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Production of Polyhydroxyalkanoates (PHAs) Using the Bacterium *Ralstonia Eutropha* to Obtain Bio-Plastic

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Abstract: The purpose of this research was to publicize the use of agro-industrial substrates in obtaining PHAs-type granules, which are synthesized intracellularly by some bacterial genera as reserve material when they encounter nutrient limitations, which would replace synthetic polymers, but the cost of elaboration of this process is expensive, for that reason in the present study different concentrations of substrate were evaluated using a DBCA experimental design. The study to obtain PHA was carried out in three concentrations of ED from cassava starch during two fermentation times. The best time was 72 hours with adding 10g/L hydrolyzed cassava starch syrup. Initiating the isolation of the bacterium from soils where the PHA-producing bacteria live, for its identification, the Gram and molecular staining technique (PCR) was used, and it was distinguished as a Gram-negative bacterium whose carbon source was the yucca flour syrup. Subsequently, the protocol of conditions that intervene in the batch-type fermentation was established. During this time, the accumulation of PHAs, the consumption of glucose, and the accumulation of cells were evaluated through a microbial growth curve. Next, the extraction of the biopolymer was carried out using solutions with acid. Finally, the lamination of the biopolymer was carried out, which does not completely cover the proposed product. Rather, it was possible to cover 10% of the entire product.

Keywords: reducing sugars, polyhydroxyalkanoates, Gram and molecular staining technique, hydrolysis.

使用细菌富养罗尔斯顿菌生产聚羟基链烷酸酯(PHAs)以获得生物塑料

摘要：本研究的目的是宣传使用农工基质获得PHAs型颗粒，这些颗粒是由某些细菌属在遇到营养限制时在细胞内合成的，作为储备材料，这将取代合成聚合物，但精心制作的成本这个过程的成本很高，因此在本研究中，使用DBCA实验设计评估了不同浓度的底物。获得PHA的研究是在两次发酵期间在木薯淀粉的三种浓度的 ED 中进行的。最佳时间为72小时，加入10g/L水解木薯淀粉糖浆。开始从产生PHA的细菌生活的土壤中分离细菌，为了对其进行鉴定，使用了革兰氏和分子染色技术（聚合酶链反应），并将其区分为革兰氏阴性细菌，其碳源是丝兰面粉糖浆.随后，建立了干预间歇式发酵的条件协议。在此期间，通过微生物生长曲线评估PHA的积累、葡萄糖的消耗和细胞的积累。接下来，使用酸溶液进行生物聚合物的提取。最后，进行了生物聚合物的层压，这并没有完全覆盖所提出的产品。相反，有可能覆盖整个产品的10%。

关键词：还原糖、聚羟基链烷酸酯、革兰氏和分子染色技术、水解。

1. Introduction

1.1. Plastics

In general, plastics are flexible, resistant, lightweight materials and insulator of electricity and color, so it is made up of macromolecules that structure a skein [1] and are made up of thermal and oxidation-resistant properties. Perdonomo et al. [2] state that,

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even if the plastic suffers some environmental adversity and pulverizes them, they will continue to be macromolecules present in the atmosphere and mixed with the air that we are naturally ingesting meanwhile. Serrato et al. [3] explain that plastic (PET bottles) are the cause of generating a large part of the polluting waste that accumulates on the planet. "Plastic - PET bottles, when discarded, remain in the environment for about 100 years or, depending on the environment, can last much longer."

Table 1 Names of each plastic, its properties, and applications [4]

Name	Properties	Applications
PVC (polyvinyl chloride)	It has a wide range of hardness. waterproof material	Pipes, shoe soles, gloves, waterproof suits, hoses
Polystyrene (PS)	Transparent pigmentable	Transparent films for packaging and wrapping food products
Polyethylene (PE)	Rigid and resistant Transparent	Household utensils (container buckets, bottles), toys. Bags, cups, and plates
Cellophane	Transparent flexible and resistant Bright and sticky	Packaging

1.2. Biodegradable Plastics

In recent decades, awareness of environmental development has had encouraging results in the production of biodegradable materials. In addition, the generation of biodegradable plastics allows designs based on these materials that aim to produce a specific environmental response before, during, or after their disposal in the surrounding soil, opening new possibilities for studies on the various uses of these materials [5]. Among these, polyhydroxyalkanoates (plastics of microbial origin) stand out.

