

Article

Not peer-reviewed version

---

# Synthesis of Polyhydroxybutyrate (PHB) by Fermentation of Whey and Pineapple Peel with *Bacillus subtilis*

---

[Jorge Amaya](#) \*

Posted Date: 10 July 2025

doi: 10.20944/preprints202507.0943.v1

Keywords: fermentación; polihidroxibutirato; *Bacillus subtilis*



Preprints.org is a free multidisciplinary platform providing preprint service that is dedicated to making early versions of research outputs permanently available and citable. Preprints posted at Preprints.org appear in Web of Science, Crossref, Google Scholar, Scilit, Europe PMC.

Copyright: This open access article is published under a Creative Commons CC BY 4.0 license, which permit the free download, distribution, and reuse, provided that the author and preprint are cited in any reuse.

Disclaimer/Publisher's Note: The statements, opinions, and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions, or products referred to in the content.

## Article

# Synthesis of Polyhydroxybutyrate (PHB) by Fermentation of Whey and Pineapple Peel with *Bacillus Subtilis*

Amaya Pinos Jorge B

Salesian Polytechnic University, Cuenca campus, Ecuador 1; jamaya@ups.edu.ec

## Abstract

Environmental pollution caused by petroleum-based plastics is a global problem due to the accumulation of waste, microplastic pollution, and greenhouse gas emissions. In this context, bioplastics, such as polyhydroxybutyrate (PHB), emerge as a sustainable alternative, as they are biodegradable and produced from renewable resources. This study evaluated the use of agro-industrial waste from Ecuador, specifically whey and pineapple peel, as carbon sources for the production of PHB through fermentation with *Bacillus subtilis*. Enzymatic hydrolysis of whey was performed with lactase, obtaining an optimal concentration of 761 ppm of glucose in 2 hours. For pineapple peel, acid hydrolysis with 6%  $H_2SO_4$  was used, achieving a concentration of 5125.81 mg/L of reducing sugars in 30 minutes. Whey fermentation was carried out at pH 6, 37 °C, and 7 days of incubation, while pineapple peel fermentation was carried out at pH 7.7 and 36.7 °C. Whey fermentation produced a higher PHB yield (0.331 g/L) compared to pineapple peel (0.272 g/L). These results demonstrate the potential of both wastes as economical and sustainable substrates for obtaining PHB, highlighting whey as a more efficient source. This study demonstrates that the use of agro-industrial waste for PHB production through fermentation with *Bacillus subtilis* is a viable and efficient strategy, highlighting whey as a more effective carbon source compared to pineapple peel, which could have a significant impact on waste reduction and environmental sustainability.

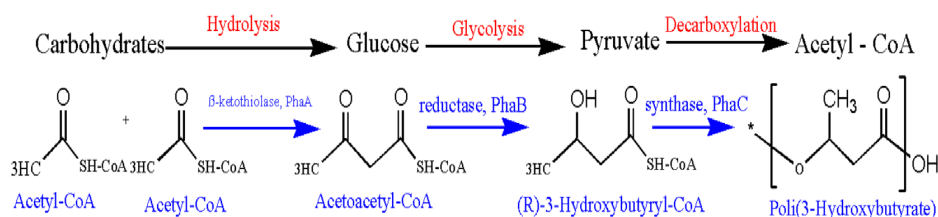
**Keywords:** fermentación ; polihidroxibutirato; *Bacillus subtilis*

## 1. Introduction

In Ecuador, whey and pineapple peel waste presents significant environmental challenges; the dairy industry generates approximately 900 thousand liters of whey per day, of which only 10% is used, since regulations limit its commercial use [1]. As a result, whey becomes a waste product, causing contamination and waste of a nutritious resource [2,3]. In turn, the high import and consumption of single-use plastics in the country aggravates the waste problem, while pineapple peels are a significant environmental problem, the processing and cultivation operations of this fruit generate a large amount of by-products, mainly peels and crowns, which are usually not used in a sustainable manner [4,5]. In this context, the use of pineapple whey and peel as carbon sources for the production of PHB biopolymer by fermentation with *Bacillus subtilis* offers a sustainable alternative [6,7]. The production of PHB, a biodegradable and biocompatible biopolymer, represents a sustainable alternative to conventional plastics, with the advantage of reducing environmental impact and taking advantage of by-products considered waste [8–10]. Although whey is a valuable dairy by-product, its use in Ecuador is limited by commercial restrictions, generating a significant surplus that contributes to pollution [11,12]. On the other hand, lignocellulosic biomass, such as pineapple peel, reduces the costs of biopolymer production, since it offers a cheap and abundant source of carbon [13]. This process converts these residues into biopolymers, promoting the reduction of agroindustrial waste and offering an environmentally friendly option to conventional plastics [14–16].

PHB is a biopolymer belonging to the family of polyhydroxyalkanoates (PHA), which are polyesters synthesized intracellularly by bacteria such as *Bacillus subtilis* in response to stress conditions, such as an excess of carbon and a limitation of other essential nutrients [17,18]. Its chemical structure is that of a linear polyester with repeating 3-hydroxybutyric acid units, characterized by ester bonds connecting the monomers (3-hydroxybutyrate units), which gives it properties of biodegradability and biocompatibility [19,20].

PHB is synthesized mainly as a result of carbon assimilation as shown in Figure 1, from sources such as glucose, starch and sources containing large amounts of polysaccharides linked by glycosidic bonds that can act as an intracellular energy reserve in certain microorganisms [21].



**Figure 1.** Synthesis of poly-3-hydroxybutyrate (P3HB). Source:

To obtain PHB, it is produced by a biochemical pathway for synthesis from carbohydrates [22]. These carbohydrates undergo metabolic processes, such as glycolysis to generate pyruvate, then pyruvate is converted to acetyl-CoA by a decarboxylation reaction [23,24]. Subsequently, two acetyl-CoA molecules are condensed to form intermediates such as acetoacetyl-CoA and then reduced to (R)-3-hydroxybutyryl-CoA and finally polymerized to form P3HB [25,26].

This study explores the use of agro-industrial wastes, such as whey and pineapple peel, in the production specifically of PHB by fermentation with the bacterium *Bacillus subtilis*. This bacterium, widely studied in biotechnology, has the ability to synthesize PHB intracellularly in response to stress conditions, such as nutrient limitations like nitrogen or phosphorus, or the presence of excess carbon. This project focuses on optimizing fermentative conditions and characterizing the PHB obtained, promoting sustainable biotechnological practices for waste management and production of biodegradable materials.

## 2. Materials and Methods

The following are the procedures that were carried out for pineapple peel and whey.

### 2.1. Pineapple Peel

In the present investigation, a finite population was studied, which is the case of pineapple residues (peel), which served as the only carbon source for *Bacillus subtilis* in the production of PHB. With these residues, the chemical proximate analysis was carried out in triplicate.

#### 2.1.1. Moisture

The Mettler Toledo HB moisture analyzer was used to measure moisture in pineapple peels, using a 4 g sample.

#### 2.1.2. Total Ash

The ash protocol contained in AOAC section 923.03 (1990) was followed, which consisted of measuring the ash in the sample with a sample particle size at 250 µm, and the results were expressed using equation 1:

$$\%Ashes = \frac{(P_2 - P_0)}{(P_1 - P_0)} \times 100 \quad (1)$$

Where:  $P_0$  = Weight of empty crucible,  $P_1$  = Weight of crucible with sample,  $P_2$  = Weight of crucible with ash.

### 2.1.3. Total Fats

To determine total fats in pineapple peels, the ether extraction method described in AOAC (1990) procedure 920.39 c was used. Initially, 1 g of the pulverized sample was weighed and 10 ml of 50%  $H_2SO_4$  was added, followed by chemical digestion to remove non-fat components. Subsequently, by extraction with petroleum ether, the ethereal phase was recovered. The percentage of fat present in the peels was calculated using equation 2:

$$\%Fats = \left( \frac{\text{final weight} - \text{empty container}}{\text{g sample}} \right) \times 100 \quad (2)$$

### 2.1.4. Hydrolysis Treatment

The substrate was subjected to acid hydrolysis with the objective of maximizing the release of sugars, especially glucose, through the decomposition of cellulose and starch. For this purpose, 10 g of the substrate was mixed in 100 ml of water, adding diluted  $H_2SO_4$  at 4 - 6% v/v. This solution was heated between 95 and 121 °C for a range of 10 - 83 min. Subsequently, the pH of the hydrolysate was adjusted to a range of 4.8 to 6.5 using a buffer prepared with citric acid ( $C_6H_8O_7$ ) and sodium acetate ( $CH_3COONa$ ).

