

# Isolation and characterization of polyhydroxyalkanoate-producing bacteria from seawater samples (Tumaco)

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## Abstract

**Introduction.** Polyhydroxyalkanoates (PHA) are a family of polyesters that comprise > 100 types of homo and heteropolymers that can be produced from renewable carbon sources by microorganisms, making it a sustainable and environmentally friendly material as a substitute for plastics. Currently, the production of biopolymers is not competitive in terms of cost and yield compared with synthetic polymers; however, such production by different bacterial strains may provide economic and ecological viability if there are investments in this area, and there is evidence that bioplastic accumulates antibiotic resistance genes (ARGs) and metal resistance genes (MRGs) in marine sediments. **Objective.** The aim of this work was to isolate and characterize molecularly and biochemically PHA-producing bacteria from water samples obtained from five sites (gas stations) in coastal regions of Tumaco Island, Nariño-Colombia, and determine the antimicrobial susceptibility of isolates because the biological role goes beyond their storage function, since their presence in cytoplasm enhances stress resistance of microorganisms. **Materials and methods.** Bacterial colonies were isolated from seawater samples. A viable colony staining method using Nile red was used to screen for PHA-producing bacteria. Colonies were isolated, characterized via biochemical, microbial, and molecular methods, and tested for antimicrobial susceptibility and fermentation. The crude extract was analyzed by GC-MS/MS techniques. **Results.** More than thirty-eight strains were identified as potential PHA-positive isolates from this screening approach but, just one isolated was viable in PHA production (T2-25A). All isolates were resistant to metronidazole, ampicillin, trimethoprim sulfamethoxazole, cephalothin, ceftriaxone, and cefazolin, and 27.3 % of isolates were resistant to novobiocin. **Conclusions.** One promising PHA-producing isolate was obtained. Nevertheless, this information will complement future studies of the conditions necessary to produce PHA. Moreover, antibiotic resistance data have attracted attention, especially because of the origin of the source waters of the isolates.

**Keywords:** Polyhydroxyalkanoate, seawater samples, fluorescence, susceptibility, granule.

## 1. Introduction

The oceans occupy a large space in the biosphere (71 % of Earth's surface) and are subject to different conditions (low temperature, high salt concentrations, luminosity, oxygen presence) and different inductor molecules; oceans are an oligotrophic environment where bacteria can adapt, multiply, and dominate in number and activity, in contrast to other organisms (Bunse and Pinhassi, 2017). Because of the vast diversity of species in the marine environment, little is



known about the biopolymers produced by marine bacteria, which may be biologically significant (El-Malek *et al.*, 2020; Javaid *et al.*, 2020). According to the standards of the American Society for Testing Materials (ASTM), polyhydroxyalkanoates (PHA) are a group of biocompatible, compostable, and biodegradable polyesters that are synthesized by bacteria, archaea, and some fungi in marine environments (Han *et al.*, 2015; El-Malek *et al.*, 2020; Javaid *et al.*, 2020). Many bacteria (archaeobacteria, Gram-positive and Gram-negative bacteria, and photosynthetic bacteria) can synthesize PHA polymers and store them as intracellular inclusion bodies. These include the genera *Pseudomonas*, *Ralstonia*, *Cupriavidus*, *Aeromonas*, *Bacillus*, *Alcaligenes*, *Enterobacter*, and *Rhodobacter* along with some *Halobacteriaceae* (Han *et al.*, 2015; Tsuge *et al.*, 2015; Li *et al.*, 2017) and Cyanobacteria (Singh und Mallick, 2017; Troschl *et al.*, 2017; Ciebiada *et al.*, 2020; Blanco *et al.*, 2021; Gomes Gradíssimo *et al.*, 2020).

When there is more carbon substrate than other nutrients, such as nitrogen, sulfur, phosphorus, or oxygen (Li *et al.*, 2017; Tsuge *et al.*, 2015; Ciebiada *et al.*, 2020; Blanco *et al.*, 2021), some microorganisms accumulate PHA as an intracellular energy source and carbon storage inclusions occur in the cytoplasm (Singh und Mallick, 2017). Bacteria can accumulate polymer amounts as high as 90 % of cell mass. This accumulation occurs naturally, so PHA can be used as an extra source of carbon and energy, when nitrogen, phosphorus or oxygen is depleted in the environment (Blanco *et al.*, 2021). Weiner (1997); Dhangdhariya *et al.* (2015) and more recently Alsaadi *et al.* (2022) revealed the significance of bacterial biopolymers, highlighting their importance and stating that marine bacteria accumulate PHA because it could produce considerable amounts of PHA. This suggests that a marine environment is a very promising source for identifying new species that synthesize this type of biopolymer.

Increasing awareness of environmental pollution has generated a resurgence of interest in biological methods to produce biodegradable polymers (Gomes Gradíssimo *et al.*, 2020). Polyhydroxybutyrate (PHB) is the simplest and most well-known PHA and can be used as a substitute for synthetic polymers. Both PHA and synthetic plastics are moldable thermoplastics and can be tailor-made for numerous applications (Rekhi *et al.*, 2021). The quality that sets PHA apart from conventional plastics is its complete biodegradability in the environment via both aerobic (enzymatically degraded to carbon dioxide and water) and anaerobic (enzymatically degraded to methane and inorganic compounds) pathways (Koller *et al.*, 2017; Rekhi *et al.*, 2021). Until now, there have been no reports of marine PHA-producing bacteria on Tumaco Island, moreover this study was confirmed that Tumaco Island could be an environment benefits to PHA-producing bacteria development, even it is necessary to do more screening studies.

Nevertheless, the oceans are increasingly polluted with plastic debris, and several studies have implicated plastic as a reservoir for antibiotic resistance genes and a potential vector for antibiotic-resistant bacteria.

Di Cesare *et al.* (2021), presented the first evidence that bioplastic accumulates ARGs and MRGs in marine sediments. Biofilms fouling ceramic, polyethylene terephthalate (PET), and PHA were investigated by shotgun metagenomic sequencing. Four ARG groups were more abundant in PHA: trimethoprim resistance (TMP), multidrug resistance (MDR), macrolide-lincosamide-streptogramin resistance (MLS), and polymyxin resistance (PMR). Further investigation (e.g., culturing, genome sequencing, antibiotic susceptibility testing) revealed that PHA biofilms were colonized by hemolytic *Bacillus cereus* group bacteria that were resistant to beta-lactams, vancomycin, and bacitracin. Taken together, this finding indicates that bioplastic, like conventional petroleum-based plastic, is a reservoir for resistance genes and a potential vector for antibiotic-resistant bacteria in coastal marine sediments.

The effects of bioplastic pollution on marine environments remain largely unknown.