According to Fernández et al. [6], polyhydroxyalkanoates are biodegradable polyesters accumulated as carbon and energy reserves, which are synthesized by various bacterial species, including *Archaea* and *Eubacteria*. These biopolymers come to have characteristics of synthetic plastics. They are reduced in a short time to carbon dioxide and water. They can also be synthesized intracellularly by various bacteria found in agro-industrial waste, which will take advantage of carbon sources and optimize the biopolymer's processes of fermentation, recovery, and purification [7].

1.3. Generalities of Polyhydroxyalkanoates

The reason why they are considered biodegradable is that their synthesis and decomposition are measured by microbial enzymes. Among these may be those of the soil. On the other hand, there are other types of enzymes (depolymerases) responsible for its hydrolysis. These can be intercellular to take advantage of this natural reserve or, in turn, extracellular that the microorganisms of the microbial community use as a carbon source [8, 9].

Currently, at least 75 different genera of PHA-producing bacteria are known, both Gram-positive and Gram-negative, which are accumulated in the cytoplasm under limited culture conditions [10].

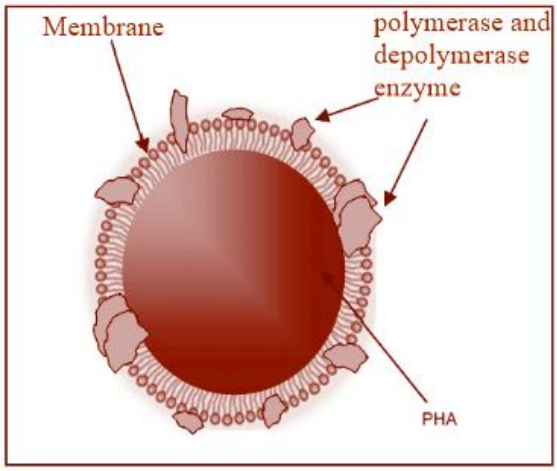

Fig. 1 Diagram of the PHA granule accumulated intracellularly

1.4. Catalyst Enzymes of Polyhydroxyalkanoates

Balseca et al. [11] state that PHA enzymes catalyze R-3-hydroxyacyl-CoA solution to polyhydroxyalkanoates, releasing CoA by joining monomers to form a biopolymer.

1.5. Microorganisms Producing PHAs

The production of PHA can be by two types of microorganisms: prokaryotes and eukaryotes, such as *Ralstonia eutropha*, *Alcaligenes latus*, *Azotobacter vinelandii*, *Pseudomonas oleovorans*, *Paracccus denitrificans*, *Protomonas extorquens*, *E. coli recombinant*, and recombinant strains of Alcaligenes eutrophus, *Escherichia*, and *Klebsiella aerogenes*. However, despite having high efficiency, it has high manufacturing costs [12]. Similarly, PHAs have been shown in animal and human tissues, which is why recent studies have determined that they help control seizures and metabolic diseases and improve heart rate [9].

1.6. *Ralstonia Eutropha*

They are Gram-negative bacteria belonging to the Burkholderiaceae family that do not form spores, which means that they have two membranes and a red or scratched spot. They have two flagella and two membranes that are generally rod-shaped Diana et al. [13]. The microorganism has great potential in bioremediation since it can degrade fermentable sugars to originate polyhydroxyalkanoate granules around the cytoplasm.

1.7. Production of Polyhydroxyalkanoates

In producing the polymer from the bacterium Ralstonia eutropha, the most used strategy is fermentation, comprising a fed-batch culture (batch type) in which the cells grow until a certain accumulation of biomass, where the concentration of

granules of PHAs desired is. For this and for a certain time, an essential nutrient concentration is limited to favor the synthesis of PHAs. Bacteria synthesize PHA through different substrates whose polymers are stored in the cytoplasm as a carbon reserve [14].

1.8. Molecular Identification (PCR)

For this reaction, the bacterium must contain in its protein structure a PhaG gene transforming fatty acids towards PHA synthesis, for which it includes the composition of the microorganism monomers and the carbon source since it can be transformed into hydroxyacyl-CoA precursors by metabolic pathways, which should be found through comparing known PhaG proteins [15]. This research aimed to produce polyhydroxyalkanoates (PHAs) using the *Ralstonia eutropha* bacterium to obtain bio-plastic.

2. Materials and Methods

2.1. Research Location

This research was developed at the State University of Bolívar, Faculty of Agricultural Sciences, Natural Resources, and the Environment, Department of Research and Linkage, in the Soil and Molecular Biology laboratories.

2.2. Sampling

Six sugar cane cultivation soil samples were collected from the Valdez mill, Babahoyo canton, in which 500 gr of each sample was taken at a depth of 15 cm. These samples were immediately transferred to the laboratory.