### 2.1.5. Substrate Conditioning

In this study, the methodology proposed by [27] for pretreatment of pineapple peels was applied. However, adjustments were made based on the recommendations of [28], who investigated obtaining glucose syrups from the same peels by enzymatic hydrolysis. The main objective was to avoid the formation of inhibitors during the process. In response to the acid pH of the hydrolysate, a buffer solution was prepared using specific compounds that do not alter the sugars present. This solution, composed of citric acid, sodium acetate and calcium carbonate, was accurately prepared: 2.10 g of citric acid and 2.94 g of sodium acetate in 100 ml volume. Then, the pH was adjusted to 6.2 by the combination of HCl and  $CH_3COONa$ . In addition, the buffer was fortified with calcium carbonate to avoid undesired changes in pH during fermentation.

### 2.1.6. Quantification of Reducing Sugars

In this study, the 3,5-dinitrosalicylic acid (DNS) method was used to quantify reducing sugars in pineapple (*Ananas comosus*) peel hydrolysis. The method, previously described by [29], was adapted by preparing specific dilutions to construct a calibration curve. To establish the standards, glucose and fructose solutions were used, each with an initial mass of 50 mg. From these, dilutions were prepared with concentrations of 5000, 500, 50 and 5 ppm. The procedure consisted of taking 1 ml of the standard solution and adding 1 ml of the DNS reagent. Subsequently, the presence of glucose and fructose in the hydrolyzed samples was analyzed using a UV-Vis spectrophotometer (Jasco V-630 equipment).

### 2.1.7. Activation and Ambient of Microorganism *Bacillus Subtilis* ATCC (6633)

*Bacillus subtilis* strain ATCC (6633) was used and seeding was performed on semi-solid nutrient Agar for 48 hours at a temperature of 37 °C in an oven. Within the framework of this study, an adaptation of the carbon source used by the microorganism was carried out. In this case, the new carbon source consisted of xylose obtained from pineapple peels. To achieve this, initial mixtures of the new substrate were made with nutrient agar, evaluating the tolerance of the microorganism until it largely replaced the traditional carbon source. These adaptations were carried out in flasks with orbital shaker (Thermo Scientific MaxQ 4000) at a pH of 6.2 and a temperature of 36.7 °C, for a period of 96 hours. Three experimental conditions were carried out as detailed in Table 1, where M1 is the

medium containing nutrient agar with 75 ml of substrate, M2 contains nutrient agar with 50 ml of substrate and M3 nutrient agar with 25 ml of substrate.

Table 1. Fermentation treatments.

Treatment	pH	Temperature °C	Medium
T1	6.20	37	M3
T2	6.20	37	M2
T3	6.20	37	M1
T4	5.67	35.8	M1
T5	5.67	35.8	M2
T6	7.77	36.7	M3
T7	7.77	36.7	M2
T8	7.77	36.7	M1

2.1.8. Fermentation Treatment

To perform the fermentation for PHB production, a one-way structure was proposed with a completely randomized design (CRD) and sequencing in transverse log, where the treatments were subjected to destructive testing. Table 1 shows the treatments modeled by the combination of: pH, temperature and medium for the fermentative production of PHB and the methodology proposed by [30] for the fermentative production of PHB using *Bacillus subtilis* bacteria was applied. Modifications were made to the operating conditions, implementing eight treatments previously described.

2.1.9. Extraction and Purification of PHB

For the extraction and purification, the methodology mentioned in the work of [31] called "Extraction, purification and modification of a biopolymer of the poly (3-hydroxybutyrate) type obtained from the fermentation of fatty acids with *B. cepacia*" was applied with the chloroform-sodium hypochlorite digestion (4% v/v). Once all the samples were purified, the product obtained was weighed in mg/mg to determine the yield obtained (SPY) in each treatment using equation 3:

$$\%SPY = \left( \frac{\text{product produced (mg)}}{\text{substrate consumed (mg)}} \right) \times 100 \tag{3}$$

2.1.10. Characterization of PHB in the FT-IR

For the characterization of PHB, the Fourier transform infrared spectroscopy technique was used, which was analyzed in Thermo Scientific Nicolet TM S10 FT-IR

2.2. Whey

2.2.1. Whey Preparation

The whey was obtained from an artisanal cheese production company located in the city of Cuenca and subsequently stored at a temperature of 2 °C. The collected material was subjected to several procedures. First, vacuum filtration was carried out in order to eliminate the proteins present. Next, a heat treatment was applied at 121 °C for 15 min to induce protein precipitation. Subsequently, centrifugation was performed to separate the proteins and remove the added water. Finally, the pH was adjusted to 6.5 using NaOH 1N in order to preserve the sugars. All procedures were carried out under aseptic conditions to avoid degradation of the sugars present.

2.2.2. Physicochemical Characterization of Spent Whey

Physicochemical characterization of the whey involved analyzing the following variables: pH, moisture content, sugar quantification, and enzymatic hydrolysis.

### 2.2.3. pH and Moisture Content

With the amount of 500 ml of substrate, pH levels were adjusted to 5, 6, 7 and 8 using 1 N NaOH. To determine the moisture content, a Mettler Toledo HB moisture analyzer with halogen lamp was used, in which 2 g of the sample was placed and placed in the analyzer tray. During the analysis the device monitored the weight loss until a constant mass was reached.

### 2.2.4. Quantification of Sugars

To carry out the validation, the DNS (3,5-dinitrosalicylic acid) method was followed, and for this, 0.8 g of NaOH was dissolved in distilled water, then 15 g of sodium tartrate was added and, finally, 0.5 g of DNS and the mixture was brought to a volume of 50 ml. For the calibration curve, absorbance readings were measured at 540 nm using 200, 400, 800 and 1000 ppm standard solutions. After obtaining the calibration curve for glucose as a standard, the samples to be analyzed were prepared: 5 ml of each sample was heated in a water bath for 5 minutes and the reaction was stopped with cold water. Subsequently, the sample was reconstituted with 5 ml of H<sub>2</sub>O, shaken and allowed to stand for 15 minutes. This procedure was repeated for each sample to be analyzed. The calibration curve was quantified by linear regression.

### 2.2.5. Enzymatic Hydrolysis

Using the methodology applied for the enzymatic hydrolysis of [32], enzymatic hydrolysis was carried out on each of the treatments using commercial LACTFLAT tablets. Six 1/2 tablets were dissolved in 18 ml of distilled water to achieve a concentration of 30 mg/ml lactase. The pH of all treatments was then adjusted to 6.5 to facilitate 99.99% enzymatic hydrolysis. To each treatment, 1 ml of the enzyme dilution was added. Three of the four treatments were incubated for one hour in a Shaker orbital incubator with agitation at 100 rpm and a temperature of 40°C. The fourth treatment was subjected to the same conditions, but for two hours. Subsequently, the pH of the four treatments was adjusted to values of 5, 6, 7 and 8, respectively. These were stored in a refrigerator at 2°C until further use for fermentation.

### 2.2.6. Activation and Microscopic Characterization of *Bacillus Subtilis*

For activation of the *Bacillus subtilis* ATCC 6051 strain, 2 ml of strain was used and inoculated in 10 ml of Trypticasein Soy Broth (TSB) for 24 hours at 37°C. The bacteria were then characterized using Gram staining to distinguish bacterial cell walls based on their response to crystal violet and iodine [33]. Initially, bacterial cells were heat fixed on a slide for adherence, subsequently stained with crystal violet followed by iodine to form a complex within the cell walls. Then a decolorization with alcohol-acetone was performed to differentiate between Gram-positive bacteria (retained staining) and Gram-negative bacteria (decolorized). Finally, a counterstain with safranin was applied to visualize Gram-negative bacteria, facilitating the identification and characterization of *Bacillus subtilis* based on its Gram-positive staining properties.

### 2.2.7. Reseeding of *Bacillus subtilis* Strain from Activation

For the reseeded process, eight test tubes were prepared, each containing 10 ml of medium. In pairs, the first two tubes each had 0.5 ml of activated bacteria, the next two had 1 ml, the next two had 1.5 ml and the last two had 2 ml of bacteria. Subsequently, the bacteria were counted and for this purpose followed the methodology proposed by [34] using the turbidity method, which analyzes the samples depending on the amount of light passing through the culture (see Table 2). Four standards were used and the absorbance at 600 nm of the previously prepared cultures at different pHs was measured.

Table 2. Bacteria count.