Specifically, the zone of Tumaco is characterized by simultaneous pollution sources, mainly the Mira River and the Patia River, which carry fluid waste from upstream agricultural practices (pesticide lixiviates, fertilizers, oil, and grease), commercial fishery plants (organic load), mining (heavy metals), and lumber yards (tannins) (Guzmán *et al.*, 2014). Other minor tributaries throughout the territory, at large municipal towns such as Tumaco (Municipality of Tumaco) and Salahonda (Francisco Pizarro's Municipality), also contribute to the discharge of fluid waste (microorganisms), domestic waste (plastic, wood, paper, organic matter, etc.) (Bonilla and Urrutia, 2022), and other unseen waste (lubricants and fuels) due to spills from pipeline ruptures on the mainland and from gasoline service stations in the area (Tejada and Afanador, 2003).

For these reasons, the purpose of this work was to isolate and characterize molecularly and biochemically PHA-producing bacteria of water samples obtained from coastal regions of Tumaco Island, Nariño-Colombia; and determine the antimicrobial susceptibility in front of conventional antimicrobial compounds. Additionally, it was doing a GC-MS analysis of crude extract producing by the isolate to verify the possible presence of PHA in the sample.

## 2. Materials and methods

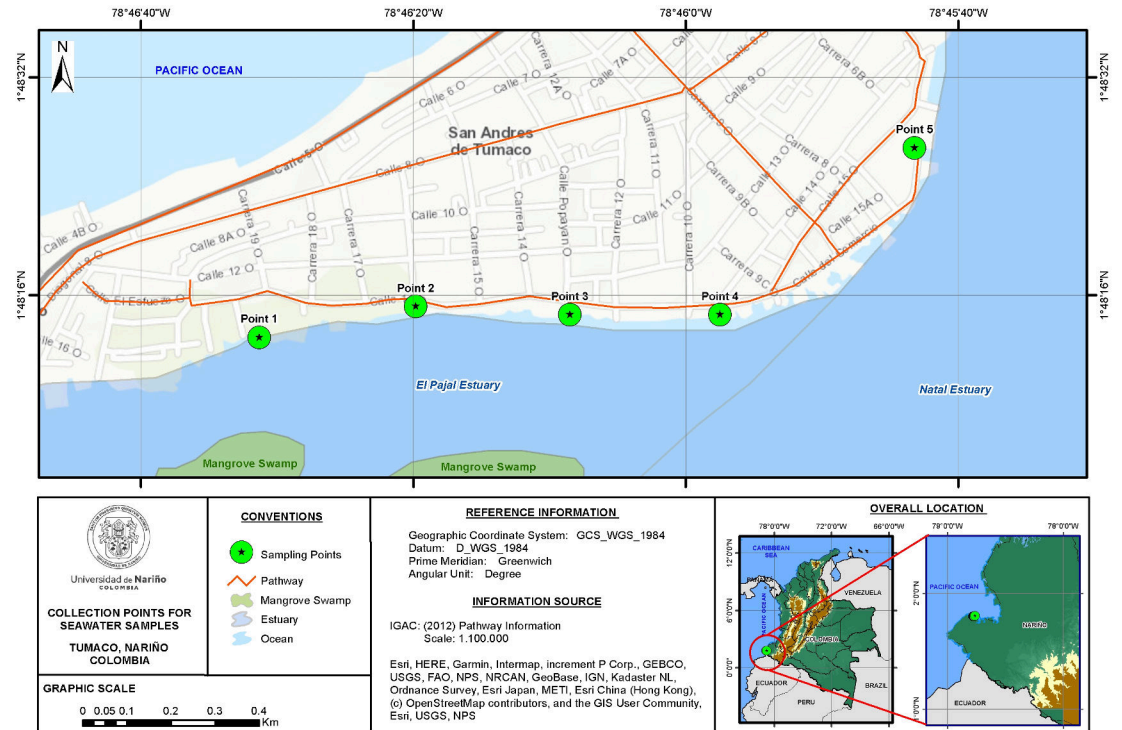
### 2.1. Description of sampling sites

The Colombian Pacific basin has a surface area of 80 000 km<sup>2</sup> and is located in the western region of the country. San Andrés de Tumaco is the second largest city on the Colombian Pacific Coast and stretches across three small islands at the south end of Tumaco Bay (Viciosa, Tumaco and El Morro Islands) (Figure 1). The main economic activities in Tumaco, agroindustry, fishing, and shrimp cultivation, supply both domestic and international demand. The physical characteristics and geographical location of Tumaco also make it a convenient port for oil export (Figure 1) (Martinez-Varela *et al.*, 2021).

### 2.2. Collection of seawater samples from coastal regions of Tumaco Island

Seawater samples were collected from five areas located near gasoline stations on the coast of Tumaco Island that presented spills. GPS coordinates of points samples collection are shown in Table 1. **Point 1** indicates a Mobil gasoline station, **Point 2** indicates the gasoline station *La Bombita*, **Point 3** indicates DistriTumaco L. D, **Point 4** indicates the gasoline station *Isla Dorada*, and **Point 5** indicates the gasoline station *Romero & Burgos* (Figure 1). At each point, two seawater samples, each one liter in size, were collected at 10 cm under the surface in sterile niskin bottles, localization of collection points was determining to obtained isolates PHA producers because the constant environment exposition to gasoline affect the bacteria populations. All samples were transported in refrigeration (4 °C) for processing at the Microbial Biotechnology Laboratory at the University of Nariño.

The table 1, show the specific GPS coordinates of points samples collection in the coastal regions of Tumaco Island, with the characteristics described previously in this paper.



**Figure 1.** Map of Tumaco Island. In the figure are indicating the sampling points corresponding to places with a close localization of gas stations. **Point 1**, Mobil gasoline station, **Point 2**, La Bombita, **Point 3**, DistriTumaco L. D, **Point 4**, Isla Dorada, and **Point 5**, the gasoline station Romero & Burgos.

**Table 1.** GPS coordinates of sample collection points in the coastal regions of Tumaco Island.

Collection Point	GPS coordinates	
Point 1	1°48'9.072"N	-78°46'30.738"W
Point 2	1°48'14.0112"N	-78°46'19.8624"W
Point 3	1°48'13.986"N	-78°46'08.436"W
Point 4	1°48'13.4496"N	-78°45'57.2004"W
Point 5	1°48'26.8956"N	-78°45'41.814"W

### 2.3. Determination of physicochemical parameters

The physicochemical parameters determined for the seawater samples were photic zone, pH, temperature, salinity, total solids, conductivity (multiparameters apparatus WTW multi 3430), dissolved oxygen (DO) (Winkler method), carbon dioxide (phenolphthalein method), alkalinity (titration with methyl orange), chloride ( $K_2CrO_4$  method), nitrates, nitrites, sulfates and reactive phosphorus (HACH DR2010) and chemical oxygen demand (COD) (Analysis was performed at the Laboratory of Water at the University of Nariño).