2.3. Isolation of Microorganisms of the Genus *Ralstonia Eutropha*

The isolation of the microorganism started from a liquid culture in BPW medium (buffered peptone water) (EMD Millipore, VM666728443, Germany) and 10 grams of each sample placed in each Erlenmeyer flask. Then, it was incubated at 38°C for 24 hours; once the time was over, serial dilutions were made until the M_1^ (-5) dilution.

For the inoculation, 1 mL of the diluted bacteria was cultivated on plates with Mc Agar. Conkey, they were subsequently incubated at 38°C for 72 hours. At the end of the incubation time, the colonies were counted in a colony count (SC6PLUS, Techne Fisher Scientific, Spain). The initial bacterial characterization was through a Gram stain [16].

2.4. DNA Extraction from Isolated Bacteria (Boiling Method)

For extracting the DNA from the strains, the cells were resuspended in 200 µL of sterile water and centrifuged for 15 s at 10,000 rpm. The cells were then resuspended in 200 µL of 1% sarcosyl, centrifuged at 14,000 rpm for 4 min, and the supernatant was discarded. Subsequently, 100 µL of 0.05M NaOH were added to the cells and heated at 100°C for 4 min. After this time, 300 µL of sterile milli-Q water were added, and the mixture was gently homogenized and centrifuged at 12,000 rpm for 3 min to remove cell debris. After centrifugation, the supernatant was transferred to a second 1.5 mL Eppendorf tube. An equal volume of phenol/chloroform/isoamyl (25:24:1) was added, homogenized with a micropipette for 10 s, and centrifuged at 12,000 rpm for 3 min.

The aqueous phase was transferred to a new tube, and an equivalent volume of chloroform/isoamyl alcohol (24:1) was added. Then it was homogenized again for 10 s and centrifuged for 3 min at 12,000 rpm.

Then, 0.07 volumes of sodium acetate pH 7 (Amresco, USA) and four volumes of absolute ethanol (Merck, Germany) were added to the reaction. It was stirred by immersion and kept at -20°C for 12h. The samples were centrifuged at 12.000 rpm for 20 min, the supernatant was removed, and the samples were dried in a SpeedVac for 5 min. Subsequently, 200 µL of sterile milli-Q water was added to each tube and heated at 42ºC for 15 min to dissolve the DNA. DNA concentration was performed using a micro-spectrophotometer (NanoDrop) (Thermo Scientific™, ND-ONE-W, USA).

2.5. Verification of the Bacteria by PCR

For PCR amplification, *R. eutropha* DNA was used as a template for the development and optimization of the amplification of the phaG-like genes, using the primers directed at the phaG-like gene H16_A0147 described by Uribe and Villa [15].

The PCR mix reaction was performed using the following reagents: 50 µL PCR buffer (-50 nM 1% volumetric KCl), 2.5 Mn MgCl, 0.2 Mm DNTP, 0.5 µM primer, 0.5 uL Taq DNA polymerase.

The sequences of the primers were the following: R. Forward: 5'-CACGCCACCAGCCGAAA-3' and R. Reverse: 5'- GATTGGATCCTCACGGAACGTCG-3. The mix solution was as follows: master mix 25 µL, primers 1 µL of each, in a total of total 27 µL, Milli-Q water 18 µL. Finally, 5 µL of bacterial DNA was added.

The amplification conditions were: 30 cycles of reaction, initial denaturation 95°C for 30 seconds, denaturation 95°C for 20 seconds, binding of primers 53°C for 45 seconds, extension 72°C for 60 seconds, final extension 72°C for 120 seconds.

2.6. Electrophoresis of the PCR Product

For electrophoresis, a 1.2% agarose gel was prepared with 6 µL of blue/orange 6X, and a 100-bp molecular weight marker (Green 6) + loading buffer was used. After loading the gel with their respective PCR product samples, it was run at 110 volts for 45 min. At the end of this time, buffer water was prepared with the diamond DNA gel dye. The agarose gel was

placed with the amplified DNA and left at rest for 30 minutes for later visualization in a transilluminator (Syngene DG1, 12824038, Spain).

2.7. Reactivation of the Bacterial Strain

For activating the *Ralstonia eutropha* strain, the methodology Diana et al. [13] recommended was used, in which 500 mL of a nutrient broth (TSB) were inoculated with 20 colonies of a pure culture on a solid medium. The incubation was at 30°C and 150 rpm for 24 hours.

