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Eco-friendly bioplastics from Cajá (*Spondias mombin*) peel waste: Polyhydroxyalkanoate production via *Brevibacterium* sp.

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ABSTRACT

Polyhydroxyalkanoates (PHA) are a family of intracellular biopolymers biodegradability and biocompatibility produced by numerous bacteria. The search for new PHA-producing microorganisms is crucial for expanding bioplastic production, as seen with *Brevibacterium* sp. In this study, hydrolysates of *Spondias mombin* peels were used as a carbon source for PHA synthesis through the fermentation of *Brevibacterium* sp. This process contributes to the bioeconomy by producing biodegradable plastic from waste materials that would otherwise be discarded. For morphological characterization of the bacterial isolate, optical microscopy, scanning electron microscopy, and transmission electron microscopy analyses were performed. Molecular identification was ensured by analysis of the 16S rRNA gene. The biopolymer was characterized by infrared spectroscopy, thermal analysis, and scanning electron microscopy. The polyhydroxybutyrate produced by *Brevibacterium* sp. showed characteristic infrared stretching. The *Spondias mombin* peels were efficient carbon sources for PHA production from the biosynthesis of a bacterium for bioplastic production: *Brevibacterium* sp. Here is the first report, to our knowledge, on the production of PHA from cajá peel (a fruit typical of the northern and northeastern regions of Brazil) using Brevibacterium sp. as the biopolymer producer.

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Introduction

Petrochemical-origin plastics are polymeric compounds considered commodities for society and used in all industrial sectors. The physicochemical properties, combined with the ability of polymers to be molded, have particularly attracted the packaging sector and the biomedical industry (Alves et al. 2022). Each year, about 11 million tons of plastic waste are accumulated in the oceans, and the packaging industry is responsible for 47% of this amount (Allan 2022).

These materials are difficult to degrade and usually accumulate in the environment for centuries (Andrady,

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2017). In the oceans, "plastic islands" have drawn the attention of researchers and environmentalists. These islands are agglomerations of plastic waste that have formed over the years and are present on five continents. One of the largest islands reported to date is called the Great Pacific Garbage Island, which moves between California and Hawaii with about 1.8 trillion floating objects (Ballejo et al. 2021; Nikiema et al. 2022).

When exposed to the environment, plastics form micro and nano plastics ranging from 5 mm to 1 nm (Yang et al. 2021). In drinking water, these particles spread through the air, being ingested by fish and marine animals, contaminating the entire food chain (Hanh et al. 2019). Due to this process, humans frequently ingest and inhale microplastics (Barboza et al. 2018; Nikiema et al. 2020). According to Jung et al. (2022), an ordinary person can consume up to 52,000 microplastic particles in food annually.

Given this scenario, the development of biodegradable polymers has been a strategy used by researchers to replace traditional plastics. Among biodegradable polymers, polyhydroxyalkanoates (PHA) stand out, as a class of biocompatible, non-toxic, thermoplastic, water-insoluble polyester with physicochemical characteristics similar to polypropylene, polystyrene, and polyethylene (Pandey et al. 2022). About 300 microorganisms biosynthesize this polymer, including the bacteria *Cupriavidus necator*, Alcaligenes latus, *Azotobacter vinelandii*, *Pseudomonas oleovorans* and *Bacillus thuringiensis*. These materials are produced intracellularly, in the form of granules, as a carbon and energy reserve for the microorganism (Kumar et al. 2020; Saratale et al. 2021). Polyhydroxyalkanoates consist of about 150 monomers possessing mechanical properties that vary according to the size of the carbon chain. The different chemical structures are made up of various functional groups such as halogens, epoxy group, hydroxyls, and carbonyls (Chee et al. 2019).

The variability in the chemical composition of the polymer depends on the microorganism and the carbon source used during fermentation, allowing its application in the production of packaging, in the composition of medical devices such as heart valves, in dressings, in the production of suture threads, in drugs delivery and tissue engineering (Aramvash et al. 2018; Sharma et al. 2021; Alves et al. 2022).

According to Sharma et al., (2021), the PHA family can be classified structurally as short-chain polymers (3-5 carbon atoms), medium (6-14 carbon atoms) and long-chain (more than 15 carbon atoms) carbon). Polyhydroxybutyrate (PHB) is an example of a short-chain polymer belonging to the PHA family, with the molecular formula $(C_4H_6O_2)_n$ that has high crystallinity and low melting point.