McFarlan standard	Absorbance (600 nm)	Bacterial suspension /mL
0.5	0.08-01	1,5x10 <sup>8</sup>
1.0	0.18-0.2	3,0x10 <sup>8</sup>
2.0	0.36-0.4	6,0x10 <sup>8</sup>
3.0	0.54-0.6	9,0x10 <sup>8</sup>

2.2.8. Fermentation

Sixteen experiments were carried out using different combinations of parameters to evaluate fermentation with *Bacillus subtilis*. Each sample consisted of 112 ml of substrate and 10% v/v bacterial inoculum, ensuring an initial cell concentration between 1.5×10<sup>8</sup> and 9×10<sup>8</sup> cells/ml. The assays were performed in an orbital shaker incubator under constant shaking at 200 rpm, with pH values adjusted to 5, 6, 7 and 8, and temperatures between 30 °C and 40 °C. Fermentation times varied between 5 and 8 days according to the specific conditions of each sample, with the objective of analyzing the combined effect of temperature, pH and cell concentration on the fermentation process.

2.2.9. Obtaining Biomass and PHB Extraction

Two parameters were evaluated, wet weight and dry weight, the biomass was separated from the medium by centrifugation at 1200 rpm for 20 minutes [35]. To determine the wet weight the biomass and medium were centrifuged in Eppendorf tubes according to the previous parameters. Then, the precipitated biomass was transferred to already weighed Petri dishes. For dry weight, the sample was subjected to a climate chamber at 60°C for 24 hours to remove all moisture. The samples were then ground at 2500 rpm using a mill with 1.5 mm sieves and the ground biomass was weighed. Next, the SDS digestion and chloroform dispersion method was used to extract and purify the biopolymer.

2.2.10. Chemical Digestion with SDS (Sodium Dodecylsulfate)

Following the methodology of [36], polymer quantification was initiated by adapting the biomass quantification procedure without the drying step. This approach aimed to ensure that residue-free biomass from the culture medium could be digested and PHB quantified. The resulting precipitate was suspended in a 20% SDS solution, as shown in Figure 2, at a concentration of 0.55 ml/g and digested at 40°C for 2 hours. Subsequently, the samples were centrifuged at 3500 rpm for 30 min to remove SDS and biomass residues. Finally, the wet biomass was transferred to Petri dishes for drying.



Figure 2. Application of SDS to wet biomass. Source: The authors.

2.2.11. Dry Sample Analysis in FTIR with SDS

The dry samples, after being treated with SDS, were measured by FTIR to analyze their content. This analysis was performed to identify and quantify the chemical composition and structural characteristics of the samples

2.2.12. Purification and Structuring of PHB with Chloroform

The cells obtained after digestion with SDS and subsequent drying were suspended in chloroform using a ratio of 20 ml of solvent per gram of biomass this according to the procedure of [37]. They were then stirred at room temperature for 24 hours. This process produced two distinct phases: a liquid phase composed mainly of polymer and chloroform, and a solid phase retained on the filter, composed of biomass residues. The polymer solution was poured into Petri dishes and left at room temperature to allow the chloroform to evaporate, resulting in a P3HB film.

3. Results

3.1. Pineapple Peel

3.1.1. Chemical Proximate Analysis of Pineapple Peel

The results obtained from the proximate analysis of pineapple peel are influenced by the variety of pineapple, its temperature, drying time, drying method used, among others. Table 3 shows the values obtained for moisture, ash and total fat.

Table 3. Proximal chemical analysis of sugarcane.

Pineapple	% Moisture	% Total ash	% Total fat
Sample 1	3.17	4.45	3
Sample 2	2.3	4.92	2
Sample 3	2.1	5.94	2
Average	2.52	5.10	2.33

The average percentage resulting from the triplicate samples gives us a value of 2.51% for pineapple peel. In the work of [38] he states that moisture is inversely proportional to temperature and time of drying and that this is the reason why the moisture percentages occupy a wide range, from 2.33% to 3.68% when working at temperatures from 70 to 80°C. In the results obtained by [39], he obtained values of 2.65% of ashes in dry basis and 9.84% of humidity, attributing as causal factor to the drying temperature at which he worked and also to the origin of the pineapple, however, the data obtained in this work do not differ from others reviewed.

The average amount of ash or inorganic residues of the three samples analyzed was 5.10%, slightly different from the value explained by [38] who in his study obtained the nutritional composition for different parts of the pineapple, corresponding to 5.2% ash.

The percentage data of the fat content present in each sample of pineapple peel in triplicate are presented in Table 9. The average value of total fat was 2.33%, which agrees with the literature as indicated by [40] that there is a range of 2-3% but that, depending on the variety of pineapple, the same is extended by 0.6 - 5.57%.

3.1.2. Hydrolysis Treatment

The acid hydrolysis process of pineapple peel, as illustrated in Figure 3, consisted of subjecting the lignocellulosic residues to the action of sulfuric acid under controlled temperature conditions. Initially, the peels are weighed and placed in flasks together with a solution of sulfuric acid at different concentrations. This system is kept under constant agitation and heating, ensuring the degradation of the polysaccharides into reducing sugars, such as glucose, which act as precursors for the production of biopolymers such as PHB.



**Figure 3.** Pineapple peel hydrolysis treatment. *Source: The authors.*

3.1.3. Quantification of Reducing Sugars

As a result of the hydrolysis of the substrate (pineapple peel), the monosaccharides (glucose and fructose) present were quantified, as shown in Table 4. The analysis of the amount of fructose present was done as a standard since it represents xylose as a pentose for later statistical analysis.

**Table 4.** Results of quantification of reducing sugars.

Conc (%) $H_2SO_4$	Time (min)	Temperature $^{\circ}C$	Glucose (mg)	Fructose (mg)
6	15	121	700,86	762,33
5	30	118	5125,81	5035,24
6	83	98	1097,87	1742,49
4	20	115	513,68	442,34
4	10	121	428,21	343,985
6	30	121	621,509	967,964
5	80	100	268,13	399,78
6	20	115	774,86	1189,54
5	25	121	887,26	1023,95
4	83	98	312,25	338,907
6	25	115	697,991	813,303
5	28	120	4782,261	5025,272
5	15	121	682,664	550,971
4	15	121	492,16	295,52
5	80	98	713,551	1513,26
5	60	105	601	450,44

3.1.4. Analysis of Hydrolysis Results

The analysis of reducing sugars presents an R-squared of 43.48%, implying that the model moderately explains the variability in the data, although the adjusted R-squared (0%) suggests limited predictive improvement when adjusting for the number of variables. The standard error of the estimate is 1774.94 and the mean absolute error is 861.8, both reflecting considerable variability in the results and limitations in the accuracy of the model. The Durbin-Watson statistic of 1.7972 and the low residual autocorrelation of Lag 1 (0.0419) suggest independence in the errors, a positive feature for the reliability of the analysis, although the high errors indicate possible areas for improvement of the predictive model in future studies.

*Response Surface Plot*

The response surface plot (Figure 4) showed an optimum hydrolysis corresponding to a value close to 5130 with an acid concentration close to 5.35% optimum in a time close to 35 min, for a temperature of 118,15°C.

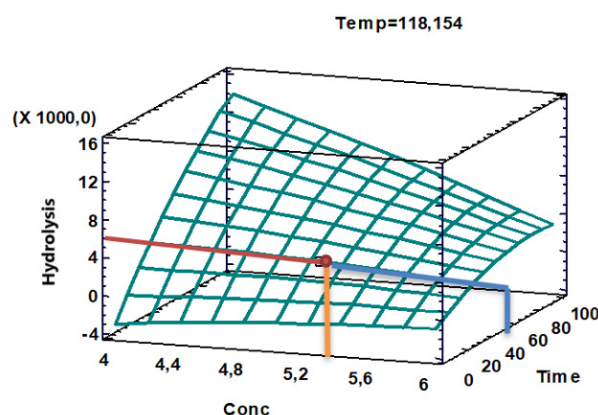


Figure 4. Response surface plot at 118.15°C. Source: The authors.

### 3.1.5. Statistical Analysis of Reducing Sugars: Fructose

In this analysis, 0 effects have a p-value less than 0.05, indicating that they are significantly different from zero at a 95% confidence level. The values obtained indicate the moderate quality of molding prediction being: The R-squared statistic corresponding to the proportion of variability in hydrolysis data is 40.26% The standard error is 1774.94 which indicates the standard deviation of the residuals. The mean absolute error (MAE) of 903.678 corresponds to the average margin of error of the residuals in the predictions from the model used. The Durbin-Watson (DW) statistic of 1.7972 for this study indicates the absence of significant autocorrelation in model errors, i.e., these data do not have a significant influence by the order in which they are presented. This is justified by the p-value which is greater than 5%, thus there is no serial autocorrelation. In Figure 4 I present the values that the main influential variables in the fructose hydrolysis process applied for the pretreatment of pineapple peel should take, in order to achieve a better separation of the lignocellulosic matrix. For the concentration factor, the acid should have a concentration of 5.25%, the fermentation time should be in the range of 45 min and the ideal temperature should be close to 118°C, values that agree with and support the Pareto graphs and response surface graphs.