2.8. Bacterial Fermentation for the Production of PHAs

For the production of PHAs, it is used according to two stages:

Stage 1: Activating the microorganism in a TSB medium for 24 hours in its exponential phase, where the adaptation of the bacteria is to favor the development of biomass;

Stage 2: 111,11 mL of the bacterial medium in the exponential phase state were placed in one liter of MSM, which are macroelements and microelements required for the fermentative process of PHAs' production, which were previously prepared.

This prepared medium was fermented for 24 hours at 30°C at 150 rpm in an orbital shaker. Once this time was over, 111.11 mL of hydrolyzed glucose from cassava starch was added, and the fermentation continued for up to 72 hours. For the analysis, 20 mL of each sample were taken every 6 hours, where biopolymer concentration, substrate consumption, and bacterial growth were evaluated.

2.9. Quantification of Fermentation Substrate Consumption

For this process, 2 µL of pure glucose diluted in 2 mL of distilled water was prepared, which was brought to a concentration of 800 ppm. From the solution made, the following concentrations were prepared: 10, 20, 50, 100, 200, and 300.

Similarly, to bring the samples to the same concentrations of standard glucose, the sample was calibrated to 250 mL with distilled water plus 100 µL of the sample extracted during the fermentation period.

2.10. Extraction of the Biopolymer

The extraction of the biopolymer was carried out through acid digestion of the non-polymeric cellular material to recover the polymer [17]:

- The final sample of aerobic fermentation is centrifuged at 5000 rpm for 12 minutes (Eppendorf brand 5804R 15amp version);
- After that, the supernatant was separated in a separate tube to centrifuge again. The pellet was washed with 200 mL of distilled water twice, and the suspension was sterilized at 121°C and 1 atm for 30 min;
- The sterilized suspension was centrifuged again under the above conditions and suspended in a 0.1 M H_2SO_4 solution;
- This solution was heated at 90°C for 2 h. After cooling to room temperature, the pH was adjusted to 10 with 5 N NaOH, followed by repeated washings with distilled water to remove excess acid;
- Next, the biomass granules were suspended in a 2:1 solution of distilled water with 6% v/v sodium hypochlorite, leaving it to decolorize for 2 hours at room temperature before centrifuging it again under the same conditions;
- Finally, the obtained polymer was washed with distilled water, then lyophilized, which will serve for its later structural analysis.

2.11. Structural Characterization of the Polymer

The characterization of the biopolymer is based on the investigation by Alcaraz Zapata et al. [17], who used the Fourier transform infrared spectroscopy (FTIR) technique in a Perkin Elmer Spectrum BX infrared spectrophotometer and an ATR module from 4000 to 400 cm-1, which resulted in identifying the functional groups describing the biopolymer produced by the bacterium *R. eutropha* whose data corroborated their research using a control sample of polyhydroxobutyrate (PHB) of the Sigma-Aldrich brand with a purity of 99%.

2.12. Statistical Analysis

Taking as reference the specific objective four, for the accumulation of PHAs in the bacteria, an experimental design was applied that used the following study factors.

Table 2 Description of study factors

Study factors		
Factor	**Code**	**Level**
DE concentration (dextrose equivalent)	A	a1: 10% a2: 7.5% a3: 5%
Time	B	b1: 72 h b2: 36 h

3. Results and Discussion

3.1. Isolation and Confirmation *of Ralstonia Eutropha*

After isolating the microorganism of interest, the characterization was carried out by Gram staining where Gram-negative bacteria could be identified, which measure between 1 and 2 µm and are characterized by having a flagellum, two membranes, and two types of pili, not fermenting lactose. Only one of the six samples was considered positive to belong to Ralstonia.

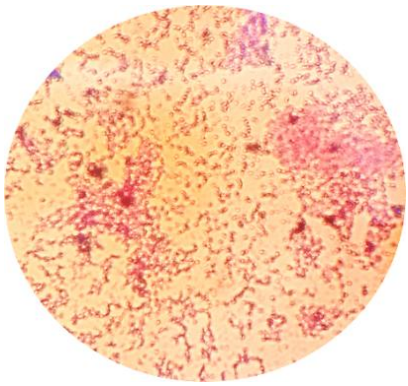

Fig. 2 Photograph of the light microscope observations at 100X

3.2. Molecular Identification by PCR

From the reanimated strains, the DNA was extracted, obtaining concentrations of 567.3 and 131.1 ng/µL.

The identification by PCR with the modified primers of the H16_A0147 gene allowed finding a low similarity of the DNA sequence of the bacterium; amplicons of band size of 800-bp were obtained (Fig. 3).