One of the limiting factors for large-scale PHA production is the cost of the carbon source used, which can account for more than 30% of the total cost of the process (Adler et al. 2021). Regard to this, the use of alternative carbon sources, such as agro-industrial wastes, is an excellent option for the production of materials with high value, in addition to being part of the bioeconomy process that uses materials that would otherwise be discarded to produce a biodegradable polymer.

To increase production, researchers have used new strategies to reduce costs, such as less explored bacterial strains or recombinant microorganisms (Taguchi et al. 2001). *Brevibacterium* is a genus composed of 25 species, a rod-shaped and gram-positive short chains bacterium ranging from 0.6 to 2.50 mm, aerobic, with a high biofilm production and growth between 30°C and 37°C. When grown on Nutrient Agar medium, it has small, opaque cream colonies, it is salt resistant and non-fastidious. The genus *Brevibacterium* can metabolize several sources of carbon and nitrogen for its growth, such as glucose, sucrose, ammonium sulfate, and amino acids (Collins 2006; Forquin 2017; Asai et al. 2019). Pandian et al., (2009) demonstrated that the isolate *Brevibacterium casei* SRKP2 synthesizes polyhydroxyalkanoate (PHB) from dairy residues, yeast extract, and seawater, thus enhancing the ability of this bacterial genus to produce biopolymer.

To minimize the cost and optimize the production of the bioplastic, many studies are being carried out in the search for microorganisms that produce polyhydroxyalkanoates and carbon sources that can be used for the synthesis of the biopolymer. This work is the first to used hydrolysates cajá peel to produce PHA from the fermentation *Brevibacterium* sp.

Materials and Methods

Bacterial isolate characterization

The bacterial isolate is available at the Bioprocess Laboratory of the Centro de Tecnologias estratégicas do Nordeste (CETENE) and was provided by the Universidade Federal de Pernambuco (UFPE) in 2018. The bacteria were grown in Nutrient Agar and incubated at 35 ºC for 24 hours. The colonies were identified by visual inspection with the aid of a colony counter with a magnifying glass. The morphological characterization was carried out by optical and scanning electron microscopy (SEM) in an FEI Quanta 200F microscope. The sample was previously metallized with a thin layer of gold.

Identification and analysis of the 16S rRNA gene sequence

Bacterial genomic DNA was obtained according to Romano and Brazilian (1999). The broth, containing the culture, was subjected to centrifugation, boiling, and chemical breakdown of the cell wall by the CTAB solution. For amplification of the 16S gene, a pair of universal primers was used (5'-AGAGTTTGATCMTGGCTCAG-3'; 5'-CTGCTGCCTYCCGTA-3'), described by Lane (1991). The gene amplicon was sequenced by Genetic Anaylzer 3500 (ABI), and the sequences were analyzed by BioEdit software (Hall 1999). The results were compared with the NCBI database, using the BlastN tool, where the best score and e-value were selected to characterize the isolate.

A similarity search was also performed on a specific bank of the 16S rRNA region in bacteria, RDP (http://rdp.cme.msu.edu/index.jsp/) (Wang et al. 2007).

Pre-treatment of peels to produce the hydrolyzate

The peel hydrolysates were produced from acid hydrolysis, adding sulfuric acid 3% (v/v) to the peels previously dried and ground in a 100:1(m/v) ratio. The mixture was placed in a water bath at 100°C for 1 hour. These hydrolysates were used as a carbon source for bioplastic production.

Bioplastic production

For PHA production, the bacterial isolate was grown in nutrient agar at 35 °C for 24 hours. The inoculum (seed culture) used in the experiments was prepared by adding a small sample of bacteria in nutrient broth (5g.L^{-1} peptone and 3g.L^{-1} meat extract) incubated at 35°C/250 rpm for 24 hours in an incubator shaker according to the modified methodology of Padian et al. (2009). After the required time, the inoculum was added at a 10% (v/v) ratio to the hydrolyzate containing 9.40 g. L^{-1} of reducing sugars previously quantified by the DNS method according to Miller et al. (1959). The samples were incubated under the same conditions mentioned above for 72 hours. Bacterial growth was monitored in an EZ Read 2000 microplate reader (600 nm) every 3 hours, with an interval of 12 hours at night. All samples were adjusted to pH 7 and the analyzes were performed in triplicate. After 72 hours of incubation, the samples were centrifuged at 10.000 rpm for 10 minutes, the supernatant was discarded, the pellet was washed with saline solution (0.9%) and lyophilized.