#### Response surface plot

The response surface plot (Figure 5) showed an optimum hydrolysis corresponding to a value close to 5035.24 with an acid concentration close to 5.35% optimum in a time range of 35- 40 min.

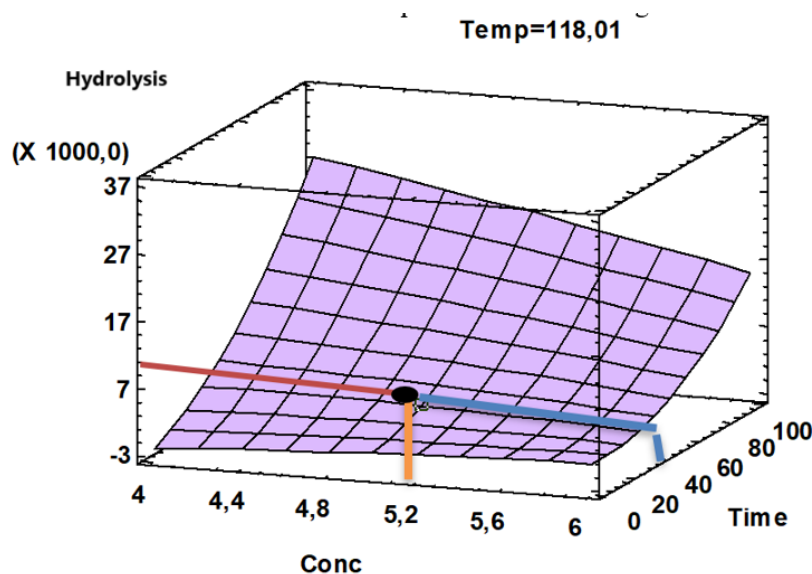


Figure 5. Response surface graph at 118.01 °C. Source: The authors.

3.1.6. Yield of PHB from Substrate

The yield was calculated by dividing the amount of product formed for the substrate consumed for all 8 treatments proposed (see Table 5) in the methodology with its triplicate to check the yield of *Bacillus subtilis* using pineapple peel as PHB producer.

**Table 5.** Treatments applied with *Bacillus subtilis* for PHB production.

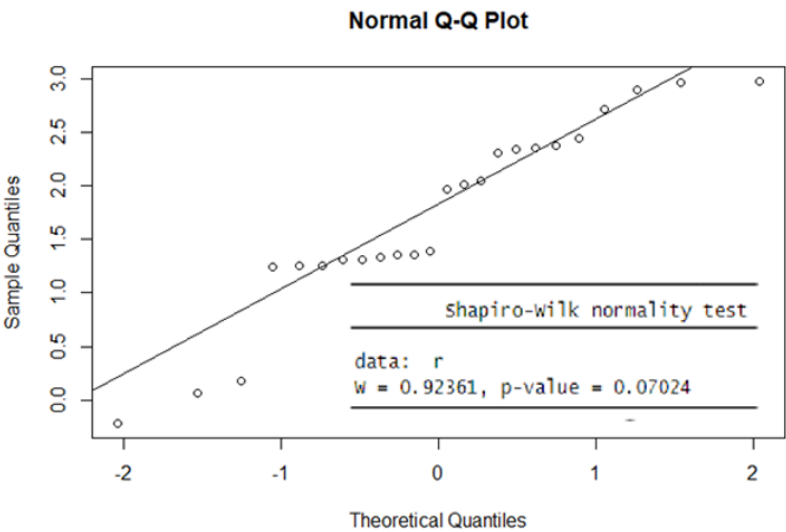
Medium	T1	T2	T3	T4	T5	T6	T7	T8
M 1	2,40	2,60	3,50	3,90	7,80	109,0	108,8	109,1
M 2	2,60	3,80	3,80	3,50	7,50	109,1	109,0	108,1
M 3	2,30	2,50	3,50	3,70	7,20	108,4	108,9	108,0
Average	2.43	2,96	3,6	3,7	7,5	108,83	108,9	108,4

Table 6 provides information corresponding to the PHB yield obtained in each treatment from the *Bacillus subtilis* bacteria, with the highest value for treatment T7, which showed the best results. At a general level, it is possible to show that the best results were obtained with the conditions of treatment 7 (T7) with 0.27 being this yield value the highest, followed by treatment 6 (T6) with 0.11 and finally treatment 8 (T8) with 0.0911.

**Table 6.** Performance of *Bacillus subtilis* for PHB production.

Treatment	Product formed (mg)	Substrate consumed (mg)	Yield of PHB from substrate (RPS)
T1	2,43	762,33	3.18x10-3
T2	2,96	5035,24	5.878 x10-4
T3	3,6	1742,49	2,066 x10-3
T4	3,7	442,34	8.36 x10-3
T5	7,5	343,985	0.068
T6	108,83	967,964	0.11
T7	108,9	399,78	0,272
T8	108,4	1189,54	0.0911

Next, in Figure 6, the result of the Shapiro-Wilk normality test suggests that there is not enough evidence to reject the null hypothesis of normality for this data set ( $p > 0.01$ ). In addition, the Q-Q plot shows a scatter of points following a straight line (see Figure 6), which supports the idea that the data could be from a normal distribution.



**Figure 6.** Shapiro-Wilk normality test. Source: The authors.

An ANOVA analysis was then performed, which, indicates that there are statistically significant differences between treatments, as the F-value is large and the p-value is extremely small. This suggests that at least one treatment mean is different from the others. In addition, Tukey’s post hoc analysis identifies specific pairs of treatments with significant differences in their means.

As can be seen from the results in Table 7 that in T7, T8 (b) and T3, T2, T4 (d) there is no significant difference between treatments; while in treatments T1 (e) and T6 there is a significant difference with respect to the rest of the treatments.

Table 7. ANOVA analysis of treatments.

	Df	Sum Sq	Mean Sq	F value	Pr(>F)	producto.t	groups	significance
tratamientos	7	18,123	2,5891	223,6	9.56e-15	T6	2,950729	a
Residuals	16	0,185	0,0116			T7	2,514396	b
						T8	2,338398	b
tratamientos	***					T5	2,014369	c
Residuals						T3	1,318426	d
						T2	1,311451	d
Signif.	codes:					T4	1,307357	d
0 "***"	0,001 "**"	0,01 "*"	0,05 ","	0,1 " "	1	T1	0,008946	e

3.1.7. Characterization of PHB Powder by FT-IR

In this analysis, the most important or representative peaks of the spectrum of all PHB products obtained were observed in order to verify their structure.

In the spectrum of Figure 7, it shows a sharp absorption band at 1723.04  $cm^{-1}$  corresponding to stretches of the C=O bond, another band was observed at 1277.87 corresponding to the C-H group, the presence of these bands have been reported as representative peaks of PHB. Intense bands were also observed between 1000 and 1300  $cm^{-1}$ , characteristic of the C-O ester group, in addition the bands at 2851.16 and 2921.07  $cm^{-1}$  show the presence of  $CH_3$  and  $CH_2$  with asymmetric and symmetric stretches respectively. The bands showing the terminal OH group are 3286.40  $cm^{-1}$ , so it can be induced that it contains the PHB peaks.

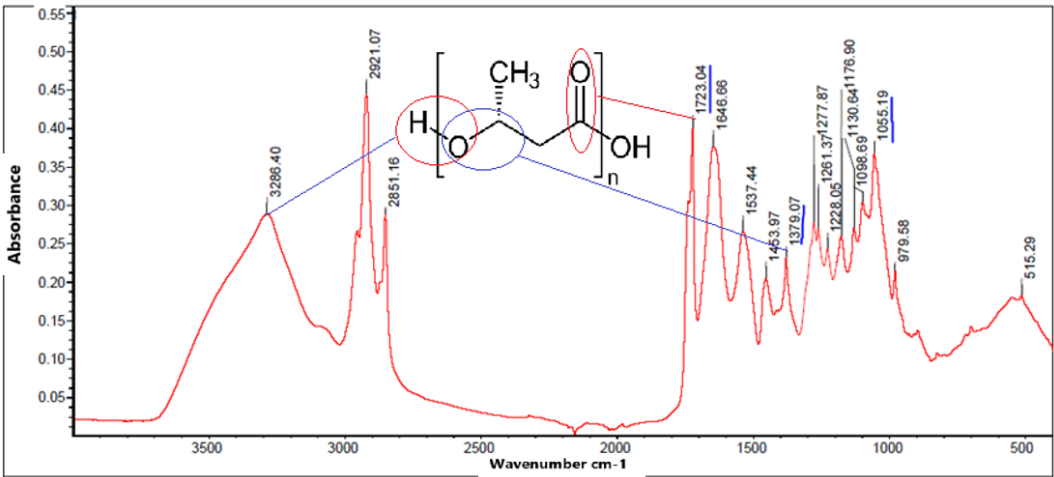


Figure 7. FTIR spectrum of PHB. Source: The authors.