## 2.4. Isolation and purification of PHA-producing bacteria

Each sample was diluted  $10^{-1}$  to  $10^{-6}$  in sterile peptone water at 0.1 %, and then 1 mL was inoculated by the pour-plate method on Plate Count medium pH 7 (Oxoid CM0325) supplemented with Nile Red 0.1 % (w/v) in acetone (SIGMA) (Higuchi-Takeuchi *et al.*, 2016; Diéguez *et al.*, 2020). Sample incubation was carried out at 30 °C. The samples were evaluated by fluorescence every 24 h for 3 days of colony growth using a transilluminator (UVP Benchtop Transilluminator 3UV) at a wavelength of 365 nm. Each isolate was described macroscopically according to nine features (shape, margin, elevation, surface, texture, optical property, size, conformation, and color). The fluorescent colonies were coded, purified, and observed under a microscope to determine differential characteristics (Gram-positive versus Gram-negative, shape, granule presence and spores). Subsequently the colonies were observed and characterized microscopically with Sudan black B staining.

## 2.5. Biochemical characterization.

The isolates were inoculated in nutrient broth (Oxoid CM0001), incubated at 37 °C for 24 h and transferred to growth medium, where they could break down carbohydrates, according to the methodology proposed by Sneath (1986).

To each liter of medium,  $(\text{NH}_4)_2\text{SO}_4$  (1 g),  $\text{KH}_2\text{PO}_4$  (1 g), KCl (0.2 g),  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  (0.2 g) and yeast extract (0.2 g) was added. The acid-base indicator was purple bromocresol (0.04 % w/v). The sugar solution was sterilized separately at 10 lb of pressure for 10 minutes and subsequently added to the broth for degradation (final concentration of 0.5 %). The inoculation was performed on microwell plates at a 2:1 ratio (broth:inoculum). Once inoculated, the broths were incubated at 37 °C for a period of 24 h. After incubation, a color change from purple to yellow was considered positive. The sugars tested for were ribose, mannose, xylose, fructose, maltose, lactose, sucrose, arabinose, galactose, and glucose. The results of biochemical characterization were analyzed, and the figures made in the software RStudio (version 2022.07.1+554).

## 2.6. Antimicrobial susceptibility testing

Estimation of the susceptibility of bacteria to commonly used antimicrobials was conducted using the Kirby-Bauer method (Biemer, 1973), in this protocol are used paper disks impregnate with the specific antibiotic. The isolates were inoculated in nutrient broth (Oxoid CM0001) and incubated at 37 °C for 24 h, then, replicated in Muller-Hinton agar (Merck Code 437.0500). Once all disks were placed, the petri dishes were incubated at 37 °C for 24 h. The antibiotic specifications for the inhibition assays are listed in Table 2, according to manufacturing.

**Table 2.** Antibiotic List used in the inhibition assays with the code number of manufacturing specifications, and quantity of antibiotic per disk.

Antibiotic	Code number	Quantity per disk
Amikacin (AK)	Oxoid CT0107B	30 µg
Ampicillin (AMP)	Oxoid CT0003B	10 µg
Cefazolin (KZ)	Oxoid CT011B	30 µg
Ceftazidime (CAZ)	Oxoid CT0412B	30 µg
Ceftriaxona (CRO)	Oxoid CT0417B	30 µg
Cephalothin (KF)	Oxoid CT0010B	30 µg
Ciprofloxacin (CIP)	Oxoid CT 0425B	5 µg

**Table 2.** Antibiotic List used in the inhibition assays with the code number of manufacturing specifications, and quantity of antibiotic per disk.

Antibiotic	Code number	Quantity per disk
Gentamicin (CN)	Oxoid CT0024B	10 µg
Metronidazole (MTZ)	Oxoid CT067B	5 µg
Novobiocin (NV)	Oxoid CT0037B	5 µg
Oxytetracycline (OT)	Oxoid CT0041B	30 µg
Tetracycline (TE)	Oxoid CT054B	30 µg
Trimetroprim Sulfametoxazol (SXT)	Oxoid CT0052B	25 µg
Vancomycin (VA)	Oxoid CT0058B	30 µg

The zones of growth inhibition around each of the antibiotic disks were measured (mm). The diameter of the zone is related to the susceptibility of the isolate, and it was interpreted using the techniques of the National Committee for Clinical Laboratory Standards (NCCLS, 2009). The disk diffusion test was assessed qualitatively in terms of susceptibility (i.e., isolates were susceptible, intermediate, or resistant). The results of antimicrobial susceptibility testing were analyzed, and the figures made in the software RStudio (version 2022.07.1+554).

### 2.7. Extraction of PHA produced by bacterial isolates

All the selected isolates were subjected to batch-type fermentation to produce PHA in liquid medium, and the relationship between source N:C was 1:4. The fermentation experiments were performed in triplicate per bacterial isolate at 30 °C for 144 h at 100 rpm. Then, 10 mL of sample was centrifuged at 8000 rpm for 10 min, and the supernatant was discarded. The pellet was treated with 1 mL of sodium hypochlorite solution (5 % w/v) and 10 mM EDTA to remove lipids and proteins. The mixture was incubated at 60 °C for 75 min and centrifuged at 8000 rpm for 5 min, and the pellet was saved. Then, the pellet was washed with 1 mL of distilled water, 1 mL of acetone and 1 mL of cold methanol. Each wash was followed by centrifugation at 8000 rpm for 5 min. Finally, the PHA extract was dried and weighed. Values are reported as the mean. Samples were then analyzed by gas chromatography coupled mass spectrometry in tandem (GC/MS/MS) (Lugg *et al.*, 2008).

### 2.8. GC-MS/MS analyses for the detection of PHA

The identification of the monomeric units of the polymer was performed by a GC/MS/MS (Shimadzu QP5000) using a fused silica capillary column thirty meters long with a 0.25 mm internal diameter and a 0.25 µm film thickness. The injector and detector temperatures were set by an ionizing flame at 250 °C. The program temperature was 60 °C for 2 min with an increase of 6 °C per minute until 220 °C (Fernández *et al.*, 2005; Huang *et al.*, 2018; Gomes Gradíssimo *et al.*, 2020). The methyl esters 3-hydroxybutyrate and 3-hydroxyvalerate, (3HB-co-3HV), were also used as reference standards (Fernández *et al.*, 2005) and exhibited two peaks with retention times (RTs) of 4850 and 7000 min, respectively, like is observed in the Figure 4. This RTs was the base to applied in GC/MS/MS analyses, when the crude extract obtain of isolates was employed in the analyze.

The polymer obtained was dissolved in 1 mL of chloroform, and then 1 mL of a mixture of methanol/sulfuric acid (85:15 v/v) was used as a derivatizing agent and heated at 90 °C for 2 h for depolymerization and methanolysis of the polyesters. Then, 1 mL of 0.1 % NaCl was added.