PHA extraction

PHA extraction was performed using the methodology proposed by Albuquerque et al. (2018), which consists of a cell lysis performed in NaOH solution (0.2 mol. L^{-1}), followed by solubilization of the polymer in chloroform and precipitation in ethanol. The total content of PHA accumulated in the cells was determined by gravimetry.

Characterization of bioplastic

The characterization of the functional groups present in the polymer was performed by infrared spectroscopy in a Bruker IF66 spectrophotometer, scanning in the region of 4000-400 cm-1 using a KBr tablet. PHA thermal characterization (TGA) was carried out using Thermogravimetric Analyzer TGA-50/50H using a scanning rate 10 °C min^{-1} under nitrogen atmosphere. Polymer morphology was checked in SEM on FEI Quanta 200F microscope.

Results and discussion

Identification and characterization of the isolated Brevibacterium sp.

The *Brevibacterium* sp isolate showed opaque and small colonies, slightly convex, with a shiny and smooth surface. In Gram stain, the colonies were Gram-positive bacilli, compatible with what is expected in this bacterial genus. Figure (1) shows the scanning electron microscopy obtained from the bacteria that showed the morphology of elongated bacilli with an average size of 1.92 µm and preserved cell structure.

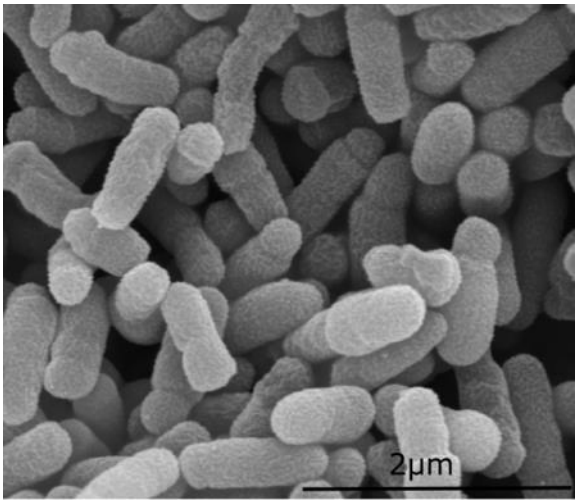

Fig 1. Scanning electron microscopy of *Brevibacterium* sp.

Additionally, 16S rRNA typing was performed to confirm bacterial identity. The generated fragment was approximately 1500 bp and the analysis obtained was compared with NCBI and RDP databases. Among the first 10 generated results, there was more than 98% similarity with the species *Brevibacterium sp.* and *Brevibacterium epidermidis*. The classification generated by the RDP showed only the genus (Brevibacterium). The molecular

characterization ratifies the data obtained in biochemical analysis and illustrates the evidence of this bacterial species in the production of PHB.

There are two types of *Brevibacterium* that are relevant to the environment and industry. *Brevibacterium linens* (Boyaval e Desmazeaud 1983; Jones e Keddie 1986; Bikash et al. 2000) and *Brevibacterium epidermidis* (Galinski 1995; Ventosa et al. 1998; Onraedt 2005) are two prominent examples. The importance of gender in the food industry was highlighted by Betts (2006), especially in the production of glutamic acid, which is useful for enhancing food products and also plays a role in lacteo processes. is required for further research to clarify its nature and investigate the metabolic pathways for the detection of various biomolecules, such as PHA.

There are about 300 species of PHA-producing microorganisms. However, only a few have been explored and characterized, such as *Alcalgenes*, *Ralstonia*, *Bacillus*, *Azotobacter*, *Rhizobium* and *Pseudomonas*. The exploration of new PHA-producing microorganisms is of great relevance for the expansion of bioplastic production and opens up possibilities for the use of other microorganisms, such as *Brevibacterium* sp. This is the first report on the production of PHB by Brevibacterium sp. using agro-industrial residues Ranganadhareddy (2022).