3.1.8. Proximal Chemical Analysis of Lacto-Serum

Moisture content analysis was performed on the whey used to produce (PHB) with Bacillus subtilis. This analysis is crucial for the efficiency of the fermentation process and the final yield of PHB. The results indicated that the whey had a moisture content of 91.64%, which is typical for whey, which naturally retains a significant amount of water

3.1.9. Quantification of Sugars

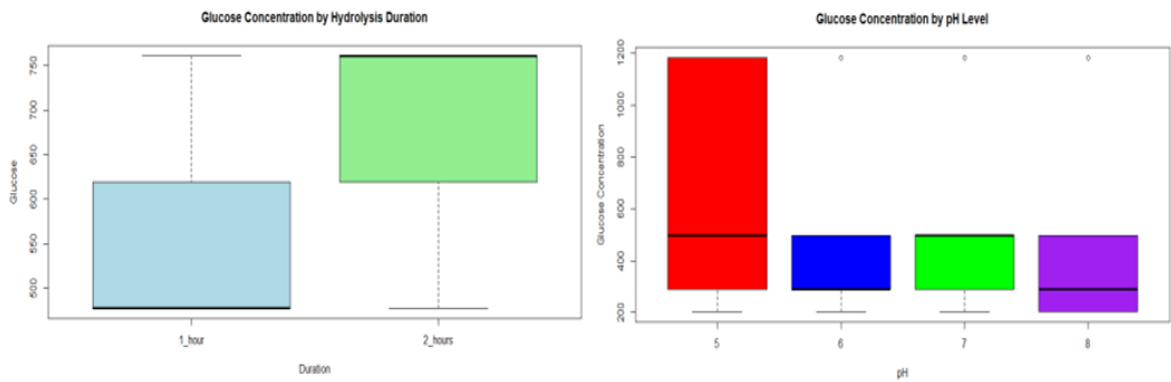
Quantitative analysis of the sugars present in the whey at different pH levels (5, 6, 7 and 8) was performed. This analysis made it possible to determine how these pH variations affect the concentration of hydrolyzed whey. The results indicated that an acidic pH (such as Sample 1, pH 5) favors the hydrolysis process, resulting in a higher concentration of fermentable sugars. On the other hand, an alkaline pH (such as Sample 4, pH 8) showed a lower concentration of sugars (see Table 8). Samples with intermediate pH (6 and 7) showed moderate concentrations and absorbance values, supported by a standard error of 51.6071, which increases the reliability and accuracy of the regression model. In addition, sugar content was evaluated under specific conditions by hydrolysis of samples with the commercial enzyme LACTFLAT. Hydrolysis was performed at pH 6.5 for 1 hour and 2 hours. The optimum result was obtained with a hydrolysis duration of 2 hours at a concentration of 761 ppm, which maximized the glucose concentration. This finding highlights the efficiency of the 2-hour duration as the most effective process for sugar release within the parameters of this research.

Table 8. Quantitative analysis of sugars in the whey.

No	Sample	Comment	Conc (ppm)	Abs	540 nm
Blanc					0
1			0	0,0568099	0,0568
2			200	0,183562	0,1836
3			400	0,314098	0,3141
4			600	0,537316	0,5373
5			800	0,505047	0,505
6			1000	0,569934	0,5699
1	pH 5	100 ul - 9 ml	1183,69	0,688157	0,6882
2	pH 6	100 ul - 9 ml	289,513	0,227826	0,2278
3	pH 7	100 ul - 9 ml	497,11	0,334698	0,3347
4	pH 8	100 ul - 9 ml	202,931	0,183253	0,1833
1	Sample N°1	1,4 - 500 ul	477,722	0,324717	0,3247
2	Sample N°2	1,4 - 500 ul	761,285	0,470698	0,4707

3.1.10. Statistical Analysis of Reducing Sugars

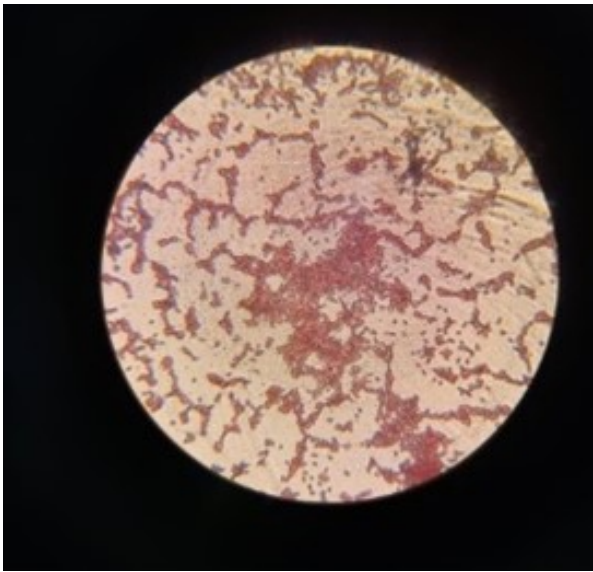
A statistical evaluation of reducing sugars (glucose) obtained from hydrolysis was performed. The objective was to determine their relevance at different durations and conditions (pH). Statistical tests such as ANOVA and Tukey HSD analysis were used to identify the optimum conditions that maximize glucose yield. Two analyses of glucose hydrolysis were performed at different pH conditions. First, at pH 6.5, it was found that the duration of hydrolysis does not significantly affect glucose concentration. Furthermore, in hydrolysis at different pH (5-6-7-8), no statistically significant differences in glucose concentration were observed between the different pH levels (see Figure 8 right). In both cases, ANOVA and Tukey HSD test were used to support these conclusions, ensuring that the observed differences were not random. Analysis of glucose hydrolysis at different pHs showed that the duration of hydrolysis and pH variations did not significantly affect glucose concentration. However, in Figure 8 (left), the graph suggests that a longer duration of the hydrolysis process (2 hours) results in a consistently higher glucose concentration. Statistical methods supported these conclusions, ensuring that the observed differences were statistically significant.



**Figure 8.** Box-plots of statistical analysis of reducing sugars.

3.1.11. Analysis of the Fermentation Process for Polyhydroxybutyrate Production

For the fermentation of whey using *Bacillus subtilis*, a microscopic characterization was performed. This characterization demonstrated the morphology and characteristics of this microorganism. Figure 9 shows a field densely populated by cells with a rod-shaped structure, in pairs and short chains, this being a typical morphological characteristic of *Bacillus subtilis*. The cell walls of this microorganism are thick, a feature typical of Gram-positive bacteria, being part of these *Bacillus subtilis*. As described by [41].



**Figure 9.** Morphology of the bacterium *Bacillus subtilis*.

3.2. Obtaining Wet Weight and Dry Weight

For PHB production, emphasizing the impact of obtaining the wet and dry weight of biomass as it influences PHB yield. Variables such as bacterial concentration, fermentation time and pH were analyzed for production. In the work of [42] it is detailed that the weight of the biomass, whether wet or dry, tends to increase or decrease in a varied manner with the change in pH. Table 9 shows the biomass weights for obtaining polyhydroxybutyrate, here an interesting relationship between pH and the wet and dry weights during fermentation is observed. At pH 5, the wet weight decreases with time, indicating that more acidic conditions may reduce accumulation. However, at pH 8, the wet weight gradually increases, suggesting that a slightly basic environment favors greater accumulation. On the other hand, dry weight also shows variations according to pH: at pH 5, it decreases from 4.14 g to 2.17 g on day 8; at pH 6, it increases steadily for four days; at pH 7, it decreases from 4.86 g to 2.10 g over the same period; and at pH 8, it varies between 4.98 g, 3.49 g, 4.13 g and 3.67 g on the corresponding days

Table 9. Biomass weights obtained at different pHs.

Biomass wet weight					Dry weight of biomass				
pH	X1, (5D)	X2, (6D)	X3, (7D)	X4, (8D)	pH	X1, (5D)	X2, (6D)	X3, (7D)	X4, (8D)
5	15.69	15.01	12.32	17.57	5	4.14	3.41	2.83	2.17
6	16.93	14.56	13.41	17.68	6	4.94	4.15	4.44	5.12
7	16.72	15.60	13.35	16.67	7	4.86	3.23	2.92	2.10
8	16.57	15.23	13.61	18.44	8	4.98	3.49	4.13	3.67

'D' represents the fermentation days used in the study and units are in g. Source:

According to the ratio of dry weight to wet weight is determined by dividing dry weight by wet weight for each data set. This ratio provides information on the proportion of dry biomass within the total sample, which is essential to evaluate the production efficiency of bioproducts, such as PHB, Table 10 shows the ratios between the weights.

