PHB-S	β-PHB synthase enzyme
PhG-K	Phosphoglycerate kinase enzyme
PLA	Polylactic acid
PTS	Lactose-specific phosphotransferase system
PVC	Polyvinylchloride
S	Substrate concentration [g L^{-1}]
t	Time [h]
Tag-K	Tagatose-6-phosphate kinase enzyme
TCA	Tricarboxylic acid cycle
TE	Trace elements
Tri-I	Triosephosphate isomerase enzyme
x	Biomass concentration [g L^{-1}]
x_{max}	Maximum biomass concentration [g L^{-1}]
Y_G	Actual growth, maximum yield
$Y_{P/S}^{E}$	Yield coefficient PHB/Substrate at the end of the exponential growth phase
$Y_{P/S}^{M}$	Yield coefficient of PHB production/substrate at the maximum PHB concentration obtained.
$Y_{X/S}^{E}$	Yield coefficient of biomass/substrate at the end of the exponential growth phase

Subindex

C	Carbon dioxide
e	In the exponential phase of growth
G	Glycerol
i	Initial, time = 0
N	Ammonium sulfate
PHB	Polyhydroxybutyrate
S	Glucose
w	Water
x	Biomass

Greek Letters

α	A specific constant of maintenance dependent on growth

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β	A specific constant of maintenance independent of growth
β-ket	β-Keotiolase enzyme
β-hBD	β-hydroxybutirate dehydrogenase enzyme
μ_{max}	Maximum specific constant growth rate

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