The polymer obtained was characterized by Fourier transform infrared spectroscopy (FTIR), according to Figure 2.

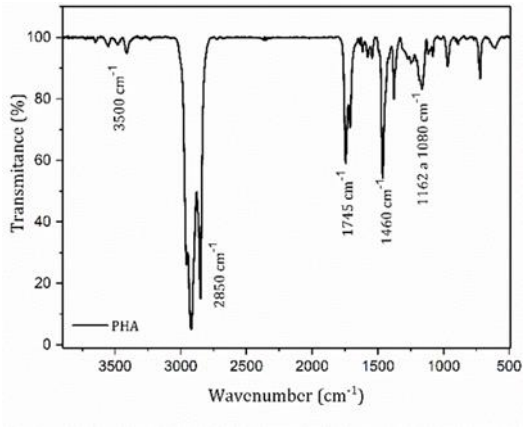

Fig 2. - Fourier transform infrared spectroscopy (FTIR) spectrum of PHB produced by *Brevibacterium sp.*

A weak band at 3500 cm-1 attributed to O-H bonds can be observed; stretching at 2920 and 2850 cm-1 referring to the C-H bond; stretches attributed to C=O ester carbonyl to 1745 cm^{-1}, stretch to 1162 cm^{-1} attributed to C-O-C bonds; stretching at 1080 cm^{-1} characteristic of C-O bonds. The presence of these stretches corroborates the results reported by Tugarova et al. (2021) and Pandian et al. (2009) confirming the production of Polyhydroxybutyrate (PHB) by *Brevibacterium sp.* Tufail et al. (2017) used *Brevibacterium halotolerance* to produce PHB with four different carbon sources: frying oil, canola oil, diesel and glucose.

The bioplastic produced by *Brevibacterium* sp. has a white coloration and an opaque aspect. The morphology of the polymer was studied by scanning electron microscopy (Figure 3). The micrograph showed a single phase surface with small defects throughout the region as described by Vahabi et al. (2019).

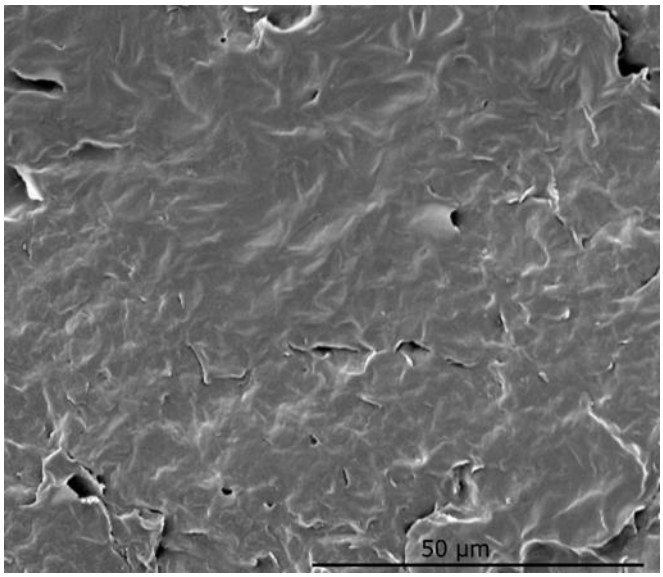

Fig 3. Scanning Electron Microscopy (SEM) of the bioplastic produced by the bacterium *Brevibacterium sp.*

The PHA granules inside the bacteria were analyzed on a MORGANI transmission electron microscope, 268 D, FEI Company. Monitoring the growth of *Brevibacterium* (Figure 4) showed that the microorganism reached the stationary phase after 21 hours of incubation. According to Khomlaem et al. (2021), PHA accumulation starts in the exponential phase and reaches a maximum during the stationary phase. The biopolymer accumulated by the bacteria was calculated as described in previous work by Shah and Kumar (2021) reaching 15 $mg.L^{-1}$ and resembles the results reported by Padian et al. (2009) who produced PHA from *Brevibacterium casei* with a maximum production (135 $mg.L^{-1}$) after 48 hours as well as Rebocho et al. (2016). Figure 4 shows the growth curve of the bacteria (a) and transmission electron microscopy containing the intracellularly grown PHA granules (b).