Table 10. Calculation of dry to wet weight ratios.

pH	Ratio 5D	Ratio 6D	Ratio 7D	Ratio 8D
5	0.264	0.227	0.230	0.124
6	0.292	0.285	0.331	0.289
7	0.291	0.207	0.219	0.126
8	0.301	0.229	0.304	0.199

In the PHB elicitation study, the relationship between dry weight and wet weight was analyzed as a function of pH and time. The results reveal significant patterns. At pH 8 on day 5, the highest ratio was observed (0.301), followed by pH 6 on day 7 (0.331) and pH 8 on day 7 (0.304). Based on this ratio, the optimal conditions for fermentation and PHB production appear to be pH 6 on day 7 (ratio 0.331) and pH 8 on day 5 (ratio 0.304). In general, acidic conditions (pH 5) are less favorable for stable biomass production. Slightly acidic (pH 6) and neutral (pH 7) conditions are more beneficial for stable production, with pH 6 being the most favorable for both dry and wet weights. Although slightly basic conditions (pH 8) improve water retention, the variability in dry weight suggests that they are not ideal for dry biomass accumulation.

3.3. Biomass Analysis by FTIR Infrared Spectroscopy

The infrared spectrum (FTIR) in Figure 10 showed several absorption peaks that are characteristic of certain PHB functional groups. The features observed in the main spectrum include peaks at 3279.63  $cm^{-1}$  which shows the presence of OH groups of alcohols or carboxylic acids, at 1633.12  $cm^{-1}$  this peak represents the C=O stretches of esters, thus indicating the presence of PHB, also at 2849.61  $cm^{-1}$  is the stretching of methyl groups.  $-CH_2$ , also at 1217.03  $cm^{-1}$ : This peak is associated with the C-O stretching vibration of the esters, which also supports the presence of PHB. The observed features suggest that the biomass obtained contains a ratio of this biopolymer.

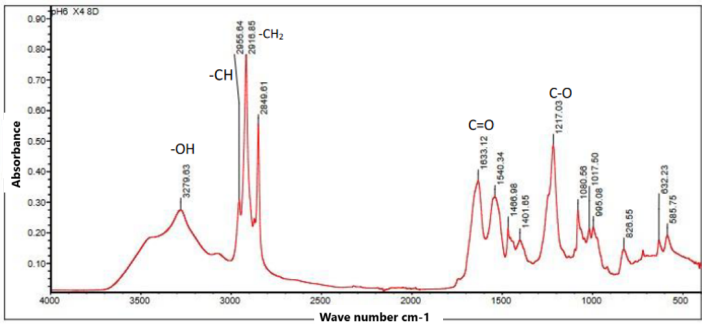


Figure 10. IR spectrum of the biomass obtained.

3.4. Interpretation of the P3HB Film Spectra

The FTIR spectrum in Figure 11 illustrates in the analysis of a plastic film made from PHB that has undergone purification with chloroform. Where it can be seen that upon transformation of PHB from powder to plastic, the FTIR spectrum and its main absorption bands show no changes during film formation. Showing that the purification with chloroform contributed to preserve the integrity of PHB. It can be distinguished that the peaks at  $1740.39\text{ cm}^{-1}$ ,  $1173.16\text{ cm}^{-1}$  and  $1104.32\text{ cm}^{-1}$  indicate the presence of ester functional groups, characteristic of PHB. Additionally, the peak at  $3280.02\text{ cm}^{-1}$  is possibly associated with O-H stretching vibrations of residual hydroxyl groups, which could be due to incomplete purification or the presence of residual moisture. Thus, it is confirmed that chloroform effectively maintains the structure.

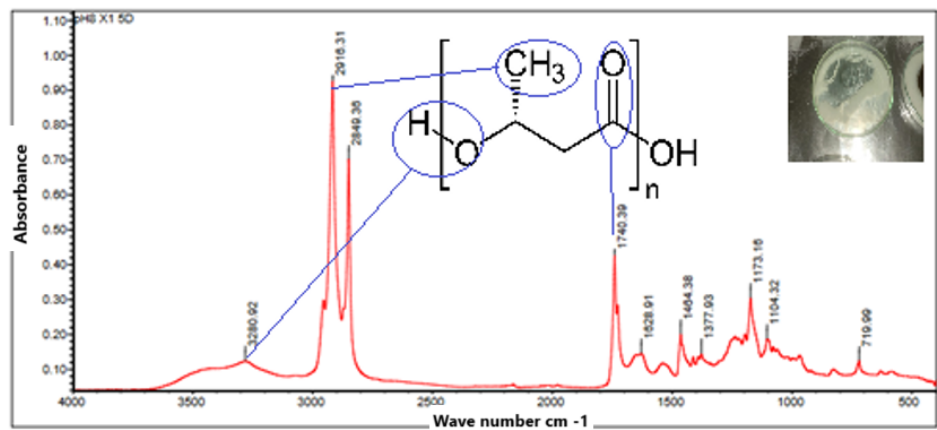


Figure 11. FTIR spectrum of the plastic poly(3hydroxybutyrate).

3.5. Yield of PHB from Substrate

In the process yield and percentage recovery are shown in Table 11; these results show that the highest yield of PHB is achieved with the following conditions: at a pH level of 7 or 8 and a fermentation period of 8 days with treatment X4. The maximum yield obtained is 0.5266 g with a porcentual purification of 53.74 which is observed at pH 7 with treatment X4 after 8 days of fermentation.

Table 11. Percentage yields for each treatment.

Treatment	Product formed (g)	Total biomass (g)	PHB yield	% Purification
pH 5 X1 5D	3.34	19.83	0.1684	19.78
pH6 X1 5D	3.61	21.87	0.1650	16.84
pH7 X1 5D	3.54	21.58	0.1641	16.75
pH8 X1 5D	3.58	21.55	0.1661	16.94
pH5 X2 6D	2.32	18.42	0.1260	12.86
pH6 X2 6D	3.12	18.71	0.1667	17.02
pH7 X2 6D	4.10	18.83	0.2177	22.22
pH8 X2 6D	8.31	18.72	0.4440	45.31
pH5 X3 7D	2.91	15.15	0.1920	19.59
pH6 X3 7D	3.35	17.85	0.1877	19.16
pH7 X3 7D	3.37	16.27	0.2072	21.14
pH8 X3 7D	3.74	17.74	0.2109	21.52
pH5 X4 8D	3.88	19.74	0.1966	20.06
pH6 X4 8D	6.89	22.88	0.3011	30.72
pH7 X4 8D	9.83	18.67	0.5266	53.74
pH8 X4 8D	3.32	22.11	0.1502	15.33

This finding suggests that higher pH levels and extended fermentation periods significantly enhance PHB production. Specifically, a pH of 7 or 8 combined with 8 days of fermentation leads to

the highest PHB accumulation, indicating that these conditions are the most effective. The optimal conditions for achieving the highest PHB yield are observed with treatment X4, at pH 7 and 8 days of fermentation. It is also important to note that treatment X2, at pH 6 and 8 days of fermentation, produces a remarkably high yield of the biopolymer.

#### 4. Discussion

The results obtained in this research showed that the whey showed a higher efficiency, in terms of yield, reaching 0.331 g/L of PHB compared to 0.272 g/L obtained with pineapple peel. This difference can be attributed to the higher availability of simple sugars after enzymatic hydrolysis of whey, which favors bacterial metabolism and intracellular accumulation of PHB. These results are consistent with previous studies such as those of [7], who reported efficient PHB production using glycerol-rich residues, and [13], who demonstrated that pineapple residues are an economical source but require more stringent pretreatment conditions to achieve high yields. Furthermore, the optimal pH (7-8) and temperature (36-37 °C) values observed are in agreement with those reported by [30], who identified that *B. subtilis* maximizes its biosynthesis of PHB under neutral to slightly alkaline conditions and in the presence of excess carbon. Furthermore, characterization of the biopolymer by FTIR spectroscopy revealed characteristic bands of the ester group (C=O) and methyl groups (CH<sub>3</sub>), confirming the structural identity of the PHB produced, in agreement with that described by [19]. These findings reaffirm that the valorization of local agroindustrial waste by microbial fermentation is not only technically and economically feasible, but also aligns with the principles of sustainability and circular economy promoted in recent research [6,18].