Finally, the aqueous phase was removed (upper phase), and anhydrous sodium sulfate was added to completely dehydrate the sample. One microliter of each sample was injected in the splitless mode at an injection temperature of 250 °C, and the column oven temperature was 100 °C (Fernández *et al.*, 2005; Gomes Gradíssimo *et al.*, 2020).

The relative amounts of monomer units of synthesized PHA were calculated from the peak areas of the methyl esters of each monomer unit in the gas chromatogram extract of the ions of methanolized samples.

## 2.9. Molecular characterization of isolate T2-25A

The characterization was done with part of the sequence of the 16S rRNA gene. Template DNA used for the amplification was obtained following the method of Rivera *et al.* (2003). Polymerase chain reaction (PCR) was carried out in a final volume of 50 µL using the following components: 1X PCR Buffer (10X 200 mM Tris-HCl [pH 8.4], 500 mM KCl) (Fermentas); 1.5 mM MgCl<sub>2</sub> (Fermentas), 0.25 mM of dNTP mix (dATP, dCTP, dGTP, and dTTP; Fermentas); 0.3 µM each of primer A Forward (5' GGAGCAAACAGGATTAGATACCC 3') and J reverse (5' TTCTCCTAGGGCTACCTTGTTAC 3') (Fermentas); 2 U of Taq DNA polymerase (Fermentas) and 1 µL of template DNA (50 ng/µL) (Rivera *et al.*, 2003).

Thermal cycling conditions were as follows: 95 °C for 2 min of initial denaturation, followed by 30 cycles of 2 min at 94 °C, 1 min at 55 °C, and 3 min at 72 °C, and a final extension for 10 min at 72 °C. Sterile water (no DNA) was used as a negative control and *Escherichia coli* DNA (ATCC 25922) was used as a positive control. All reactions were performed with a LabNet Multigene Gradient Thermocycler. The expected size for the T2-25A fragment amplified with primers A and J was 711 bp; the PCR products were analyzed on 1% agarose gels-1X TBE and run at 70 V for 2 h. The PCR products (50 ng/µL) were sequenced using primers A and J by MacroGen DNA Sequencing Service (Korea) with the Applied Biosystems 3730 instrument.

The 16S rRNA partial sequences were deputed with the software Chromas (Version 2.6.6, Copyright 1998-2018), and then, compared to the sequences available from the Ribosomal Database Project (RDP - <http://rdp.cme.msu.edu/>) (Cole *et al.*, 2014). The analysis included the Classifier tool to assign sequences to taxonomic levels and the Sequence Match tool, and finally was assigned one representative of each cluster to the closest type of strain neighbor. Additionally, the same sequences were compared with 16S rRNA sequences deposited at the National Center for Biotechnology Information (NCBI – GenBank) using the BlastN tool (Altschul *et al.*, 1990).

## 3. Results

### 3.1. Physicochemical characteristics

A total of five seawater samples, and duplicates, were analyzed from the described areas of Tumaco Island, Nariño-Colombia, the results are shown in the Table 3. According to results, the temperature, pH, conductivity, and salinity showed no significant differences between sampling points. The maximum values observed for these parameters were a temperature of 31.15 °C (Point 3), a pH of 7 (Point 5), a conductivity of 34.05 µS (Point 4), a salinity of 27.25 ‰ (Points 1 and 2), and total solids of 19250 mg/L (Point 4) (Table 3). The concentration of dissolved oxygen ranged from 5.995 mg/mL (Points 1, 2 and 4) to 7.665 mg/mL (Points 3 and 5), CO<sub>2</sub> saturation varied between 3.3 (Point 5) and 5.5 (Points 1, 2, and 3), and alkalinity ranged from 62 (Point 5) to 95 mg/L (Points 1 and 2). The concentration of chloride (mg Cl/L) ranged from 16087 (Point 3) to

17868.5 (Point 5), nitrate concentration (mg/L  $\text{NO}_3^-$ -N) varied from 2.4 (Point 5) to 8.5 (Point 3), and nitrite concentration (mg/L  $\text{NO}_2^-$ -N) varied from 0.065 (Point 3) to 0.036 (Points 1 and 2). The concentration of sulfates (1/100) (mg/L  $\text{SO}_4^{2-}$ ) ranged from 33.5 (Point 4) to 41.5 (Point 5), the reactive phosphorus concentration (mg/L  $\text{PO}_4^{3-}$ ) varied between 1.7 and 1.875, and the COD (mg  $\text{O}_2$ /L) varied from 530 (Point 5) to 1833.5 (Point 3) (See Table 3).

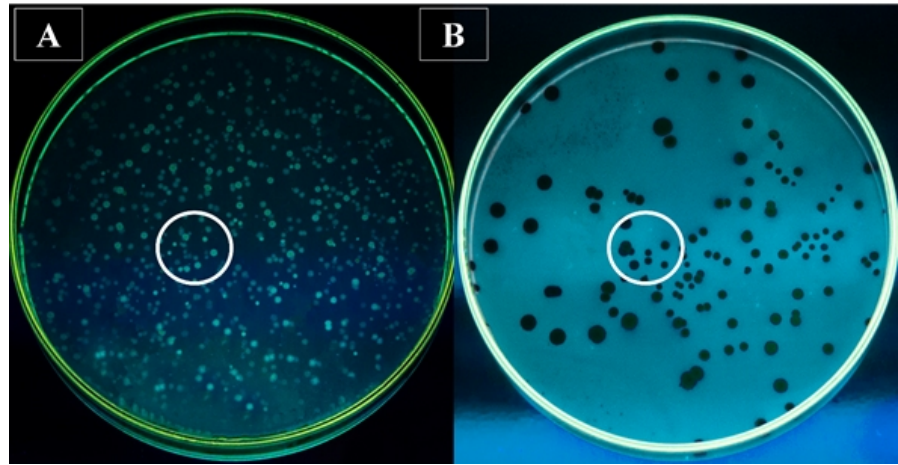
**Table 3.** Results of physiochemical analyses parameters of seawater samples collected from five points of the coastal regions of Tumaco Island, Nariño