Using residual substrates, such as agricultural residues, industrial waste, and wastewater effluents, aligns with circular economy concepts by reducing resource use

and waste output. Extensive research has demonstrated the feasibility of using these substrates for PHA synthesis, resulting in cost-effective and ecologically friendly production. Nonetheless, there is still a need to optimize substrate-specific yield, examine its impact on polymer characteristics, and develop metabolic pathways to deal with substrate variability. Addressing these restrictions is critical for increasing PHA production and widening its commercial applications.

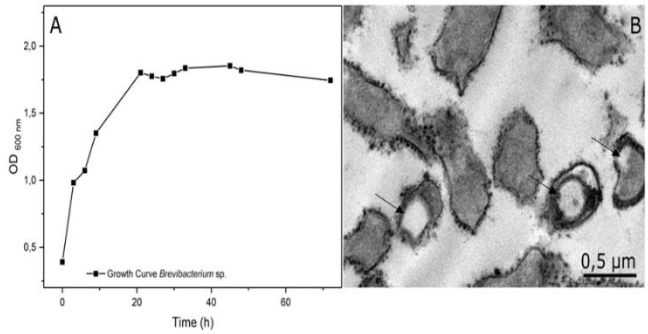

Fig 4. Growth curve of the bacteria (a) and image transmission electron microscopy containing the intracellularly grown PHA granules

Thermogravimetric analysis determines the thermal decomposition of the polymer, as can be seen in figure 5.

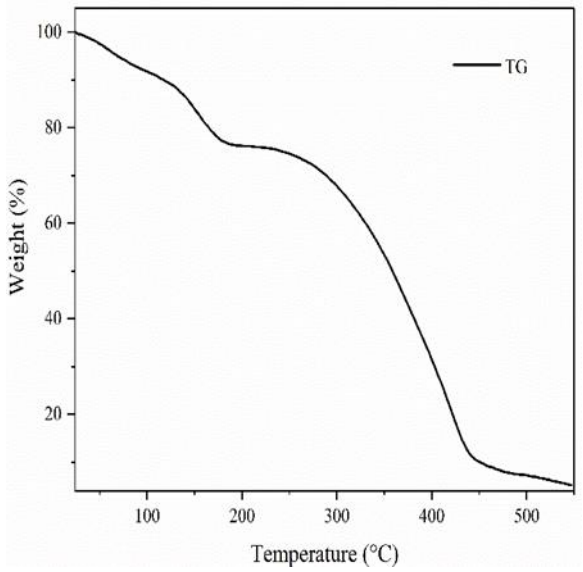

Fig 5. Thermal analysis of the bioplastic produced by *Brevibacterium* sp.

The TG curve has two thermal events, the first referring to the loss of water from the bioplastic (T_{onset} 120°C) and the second event corresponding to polymer degradation with T_{onset} (224°C) being the maximum degradation temperature at 288°C. According to Suzuki et al. (2021) PHB has thermal and mechanical properties similar to isotactic polypropylene, which is widely used industrially. Similar results were previously reported by Trakunjae et al. (2021). PHB is a thermoplastic that can be molded industrially by polymer processing techniques such as extrusion, injection and fiber formation, which enables its application in several segments, in addition to being completely biodegradable when released into the environment (Saratale et al. 2021; Vahabi et al. 2019).

Conclusion

The peel hydrolysates from *Spondias mombin* are efficient and promising alternative carbon sources for the production of PHA, in addition to being part of the bioeconomy process, using materials that would be discarded to produce a biopolymer with high added value. This production strategy is current, very relevant and extremely important for the development of environmentally sustainable materials. Infrared spectroscopy showed characteristic stretches confirming the production of polyhydroxyalkanoate by *Brevibacterium* sp.

The use of the 16S rRNA gene proved to be a safe and effective tool for typing bacterial microorganisms. In a simple, fast and low-cost way, it was possible to confirm the biological identity of the isolate of *Brevibacterium* sp. Molecular methods aimed at understanding the PHA biosynthetic pathway in this isolate, may lead to an improvement in PHA production by the *Brevibacterium* sp.

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Conflict of interest

The authors declare there is no conflict of interest.

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