#### 5. Conclusions

Regarding the study of sugarcane, this research has succeeded in optimizing the pH conditions and fermentation time to maximize the yield of PHB. Statistical tests, such as analysis of variance (ANOVA) and Tukey HSD test, indicate that pH variation during enzymatic hydrolysis does not significantly affect glucose concentration, which simplifies the optimization of fermentation conditions. In addition, pH has been shown to influence biomass wet weight, with a slightly basic pH (7) being the most stable. In terms of PHB yield, treatments at pH 7 and 8 for 8 days of fermentation were the most effective, reaching maximum yields of 53.74% and 45.31%, respectively. These findings suggest that slightly basic pH levels and prolonged fermentation periods are essential to improve PHB production. However, in the pineapple peel study, PHB production was determined and optimized using *Bacillus subtilis* and whey as substrate. Statistical analysis revealed significant differences according to the physicochemical parameters evaluated. When experimenting with critical points, it was observed that the yield was minimal in treatments T4 and T5, where the microorganism showed high sensitivity at a pH of 5.67 and a temperature of 35.8 °C. The best treatment (T6) achieved a yield of 0.11, with optimal conditions of temperature (36 °C), pH (7.77), agitation (115 rpm) and culture medium (75 ml substrate, 25 ml agar, inoculum 6 ml). Treatment T2 obtained the lowest yield ( $5.878 \times 10^{-4}$ ) with conditions of pH 5.67, temperature 36.4 °C and medium (25 substrate, 75 agar, inoculum 6 ml). Overall, pH was identified as one of the most influential factors in the PHB production capacity by *Bacillus subtilis* bacteria.

**Author Contributions:** Conceptualization: Jorge Amaya. and Y.Y.; methodology: Jorge Amaya.; software: Jorge Amaya; validation, Jorge Amaya; formal analysis: Jorge Amaya; investigation: Jorge Amaya; resources: Jorge Amaya; data curation: Gabriela Vaca.; writing—original draft preparation: Jorge Amaya; writing—review and editing: Jorge Amaya.; visualization: Jorge Amaya; supervision: Jorge Amaya.; project administration: Jorge Amaya. All authors have read and agreed to the published version of the manuscript.

**Funding:** Esta investigación fue financiada por la Universidad Politécnica Salesiana, Sede Cuenca". y "El APC fue financiado por el Grupo de Investigación y Evaluación de Biodiversidad (GIVABI) y la Universidad Politécnica Salesiana, Sede Cuenca".

**Institutional Review Board Statement:** Not applicable.

**Informed Consent Statement:** Not applicable.

**Data Availability Statement:** The data supporting the results of this study are not publicly available due to institutional and confidentiality restrictions, but can be provided by the corresponding author upon reasonable request.

**Acknowledgments:** During the preparation of this study, the authors used the facilities of the Life Sciences Laboratory of the Universidad Politecnica Salesiana, Cuenca, for which they offer their sincere thanks to the university and the GIBAVI research group for the help provided. The authors have reviewed and edited the results and assume full responsibility for the content of this publication.

**Conflicts of Interest:** "The authors declare that they have no conflict of interest" and "The funders had no role in the design of the study; in the collection, analysis, or interpretation of the data; in the writing of the manuscript; or in the decision to publish the results."

## Abbreviations

The following abbreviations are used in this manuscript:

PHB	Polyhydroxybutyrate
ANOVA	Analysis of Variance
FTIR	Fourier Transform Infrared Spectroscopy
GR	Reagent grade methanol
DNS	3,5-dinitrosalicylic acid
ATCC	American Type Culture Collection
CRD	Completely Randomized Design
TBS	Tryptic Soy Broth
SDS	Sodium Dodecyl Sulfate
HSD	Honestly Significant Difference
SPY	Substrate Product Yield

## References

1. Coba, G. "Prohibición del suero líquido provocaría el desperdicio del 90% de insumos en la industria lechera", 2019.
2. Addai, F.P.; Lin, F.; Wang, T.; Kosiba, A.A.; Sheng, P.; Yu, F.; Gu, J.; Zhou, Y.; Shi, H. Technical integrative approaches to cheese whey valorization towards sustainable environment. *Food & Function* **2020**, *11*, 8407–8423. <https://doi.org/10.1039/D0FO01484B>.
3. de Almeida, M.P.G.; Mockaitis, G.; Weissbrodt, D.G. Got Whey? Sustainability Endpoints for the Dairy Industry through Resource Biorecovery. *Fermentation* **2023**, *Vol. 9*, Page 897 **2023**, *9*, 897. <https://doi.org/10.3390/FERMENTATION9100897>.
4. Sarangi, P.K.; Singh, A.K.; Srivastava, R.K.; Gupta, V.K. Recent Progress and Future Perspectives for Zero Agriculture Waste Technologies: Pineapple Waste as a Case Study. *Sustainability* **2023**, *Vol. 15*, Page 3575 **2023**, *15*, 3575. <https://doi.org/10.3390/SU15043575>.
5. Hamzah, A.F.A.; Hamzah, M.H.; Man, H.C.; Jamali, N.S.; Siajam, S.I.; Ismail, M.H. Recent Updates on the Conversion of Pineapple Waste (Ananas comosus) to Value-Added Products, Future Perspectives and Challenges. *Agronomy* **2021**, *Vol. 11*, Page 2221 **2021**, *11*, 2221. A su vez, la elevada importación y consumo de plásticos de un solo uso en el país agrava el problema de residuos, mientras que la cáscara de piña, son una problemática ambiental significativa, las operaciones de procesamiento y cultivo de esta fruta generan una gran cantidad de subproductos, principalmente cáscaras y coronas, que usualmente no se aprovechan de manera sostenible, <https://doi.org/10.3390/AGRONOMY11112221>.
6. Albuquerque, P.B.; Malafaia, C.B. Perspectives on the production, structural characteristics and potential applications of bioplastics derived from polyhydroxyalkanoates. *International Journal of Biological Macromolecules* **2018**, *107*, 615–625. <https://doi.org/10.1016/J.IJBIOMAC.2017.09.026>.
7. Cavalheiro, J.M.; de Almeida, M.C.M.; Grandfils, C.; da Fonseca, M.M. Poly(3-hydroxybutyrate) production by *Cupriavidus necator* using waste glycerol. *Process Biochemistry* **2009**, *44*, 509–515. <https://doi.org/10.1016/J.PROCBIO.2009.01.008>.