PARAMETERS	Point 1	Point 2	Point 3	Point 4	Point 5
Secchi Disc Depth (cm)	120	120	54.5	167	174.5
Temperature (°C)	30.5	30.5	29.9	30.7	31.15
pH	6.5	6.5	7	6.5	6
Conductivity (µS)	27.4	27.4	32.75	34.05	33.9
Salinity (‰)	27.25	27.25	20.95	21.45	21.5
Total solids (mg/L)	18650	18650	18550	19250	18950
Dissolved oxygen (mg/L $\text{O}_2$ ) – % Saturation	5.995 (85)	5.995 (85)	7.665 (101)	5.995 (85)	7.665 (106.5)
Carbondioxide (mg/L)	5.5	5.5	5.5	4.4	3.3
Alkalinity (mg/L $\text{CaCO}_3$ )	95	95	70	65	62
Chloride (mg/L Cl)	16977.5	16977.5	16087	17024.5	17868.5
Nitrates (mg/L $\text{NO}_3^-$ -N)	3.55	3.55	8.5	3.35	2.4
Nitrites (mg/L $\text{NO}_2^-$ -N)	0.036	0.036	0.0165	0.025	0.0175
Sulfate (1/100) (mg/L $\text{SO}_4^{2-}$ )	37	37	37.5	33.5	41.5
Phosphorus (mg/L de $\text{PO}_4^{3-}$ )	1.75	1.75	1.875	1.71	1.7
COD (mg/L $\text{O}_2$ )	769.5	769.5	1833.5	568	530

### 3.2. Isolation and purification of PHA-producing bacteria

Using an acetone solution of Nile red, colonies of PHA-producing bacteria were identified on agar plates via their bright orange fluorescence upon irradiation with UV light (Figure 2). In the fluorescent emission spectra, the intensity of fluorescence increased with the PHA content of bacterial cells, and the reached a steady-state concentration after 48 h of incubation in all strains assayed (Figure 2). In our experiments, dyes were directly incorporated in the medium at concentrations of only 0.5 mg/mL, and the growth of the cells occurred in the presence of the dyes. Fluorescence testing over three days allowed us to gauge the presence of PHA in viable colonies at any time during the growth experiment, which was a powerful way to discriminate between PHA-negative and PHA-positive strains, the Figure 2 show the PHA-producing bacteria growing and fluorescence.



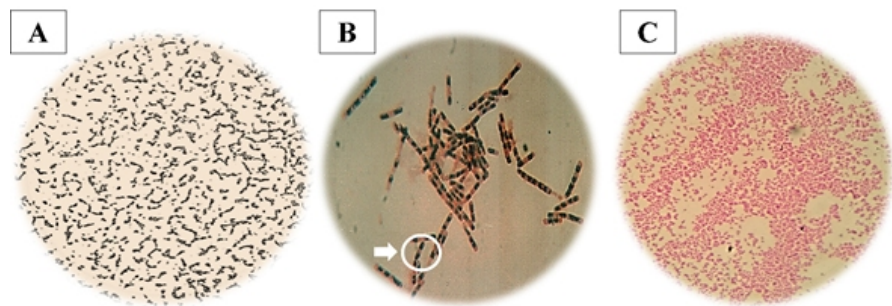


**Figure 2.** Fluorescent of PHA-producing bacteria colonies sowing on Plate Count medium pH 7 supplemented with Nile Red 0.1 % (w/v) in acetone after 48 h of incubation. **B.** Negative control *Escherichia coli* ATCC 25922 without fluorescence. The colonies were marked in white circles to show the differences.

Thirty-nine (39) possible PHA-producing bacteria were isolated in this study, and the isolates were coded T1 to T5 depending on the isolation point. The observed fluorescence was yellow with a red border in 56.41 % of isolates, pink (25.64 %), orange (5.13 %), red (2.56 %), and other (5.13 %), or negative (5.13 %). Initially, Sudan black B staining was performed to detect lipid cellular inclusions; this method showed granules in 26 % of isolates and no cellular inclusions in 74 % (Figure 3).

### 3.3. Macroscopic and microscopic characterization

The macroscopic and microscopic characteristics analyzed in this study allowed us to determine the diversity of the 39 isolates. The main colony forms were irregular, circular, punctate and filamentous. Three types of margins were found: entire, wavy and filamentous. The isolated bacteria showed flat, raised and convex types of elevation. The bacterial surface type was smooth, rough or dry. The bacterial consistency was butyrous, viscous, rubbery, or membranous. The opacity was brilliant, translucent or opaque. The size of the colonies varied between small, medium and large. The most frequent colony conformation was round (97.44 %), and the chromogenesis in most colonies was white (92.31 % of the isolates). Only three colonies presented beige, brown,

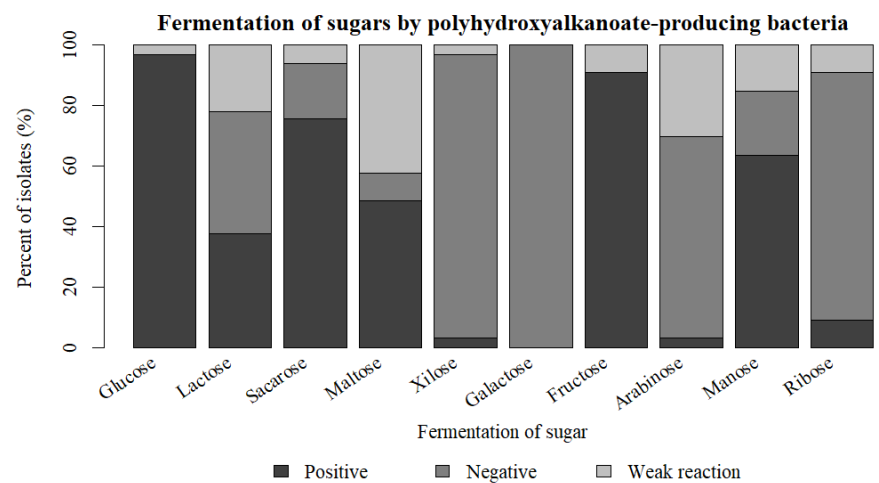


**Figure 3.** **A, B** Microscopic structure of possible PHA-producing bacteria. PHA granules stand out with a black color on a pink background and a white circle in B. **C.** Negative control of PHA-producing bacteria (*Escherichia coli* ATCC 25922).

and red colors. In microscopic morphology, thirty-five isolates were Gram-positive, and only four isolates were Gram-negative. The most frequently observed shapes were bacilli, coccobacilli, diplobacilli and cocci. Twenty-five isolates showed spore presence, and fourteen isolates did not exhibit spores.

### 3.4. Biochemical characterization

According to the data obtained regarding the fermentation of the tested monosaccharides, we established that some sugars were assimilated by the isolates at different rates. The results can be observed in the Figure 4. The rates of monosaccharide assimilation by isolates are shown in Figure 4. Sugars like glucose, fructose, sucrose, and mannose were assimilated by the highest percentage of isolates, and maltose, lactose, ribose, xylose, and arabinose were assimilated by a lower percentage of isolates. Interestingly, galactose was not assimilated by any isolates.