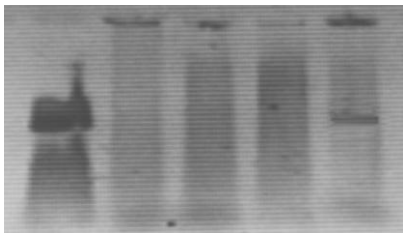

Fig. 3 Electrophoresis of the amplified DNA

Uribe & Villa [15] carried out a successful detection by PCR of the PHAs-producing gene of the bacterium, comparing it with some similar primers. Mora et al.'s [18] samples were collected from contaminated soils so that, in comparison between the PHA homologs of the gene of the bacteria isolated from this research and modified H16_A0147, it was found that they could share active catalytic sites, optimal for producing PHAs.

3.3. Bacterial Fermentation with *Ralstonia Eutropha*

With the hydrolyzed cassava flour ready and microorganism isolated in the same way, it is determined that the amount of MSM goes according to the growth of the biomass and cell. Therefore, for the experimentation, it was prepared by adding the corresponding amount of each solution. saline for 20 g/L of sugar (glucose), provided by the hydrolyzed cassava flour: $Na_2HPO_4.7H_2O$, 6.7g/L; KH_2PO_4, 1.5g/L; $(NH_4)2SO_4$, 1.0g/L; $MgSO_4.7H_2O$, 0.2 g/L; iron ammonium citrate, 60 mg/L; $CaCl_2.2H_2O$, 10 mg/L; 1 mL of trace elements (0.3 g/L de H_3BO_3; 0.2 g/L of $CoCl_2.6H_2O$; 0.1 g/L of $ZnSO_4.7H_2O$; 30 mg/L of $MnCl_2.4H_2O$; 30 mg/L of $NaMoO_4.2H_2O$; 20 mg/L of $NiCl_2.6H_2O$; 10 mg/L of $CuSO_4.5H_2O$).

3.4. Factorial Analysis

The weights of the dry biomass obtained during the entire time of bacterial fermentation were evaluated according to the quantities of carbon source supplied. For this, the three concentrations of reducing sugars in the hydrolysis in 2 different fermentation times were evaluated.

Table 3 Analysis of variance

Source	Sum of squares	Gl	Middle Square	F-reason	P-valor
A:Glucose	0.02968	2	0.01484	1.03	NS 0.385
B:Time	0.13176	1	0.13176	9.18	**0.0105
AB	0.85314	2	0.42657	29.71	**
RESIDUE	0.17227	12	0.01436		
Total	1.18684	17			

** Highly significant statistical difference; NS - non-significant statistical difference

The P-values test the statistical significance of each of the factors. Since 2 P-values are less than 0.05, these factors have a statistically significant effect on biomass with a 95.0% confidence level. After the analysis of variance, it can be considered that there is no significant difference in Factor A. However, Factor B (reaction time) and AxB interaction have a highly significant statistical difference.

3.5. Evaluation of the Accumulation of PHA Granules

Alcaraz Zapata et al. [17] describe the differential lipophilic identification with Sudan Black B, using a strategy for detecting PHA granules formed intracellularly in bacteria, followed during aerobic fermentation. They concluded that the optimal time of accumulation is 72 hours. These data were similar to what was obtained in this investigation, which used different samples at different fermentation times that were seeded in Petri dishes with a TSB agar medium and, once incubated, identified by performing a bacterial smear on an object slide (Fig. 4 and 5).