8. Noble, R.; Elphinstone, J.G.; Sansford, C.E.; Budge, G.E.; Henry, C.M. Management of plant health risks associated with processing of plant-based wastes: A review. *Bioresource Technology* **2009**, *100*, 3431–3446. <https://doi.org/10.1016/J.BIORTECH.2009.01.052>.
9. Wu, W.; Geng, P.; Li, G.; Zhao, D.; Zhang, H.; Zhao, J. Influence of Layer Thickness and Raster Angle on the Mechanical Properties of 3D-Printed PEEK and a Comparative Mechanical Study between PEEK and ABS. *Materials* **2015**, Vol. 8, Pages 5834–5846 **2015**, *8*, 5834–5846. <https://doi.org/10.3390/MA8095271>.
10. Papagianni, M. Fungal morphology and metabolite production in submerged mycelial processes. *Biotechnology Advances* **2004**, *22*, 189–259. <https://doi.org/10.1016/J.BIOTECHADV.2003.09.005>.
11. Pavel, C.C.; Popa, K. Investigations on the ion exchange process of Cs<sup>+</sup> and Sr<sup>2+</sup> cations by ETS materials. *Chemical Engineering Journal* **2014**, *245*, 288–294. <https://doi.org/10.1016/J.CEJ.2014.02.036>.
12. Papademas, P.; Kamilari, E.; Aspri, M.; Anagnostopoulos, D.A.; Mousikos, P.; Kamilaris, A.; Tsaltas, D. Investigation of donkey milk bacterial diversity by 16S rDNA high-throughput sequencing on a Cyprus donkey farm. *Journal of Dairy Science* **2021**, *104*, 167–178. <https://doi.org/10.3168/JDS.2020-19242>.
13. Suwannasing, W.; Tanamool, V.; Singhaboot, P.; Kaewkannetra, P. Valorisation of Pineapple Cannery Waste as a Cost Effective Carbon Source for Poly 3-hydroxybutyrate (P3HB) Production. *Polymers* **2023**, Vol. 15, Page 3297 **2023**, *15*, 3297. <https://doi.org/10.3390/POLYM15153297>.
14. Yashas, Y.G.; Nagaraju, S.B.; Puttegowda, M.; Verma, A.; Rangappa, S.M.; Siengchin, S. *Journal of Composites Science* **2023**, Vol. 7, Page 2426. <https://doi.org/10.3390/JCS7060242>.
15. Valle, C.; Voss, M.; Gaudino, E.C.; Forte, C.; Cravotto, G.; Tabasso, S. Harnessing Agri-Food Waste as a Source of Biopolymers for Agriculture. *Applied Sciences* **2024**, Vol. 14, Page 4089 **2024**, *14*, 4089. <https://doi.org/10.3390/APP14104089>.
16. Blasi, A.; Verardi, A.; Lopresto, C.G.; Siciliano, S.; Sangiorgio, P. Lignocellulosic Agricultural Waste Valorization to Obtain Valuable Products: An Overview. *Recycling* **2023**, Vol. 8, Page 61 **2023**, *8*, 61. <https://doi.org/10.3390/RECYCLING8040061>.
17. Kourmentza, C.; Plácido, J.; Venetsaneas, N.; Burniol-Figols, A.; Varrone, C.; Gavala, H.N.; Reis, M.A. Recent Advances and Challenges towards Sustainable Polyhydroxyalkanoate (PHA) Production. *Bioengineering* **2017**, Vol. 4, Page 55 **2017**, *4*, 55. <https://doi.org/10.3390/BIOENGINEERING4020055>.
18. Koller, M.; Maršálek, L.; de Sousa Dias, M.M.; Braunegg, G. Producing microbial polyhydroxyalkanoate (PHA) biopolyesters in a sustainable manner. *New Biotechnology* **2017**, *37*, 24–38. <https://doi.org/10.1016/J.NBT.2016.05.001>.
19. Sudesh, K.; Abe, H.; Doi, Y. Synthesis, structure and properties of polyhydroxyalkanoates: biological polyesters. *Progress in Polymer Science* **2000**, *25*, 1503–1555. [https://doi.org/10.1016/S0079-6700\(00\)00035-6](https://doi.org/10.1016/S0079-6700(00)00035-6).
20. Tomietto, P.; Loulergue, P.; Paugam, L.; Audic, J.L. Biobased polyhydroxyalkanoate (PHA) membranes: Structure/performance relationship. *Separation and Purification Technology* **2020**, *252*, 117419. <https://doi.org/10.1016/J.SEPUR.2020.117419>.
21. Berezina, N.; Martelli, S.M. Polyhydroxyalkanoates: Structure, Properties and Sources. *RSC Green Chemistry* **2014**, 2015-January, 18–46. <https://doi.org/10.1039/9781782622314-00018>.
22. Echavarría, A.C.C.; Martínez, A.L.M.; Montoya, M.M. Identificación Molecular de Bacterias Productoras de Polihidroxicanoatos en Subproductos de Lácteos y Caña de Azúcar. *Revista Facultad Nacional de Agronomía Medellín* **2013**, *66*, 7129–7140.
23. Campbell, I.; Campbell, H. A pyruvate dehydrogenase complex disorder hypothesis for bipolar disorder. *Medical Hypotheses* **2019**, *130*, 109263. <https://doi.org/10.1016/J.MEHY.2019.109263>.
24. Kocharin, K.; Chen, Y.; Siewers, V.; Nielsen, J. Engineering of acetyl-CoA metabolism for the improved production of polyhydroxybutyrate in *Saccharomyces cerevisiae*. *AMB Express* **2012**, *2*, 1–11. <https://doi.org/10.1186/2191-0855-2-52/FIGURES/5>.
25. Chavan, S.; Yadav, B.; Tyagi, R.D.; Drogui, P. A review on production of polyhydroxyalkanoate (PHA) biopolyesters by thermophilic microbes using waste feedstocks. *Bioresource Technology* **2021**, *341*, 125900. <https://doi.org/10.1016/J.BIORTECH.2021.125900>.
26. Tan, G.Y.A.; Chen, C.L.; Li, L.; Ge, L.; Wang, L.; Razaad, I.M.N.; Li, Y.; Zhao, L.; Mo, Y.; Wang, J.Y. Start a Research on Biopolymer Polyhydroxyalkanoate (PHA): A Review. *Polymers* **2014**, Vol. 6, Pages 706–754 **2014**, *6*, 706–754. <https://doi.org/10.3390/POLYM6030706>.
27. Amador, K.R.; Óscar Rojas Carrillo.; Aguilar, P.A.; Vega-Baudrit, J. OBTENCIÓN DE XILOSA A PARTIR DE DESECHOS LIGNOCELULÓSICOS DE LA PRODUCCIÓN Y PROCESO INDUSTRIAL DE LA PIÑA (*Ananascomusus*). *Uniciencia* **2012**, *26*, 75–89. En este estudio, se aplicó la metodología propuesta por Ramírez (2012) para el pretratamiento de las cáscaras de piña.

28. Egúsquiza, A.D. Obtención de jarabes glucosados a partir de cáscaras de Ananas comosus (Piña) mediante hidrólisis enzimática de Celulasas. *Ciencia y Tecnología* **2016**, *21*, 36–42.
29. Montañez, L.J.B. Cuantificación de azúcares reductores del sustrato en residuos de piña con el método del ácido 3,5-dinitrosalicílico. *Revista de Investigación* **2020**, *13*, 57–66. <https://doi.org/10.29097/23461098.308>.
30. Yüksekdağ, Z.N.; Aslim, B.; Beyatli, Y.; Mercan, N. Effect of carbon and nitrogen sources and incubation times on poly-beta-hydroxybutyrate (PHB) synthesis by *Bacillus subtilis* 25 and *Bacillus megaterium* 12. *African Journal of Biotechnology* **2004**, *3*, 63–66. <https://doi.org/10.5897/AJB2004.000-2011>.
31. Farfán, A.F.R. Extracción, purificación y modificación de un biopolímero del tipo poli (3-hidroxibutirato) obtenido de la fermentación de ácidos grasos con *B. cepacia*. Master's thesis, Universidad Nacional de Colombia - Sede Bogotá, 2019.
32. Óscar Julián Sánchez Toro.; Buriticá, M.C.O.; Garcés, A.L.B. Obtención de ácido cítrico a partir de suero de leche por fermentación con *Aspergillus* spp. *Revista Colombiana de Biotecnología*, ISSN 0123-3475, ISSN-e 1909-8758, Vol. 6, N°. 1, 2004, págs. 43-54 **2004**, *6*, 43–54.
33. 2018.
34. Aryal, S. Normas McFarland: Principio, preparación, usos, limitaciones, 2021.
35. Campuzano, C.A. Análisis de la producción de Polihidroxibutirato usando lactosuero como materia prima. Master's thesis, Universidad Nacional de Colombia, 2015.
36. Reyes, D.A.M. Modelamiento matemático y optimización del proceso de producción de polihidroxialcanoatos empleando la bacteria *Burkholderia cepacia* B27 a partir de ácidos grasos. Master's thesis, Universidad Nacional de Colombia, 2016.
37. Quines, L.K.; Schmidt, M.; Zanonato, K.; Schmidell, W.; Aragão, G.M. Métodos de extração de polihidroxialcanoatos a partir de biomassa bacteriana. *Química Nova* **2015**, *38*, 1207–1218. <https://doi.org/10.5935/0100-4042.20150129>.
38. del Mar López-Fernández, M.; Franco-Mariscal, A.J. Indagación sobre la degradación de plásticos con estudiantes de secundaria. *Educación Química* **2021**, *32*, 21–36. <https://doi.org/10.22201/FQ.18708404E.2021.2.76553>.
39. Castrejón, L.J. Elaboración de galletas con un edulcorante natural stevia (*Stevia rebaudiana* Bertoni) enriquecida con harina de cáscara deshidratada de piña (*Ananas comosus*). *Universidad Nacional de Cajamarca* **2019**.
40. Kosseva, M.R.; Rusbandi, E. Trends in the biomanufacture of polyhydroxyalkanoates with focus on downstream processing. *International Journal of Biological Macromolecules* **2018**, *107*, 762–778. <https://doi.org/10.1016/J.IJBIOMAC.2017.09.054>.
41. de Jesús San Miguel González, G.; Huerta, M.E.A.; Sánchez, G.B.R.; Díaz, D.P.N. *Bacillus*: Una bacteria versátil, multifuncional y ampliamente aplicada. *Biología y Sociedad* **2024**, *7*, 73–81. <https://doi.org/10.29105/BYS7.13-108>.
42. Philip, S.; Sengupta, S.; Keshavarz, T.; Roy, I. Effect of impeller speed and pH on the production of poly(3-hydroxybutyrate) using *Bacillus cereus* SPV. *Biomacromolecules* **2009**, *10*, 691–699. [https://doi.org/10.1021/BM801395P/ASSET/IMAGES/MEDIUM/BM-2008-01395P\\_0008.GIF](https://doi.org/10.1021/BM801395P/ASSET/IMAGES/MEDIUM/BM-2008-01395P_0008.GIF).

**Disclaimer/Publisher's Note:** The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.