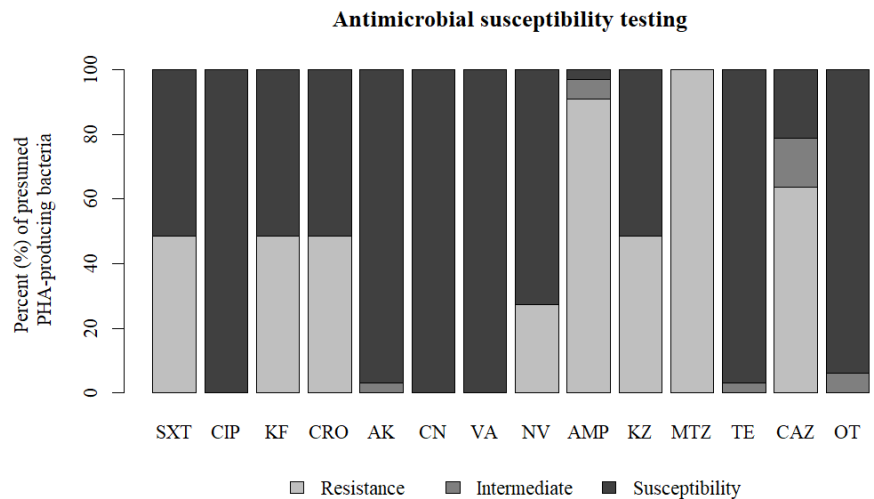
**Figure 4.** Percentage of isolates that assimilated monosaccharides in biochemical characterization. The sugars tested were ribose, mannose, xylose, fructose, maltose, lactose, sucrose, arabinose, galactose, and glucose. The glucose was the monosaccharide assimilated by the major percentage of isolates and galactose was not assimilated for any isolate.

### 3.5. Antimicrobial susceptibility testing

All the presumed PHA-producing bacteria isolates were susceptible to the antibiotics CIP, CN and VA (Figure 5). However, isolate T-10 was intermediate in susceptibility to the antibiotics AK and TE, and isolates T2-10 and T2-33 were intermediate in susceptibility to OT. All isolates were resistant to MTZ, near to 95 % of isolates were resistant to AMP, 60 % to CAZ, 50 % to SXT, KF, CRO, and KZ, and near to 25 % to NV (See Figure 5).

### 3.6. GC-MS/MS analyses of PHA

The analyses were doing for all the crude extracts obtained by presumed PHA-producing bacteria, but only the extract produced by the isolate T2-25A exhibited a peak similar to the PHA standards used (figure 4). Under the conditions used and applied the RTs determined for the standards of methylated monomers of PHA, the extract produced by this isolate have a similar peak in RTs beside the copolymer hydroxybutyrate methyl-cohydroxyvalerate methyl (3HB-co-3HV).



**Figure 5.** Results of antibiotic susceptibility in percentage bacteria isolates. The resistance level was independent of antibiotic class.

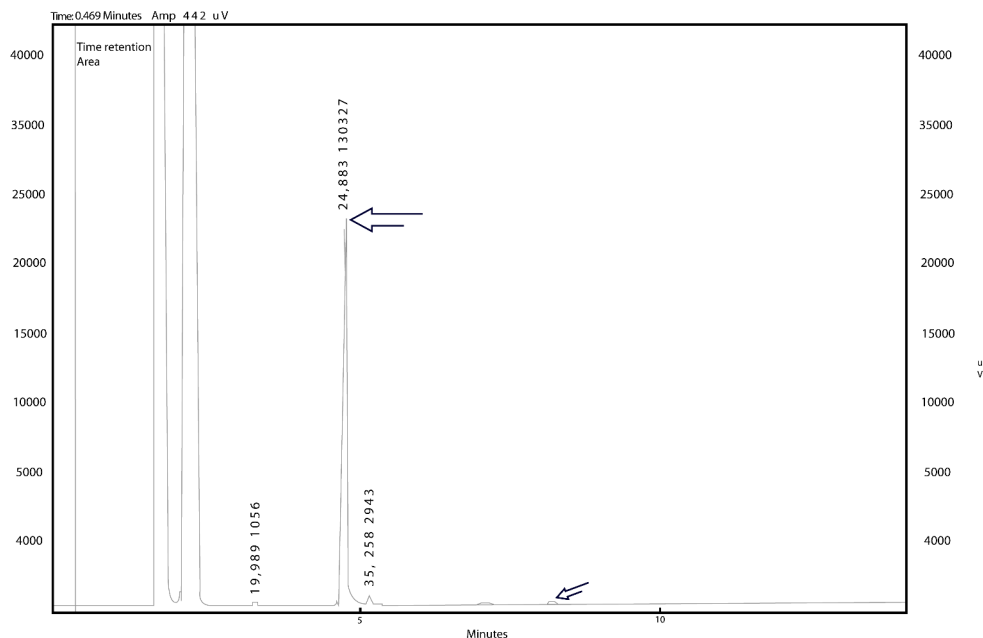
Also, in the figure 4 can be observed that the PHA crude extract obtained from isolate T2-25A showed one peak with an RT of 4.883 and another one with RT of 7.000, which were similar to the standard. The methylated derivatives of the sample exhibited one peak that matched the retention time of methylated monomers of 3-HB (Fernández *et al.*, 2005). Nevertheless, the results obtained by GC-MS/MS evidence the presence of PHA in the crude extract producing by isolate T2-25A (Figure 6).

However, in the chromatographic profile there are other peaks because we work with the crude extract, is important emphasize in the characteristics peaks and RTs observed for the PHA standards. With this protocol, we were able to found and separated compounds with similar characteristics than the standard as of complex samples. Due to other peaks in the samples, it is necessarily to improve the purification method, however, overall, it is important to focus on the presence of compounds in large-scale proportions.

### 3.7. Molecular characterization of isolate T2-25A

The partial sequence (700 bp) of the 16S rRNA ribosomal gene allowed us to classify isolate T2-25A as a bacterium in the family *Beggiatoaceae* (ability to oxidize sulfide to elemental sulfur that is stored as intracellular sulfur globules, filamentous or chain-like morphology, and aerobic, or nitrate-dependent sulfide-oxidizing bacteria)

Despite having compared the T2-25A sequence with various open access databases, it was not possible to characterize it completely with a reliable score; the highest genetic similarity found was 16.1 % when the T2-25A sequence was compared with another sequence (accession code FR690980). It is therefore necessary to use other molecular methodologies to verify whether a sequence has already been reported in databases or if isolate T2-25A is a new sequence that has not previously been studied.



**Figure 6.** Chromatogram of extract obtain of T2-25A, corresponding with the RTs of methyl ester 3-hydroxybutyrate. The peak observed in RT of 4.850 min has the major intensity value and the peak for 7.000 is low but it can be observed. The RTs patterns are similar with the observed in the GC/MS/MS extract.