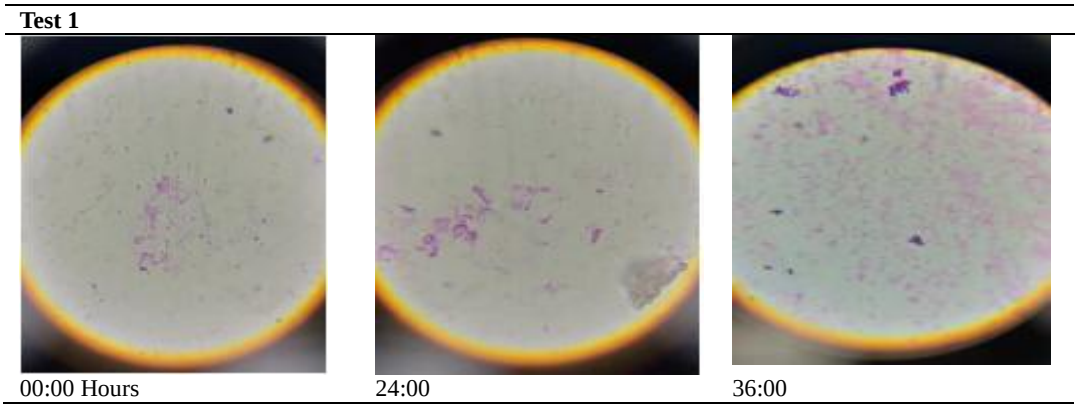

Fig. 4 Verification of accumulation of PHAs in the assay1 by microscopy

Test 1 shows that biopolymer accumulation in the bacteria is scarce, which is due to the fermentation time and the carbon source that was injected at the beginning of the reaction, and this means that the fermentation time was not respected for the adaptation of the bacteria so that it can assimilate the carbon source.

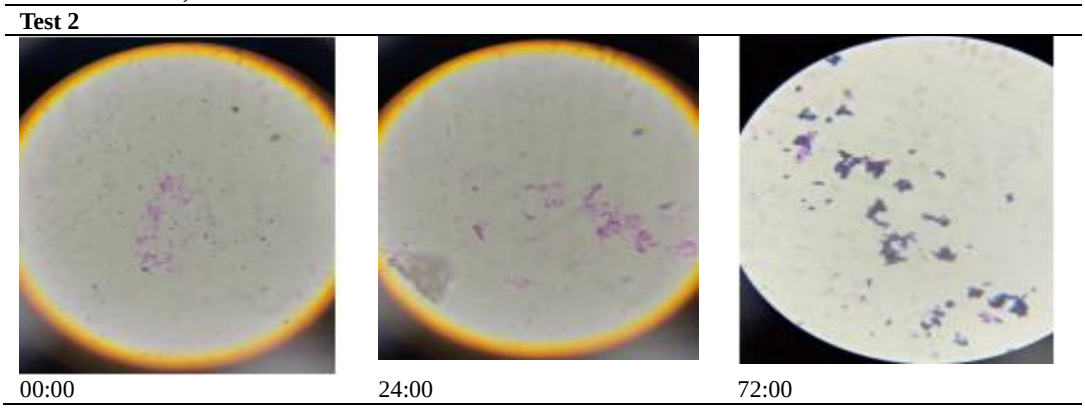

Fig. 5 Verification of accumulation of PHAs in Test 2 by microscopy

According to Test 2, the microscopic analysis shows an appearance of the biopolymer within the bacteria. Therefore, 24 hours after injecting a pulse of the carbon source, there is an accumulation of PHA granules, and, as the fermentation time passes, the accumulation of the biopolymer becomes clearer. This corroborates the research by Alcaraz Zapata et al. [17], who explain that, after 30 hours of fermentation, there is an accumulation of biopolymer.

3.6. Extraction of the Biopolymer

The weight obtained from the PHAs is according to the amount of carbon source supplied at the beginning. In this investigation, 10 g of glucose was added to one liter of MSM, thus obtaining 0.20 g of polymer per liter of the cultivation medium. The data is similar to the weight of PHAs obtained by Fernandez et al. [6], in which they used 66.36 g of glucose with a DE percentage of 79% per liter of culture and therefore obtained 0.62 g of PHAs. While the data reported by Alcaraz Zapata et al. [17], who used 10 g of glucose per liter of solution as a carbon source and, in 72 hours of fermentation, obtained a weight of PHAs of 0.383 g/L of the medium. In turn, Toro et al. [19] used different culture media enriched with glucose and obtained the best weight, 0.0624 g of PHAs, in one liter of solution. Therefore, it follows that, by having a good percentage of reducing sugars in the carbon source supplied to the bacteria, the formation of PHAs, which is intracellular, is high, and, in turn, these values are directly related to the biomass generated.

Fig. 6 Bioplastic obtained and degraded at 25 days

The bioplastic obtained was 20 mg per liter of fermented culture medium. Therefore, the amount of polymer obtained was not enough to achieve complete apple coverage but rather 10 percent of the total surface. One of the reasons is lacking material and information in the laboratory and country, for which the lamination of the polymer was not completed.

4. Conclusion

From one of the collected soil samples, it was possible to identify the same *Ralstonia eutropha* bacteria used for bacterial fermentation. After the tests, the data showed that, during the synthesis of biopolymer production, this reaction yielded an amount of 220 mg/L of PHA, representing the maximum production of the *Ralstonia eutropha* bacterium with an amount of reducing sugars greater than 30%.

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