#### 4. Discussion

The marine ecosystem is an attractive and rich source of novel strains of bacteria that have the potential to produce new bioactive compounds for important and sustainable biotechnology development (Giordano, 2020). This ecosystem is characterized by both seasonal and daily fluctuations in several physicochemical variables that have an impact on resident microorganisms who must frequently respond to the changing conditions of their environment, even the electrical conductivity of seawater samples gives an overview of the soluble salts present in seawater.

It has been pointed out that under nutritional stress conditions, bacterial cells with higher PHA contents survive longer than those with lower PHA contents (Tufail *et al.*, 2017). PHA granules serve as a carbon and energy source during starvation and are crucial for bacterial survival under harsh conditions caused by fluctuating osmotic pressure and radiation (Urtuvia, 2014; Shah and Kumar, 2021). PHA degrades naturally and completely into carbon dioxide and water under aerobic conditions and into methane under anaerobic conditions (Tsuge *et al.*, 2015; Tufail *et al.*, 2017). In that way, PHA can be considered biodegradable plastics and they are less problematic than petroleum-based plastics for the environment (Urtuvia, 2014; Shah and Kumar, 2021).

About the PHA accumulation, in this study was measured as relative fluorescence intensity, reached a steady-state concentration after 48 h of incubation in all strains assayed. At 72 h, the fluorescence observed in each strain incubated with glucose and fructose was usually similar. Our results show that that seawater samples could be a source for the isolation of PHA-producing strains with possible industrial applications for producing intracellular nanostructures, as described in other studies (Higuchi-Takeuchi *et al.*, 2016; Diéguez *et al.*, 2020). Bacterial PHA production occurs even at high environmental salt concentrations (Cristea *et al.*, 2018).

In this study, we evaluated over fifty isolates for PHA accumulation based on a colony staining method using Nile red. Of these, thirty-nine isolates were selected based on the intensity of Nile red fluorescence, and then the cells were analyzed for their microscopic and macroscopic characteristics, biochemical characterization, and antimicrobial susceptibility. The maximum PHA accumulation was observed in isolate T2-25A compared with the other strains analyzed in this study, which presented red fluorescence, the presence of granules, and the ability to ferment glucose, maltose, lactose, fructose, mannose, and ribose. The isolate could also be considered a promising source of accumulated polymers in sucrose (Koller *et al.*, 2017). All these qualities make isolate T2-25A a good candidate for the industrial production of PHA due to the possibility of using complex and economical substrates. This is an advantage, since the cost of the substrate becomes reduced when a microorganism uses materials other than carbon (Koller *et al.*, 2017).

Additionally, T2-25A was resistant to seven antibiotics (SXT, KF, CRO, AMP, KZ, MTZ, CAZ) and susceptible to seven antimicrobials (CIP, AK, CN, VA, NV, TE, OT). Resistance was probably due to anthropogenic activities that have frequently been implicated in altering natural microbial community structure in aquatic ecosystems (Blair *et al.*, 2015; Hall and Mah, 2017). These activities have directly or indirectly caused irreversible changes in microbial biodiversity. Resistance in PHA-producing bacteria could be a consequence of anthropogenic pollution observed at the different sampling points (Lupo *et al.*, 2012; Shah and Kumar, 2021). It has been suggested (Blair *et al.*, 2015; Hall and Mah, 2017) that in tropical environments, microbial populations resistant to antibiotics may be selected from the natural microbiota upon contact with these compounds. Resistance to antibiotics would be an advantage for industrial PHA production because a bacterium that produces this compound could be cultivated free of competitors.

Recent research has demonstrated that their biological role goes beyond their storage function, since their presence in cytoplasm enhances stress resistance of microorganisms (Obruca *et al.*, 2018). The possible relationship between environments under stress or strong perturbation and biodiversity increase is another ecological parameter that has been investigated by some authors (Tufail *et al.*, 2017; Milan *et al.*, 2018; Patin *et al.*, 2018) and is also considered in the present study. In our screening of PHA-producing bacteria living in environments under anthropic pressure in the marine ecosystem, we searched for a) strains with the potential to produce PHA, b) PHA-producing strains with resistance to multiple antibiotics, and c) the microbial diversity of PHA-producing isolates in the Tumaco marine ecosystem.

Domestic activities have become direct sources of pollution in the marine environment, a consequence of the lack of urban planning in the municipality of Tumaco (Guzmán *et al.*, 2014). The inadequate management of wastewater domestic has led to approximately 35 % to 40 % of solid residues and 50 % of fluid wastes being directly discharged, untreated, into the sea, resulting in considerable pollution and deterioration of the receiving water bodies, marine ecosystems, and associated resources (Ramaiah *et al.*, Ramaiah 2002; Zouch *et al.*, 2018; Martinez-Varela *et al.*, 2021). Furthermore, deficient management of landfills has caused problems with infectious vectors and surface and underground water pollution due to lixiviates.

Our GC–MS/MS analysis of PHA, focused on the PHA-producing isolate, provided an identification of the compound extraction, because the RT of the related peak was very similar to the patterns. However, more specific biochemistry methods are required to refine the PHA because we obtained the extract with other compounds, which can be seen in by the other peaks in the sample (Figure 4). More studies are required to improve the performance of these techniques.

Additionally, the morphological and biochemical characteristics of isolate T2-25A were observed. Our experimental procedure showed that the isolate is close to the *Bacillus* genus, but according to 16S rRNA sequence analysis, it is related to a bacterium in the family *Beggiatoaceae*, described as a sulfide-oxidizing bacterium (Winkel *et al.*, 2016) in freshwater and marine habitats, such as lake sediments, hydrothermal vents, and springs (Ramaiah *et al.*, Ramaiah2002; Winkel *et al.*, 2016; Gureeva *et al.*, 2019), and in eutrophic coastal sediments in areas of coastal upwelling. This bacterium is also associated with black band disease of corals; however, we have not found published articles that demonstrate the production of PHA by this bacterium, as we observed in this research, and the genetic similarity between isolate T2-25A and this bacterium was a low value for ensuring correct identification. In addition, recent sequencing of the 16S rRNA gene from large sulfur bacteria revealed frequent inconsistencies between the morphologically determined taxonomy of genera and the genetically derived classification. Only a few 16S rRNA gene sequences have been produced, and, in many cases, only rather distant relationships between the identified organisms and the type species of the respective assigned genus have been found. (Suriyamongkol *et al.*, 2007) detected inconsistencies in the identification of large sulfur bacteria. Within the group of large filamentous sulfur bacteria, the genus *Beggiatoa* with the type species *B. alba* is defined by its existence as single free-living filaments, the formation of sulfur inclusions and gliding motility. The genus *Thioploca* with the type species *Thioploca schmidlei* differs from the genus *Beggiatoa* in only one property: instead of being free-living, the trichomes of *Thioploca* are bundled in a common sheath. Difficulties in the morphological differentiation of the two genera are caused by the fact that trichomes leaving the common sheath of a *Thioploca* filament are morphologically indistinguishable from single *Beggiatoa* filaments (Gureeva *et al.*, 2019). The classification and identification of large sulfur bacteria based on morphological features is ambiguous (Gureeva *et al.*, 2019). When considering possible polymorphisms in individual cells (dimorphic lifestyle), some morphologies may even be inducible. Therefore, Salman *et al.* (2011, 2013) suggested that the classification and identification of large sulfur bacteria should be primarily based on phylogenetic data. To confirm our results, it is necessary to conduct other tests and find accurate and reliable molecular analysis methods.

## 5. Conclusion

This study presents a partial characterization of PHA-producing bacteria in seawater samples collected from five sites on the coast of Tumaco Island, Nariño-Colombia. This study provides insights into the feasibility of isolating PHA-producing bacteria in marine ecosystems and into the potential for isolating new species of microorganisms capable of producing the biopolymer in large quantities yet having zero impact on our ecosystem. However, more studies are necessary to determine molecular diversity and the environmental and biotechnological impacts of bioplastic-producing bacteria. It is very important to remember that a small fraction of microorganisms can be known by culture-dependent methods.

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## 7. Conflict of interest

The authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent arrangements), or non (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript. Potential conflicts of interest related to individual authors' commitments. Potential conflicts of interest related to commitments of editors, journal staff, or reviewers.

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### Aislamiento y caracterización de bacterias productoras de polihidroxialcanoato a partir de muestras de agua de mar (Tumaco)

**Resumen: Introducción.** Los polihidroxialcanoatos (PHA) son una familia de poliésteres que comprenden más de 100 tipos de homopolímeros y heteropolímeros que pueden ser producidos por microorganismos a partir de fuentes de carbono renovables, lo que los convierte en materiales sustitutos de los plásticos, sostenibles y respetuosos con el medio ambiente. Actualmente, la producción de biopolímeros no es competitiva en términos de costos y rendimiento en comparación con los polímeros sintéticos; sin embargo, la producción por diferentes cepas bacterianas puede proporcionar viabilidad económica y ecológica si se realizan inversiones en esta área, ya que hay evidencia de que el bioplástico acumula genes de resistencia a antibióticos (ARG) y genes de resistencia a metales (MRG) en sedimentos marinos. **Objetivo.** El objetivo de este trabajo fue aislar y caracterizar molecular y bioquímicamente bacterias productoras de PHA presentes en muestras de agua obtenidas de cinco sitios (estaciones de servicio) en las regiones costeras de la Isla de Tumaco, Nariño-Colombia, y determinar la susceptibilidad antimicrobiana de las muestras obtenidas, ya que su papel biológico va más allá de su función de almacenamiento porque su presencia en el citoplasma mejora la resistencia al estrés de los microorganismos. **Materiales y métodos.** Se aislaron colonias bacterianas a partir de muestras de agua. Se utilizó un método de tinción de colonias viables utilizando Rojo Nilo para seleccionar bacterias productoras de PHA. Las colonias aisladas se caracterizaron mediante métodos bioquímicos, microbiológicos y moleculares, y se sometieron a pruebas de susceptibilidad antimicrobiana y fermentación. El extracto crudo se analizó mediante técnicas de GC-MS/MS. **Resultados.** Más de treinta y ocho cepas fueron identificadas como posibles aislados positivos para PHA siguiendo el enfoque de selección previamente expuesto, pero solo un aislado resultó viable para la producción de PHA (T2-25A). Todas las muestras aisladas fueron resistentes a metronidazol, ampicilina, trimetoprima sulfametoxazol, cefalotina, ceftriaxona y cefazolina, y el 27.3 % fueron resistentes a novobiocina. **Conclusiones.** Se obtuvo una cepa con potencial para la producción de PHA. No obstante, esta información complementará estudios futuros sobre las condiciones necesarias para producir PHA. Adicionalmente, los datos sobre resistencia a los antibióticos llamaron nuestra atención, especialmente debido al origen de las aguas donde obtuvimos los aislados.

**Palabras Clave:** Polihidroxialcanoato; Muestras de agua de mar; Fluorescencia; Susceptibilidad; Gránulo.

### Isolamento e caracterização de bactérias produtoras de polihidroxialcanoato a partir de amostras de água do mar (Tumaco)

**Resumo:** **Resumo. Introdução.** Os polihidroxialcanoatos (PHA) são uma família de poliésteres que abarca mais de 100 tipos de homopolímeros e heteropolímeros que podem ser produzidos por microrganismos a partir de fontes renováveis de carbono, tornando-se um material sustentável e ambientalmente amigável como substituto dos plásticos. Atualmente, a produção de biopolímeros não é competitiva em termos de custo e rendimento em comparação com os polímeros sintéticos; no entanto, a produção por diferentes cepas bacterianas pode fornecer viabilidade econômica e ecológica se houver investimentos nessa área, já que existe evidência de que o bioplástico acumula genes de resistência a antibióticos (ARGs) e genes de resistência a metais (MRGs) em sedimentos marinhos. **Objetivo.** O objetivo deste trabalho foi isolar e caracterizar molecular e bioquimicamente bactérias produtoras de PHA presentes em amostras de água obtidas de cinco locais (postos de gasolina) nas regiões costeiras da Ilha de Tumaco, Nariño-Colômbia, e determinar a suscetibilidade antimicrobiana das amostras coletadas, uma vez que seu papel biológico vai além da função de armazenamento, pois sua presença no citoplasma melhora a resistência ao estresse dos microrganismos. **Materiais e métodos.** Foram isoladas colônias bacterianas a partir de amostras de água. Foi utilizado um método de coloração de colônias viáveis utilizando o Vermelho de Nilo para selecionar bactérias produtoras de PHA. As colônias isoladas foram caracterizadas por métodos bioquímicos, microbiológicos e moleculares, e foram submetidas a testes de susceptibilidade antimicrobiana e fermentação. O extrato bruto foi analisado por técnicas de GC-MS/MS. **Resultados.** Mais de trinta e oito cepas foram identificadas como possíveis isolados positivos para PHA por meio da abordagem de seleção previamente exposta, mas apenas um isolado mostrou-se viável para a produção de PHA (T2-25A). Todas as amostras isoladas foram resistentes a metronidazol, ampicilina, trimetoprima sulfametoxazol, cefalotina, ceftriaxona e cefazolina, e 27,3% foram resistentes a novobiocina. **Conclusões.** Foi obtida uma cepa com potencial para a produção de PHA. No entanto, esta informação complementar estudos futuros sobre as condições necessárias para a produção de PHA. Adicionalmente, os dados sobre resistência a antibióticos chamaram nossa atenção, especialmente devido à origem das águas onde obtivemos os isolados.

**Palavras-chave:** Polihidroxialcanoato; Amostras de água do mar; Fluorescência; Suscetibilidade; Grânulo.

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