Juliana Paula da Costa Dinis de Oliveira

Degree in Biochemistry

Plastic biodegradation by marine-derived actinobacteria

Dissertation to obtain Master Degree in Biotechnology

Supervisors: Dr. Susana P. Gaudêncio, UCIBIO DQ-FCT/UNL

Co-supervisor: Dr. Nídia Lourenço, UCIBIO DQ-FCT/UNL

Jury:

President: Isabel Maria Godinho de Sá Nogueira, PhD

Opponent: Marta Susana Silvestre Gouveia Martins, PhD

Supervisor: Susana Maria Pereira Gaudêncio de Matos, PhD

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FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

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Ao meu Avô

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Abstract

Plastic is an integral part of our life, being present in most of the products that we use daily. Due to the production and consumption patterns of society, plastics are accumulating in the environment, which causes pollution issues, and intergenerational impacts on the life and health of organisms.

We hypothesize that different actinobacteria strains are capable of degrading different types of (micro)plastics by using them as a carbon source, and then transformed them into bioplastics. In this way, this study aimed to find and develop solutions and sustainable methods to mitigate this environmental problem, focusing on the study of the ability of marine actinobacteria to accelerate plastics biodegradation.

In this work, the screening of thirty-six marine-derived actinobacteria strains was performed to evaluate their polyvinylidene fluoride, polystyrene, and polylactic acid biodegradability potential. The selected actinobacteria (*Streptomyces gougerotti*, *Micromonospora matsumotoense*, *M. terminaliae*, and *Nocardiopsis prasina*) were used in the plastic biodegradation assays using plastic films and testing different conditions. Half of the plastic films used in the assays were pre-treated with UV irradiation and yeast extract was added to culture media to perceive its influence in biodegradation. In both cases, enhanced degradation by the microorganisms was observed.

Biodegradation of films was monitored by weight loss, which was detected in all the inoculated films, except for LDPE UV films inoculated with *M. terminaliae*. The maximum weight loss percentage was 1.27% for PLA inoculated with *N. prasina*. *Nocardiopsis* was identified as a new genus with the ability to degrade PLA. Biodegradation was also accessed based on changes in surface chemical structure (by infra-red spectroscopy) and mechanical properties (tensile strength). The absorptions bands of carbonyl groups are the most related to biodegradation. The maximum decrease in Young modulus was 59% for polystyrene films inoculated with *S. gougerotti*.

We conclude that *S. gougerotti*, *M. matsumotoense*, and *N. prasina* had the potential to use conventional plastics as a carbon source. Furthermore, *S. gougerotti* and *M. matsumotoense* were able to biodegrade conventional plastics and possibly transform them into bioplastics, through the production of PHA inclusions.

Keywords: Plastic pollution; Actinobacteria; Biodegradation; Pre-treatment; Methods to detect biodegradation.

Resumo

O plástico está presente na nossa vida e na maioria dos produtos que utilizamos no dia-a-dia. Devido aos padrões de produção e consumo da sociedade, os plásticos estão a acumular-se no ambiente, criando impactos intergeracionais na vida e saúde dos organismos.

Colocou-se a hipótese de que diferentes espécies de actinobactérias seriam capazes de degradar diferentes tipos de (micro)plásticos, usando-os como fonte de carbono, transformando-os, posteriormente, em bioplásticos. Desta forma, este estudo teve como objetivo encontrar e desenvolver soluções e métodos sustentáveis para mitigar este problema ambiental. Assim, esta dissertação focou-se no estudo da capacidade de actinobactérias marinhas em degradar plásticos.

Trinta e seis actinobactérias foram avaliadas pelo seu potencial de biodegradar fluoreto de polivinilideno, poliestireno e ácido poliláctico. As espécies selecionadas (*Streptomyces gougerotti*, *Micromonospora matsumotoense*, *Micromonospora terminaliae*, *Nocardiopsis prasina*) foram utilizadas em ensaios de biodegradação com diferentes tipos de filmes plásticos (LDPE, PS e PLA), testando diferentes condições de ensaio. Metade dos filmes poliméricos foram pré-tratados com radiação UV e foi adicionado ao meio de cultura extrato de levedura, para perceber que influência tinham na biodegradação. Ambos aumentaram a biodegradação dos filmes.

A degradação dos filmes foi monitorizada por perda de peso, observada em todos os filmes inoculados com actinobactérias, exceto nos filmes de LDPE UV inoculados com *M. terminaliae*. A percentagem máxima de perda de peso foi de 1.27% para o PLA inoculado com *N. prasina*. *Nocardiopsis* foi identificado neste estudo como tendo potencial para degradar PLA. As alterações na biodegradação foram ainda estudadas com base nas mudanças da estrutura química da superfície (espectroscopia de infravermelho) dos filmes e nas suas propriedades mecânicas (ensaios de tração). As bandas de absorção dos grupos carbonilo são as mais relacionadas à biodegradação. A diminuição máxima do módulo de *Young* foi de 59% para filmes de PS inoculados com *S. gougerotti*.

Concluiu-se, com este trabalho, que *S. gougerotti*, *M. matsumotoense* e N. prasina conseguem utilizar (micro)plásticos convencionais como fonte de carbono. Para além disto, *S. gougerotti* e *M. matsumotoense* conseguiram degradar plásticos convencionais e possivelmente transformá-los em bioplásticos, através da produção de vesículas de PHA.

Palavras-chave: Poluição por plásticos; Actinobactéria; Biodegradação; Pré-tratamento; Métodos para detetar a biodegradação.

List of Publications

Juliana Oliveira, Afonso Belchior, Verônica D da Silva, Ana Rotter, Zeljko Petrovski, Pedro L Almeida, Nídia D Lourenço, Susana P. Gaudêncio, Marine environmental plastic pollution: mitigation by microorganism degradation and recycling valorization, (2020) Front. Mar. Sci. doi: 10.3389/fmars.2020.567126.

Vita Rudovica, Ana Roter, Susana P.Gaudêncio, Lada Lukić Bilela, Valdis Bisters, Juris Burlakovs, Inga Grinfelde, Cristiana Torres, Maria F. Carvalho, Diogo A. M. Alexandrino, Fusun Akgul, Jovita Pilecka-Ulcugaceva, Juliana Oliveira, Olga Anne, Roy Hendroko Setyobudi, Yahya Jani, Zane Vincevica-Gaile, Toomas Tamm, Mait Kriipsalu, Merrit Shanskiy, Ruta Ozola-Davidane, Oskars Bikovens, Laura Ferrans, Arita Dubnika, Ivo Safarik, Kristina Pospyskova, Stefanos Kikionis, Efstathia Ioannou, Vassilios Roussis, Giovanna Christina Varese, Katja Klun, Deniz Tasdemir, Marta Martinez-Sanz, Amparo Lopez Rubio, Karina Stankevica, Maris Klavins, Aysegul Erdogan, Berat Haznedaroglu, Lucie Novoveska, Celine Rebours, Amit Bhatnagar, Linn Kristin Akslen-Hoel, Miroslava Atanassova, Jonne Kotta, Marija Katarzyte, Marine waste, industrial by-products and biopolymers valorization, Submitted.

António Pinto-Almeida, Anelize Bauermeister, Inês R. Grilo, Luca Luppino, Juliana Oliveira, Joana Sousa, Daniel Petra, Yessica Parera-Valadez, Alejandra Prieto-Davó, Clara F. Rodrigues, Deniz Tasdemir, Pieter Dorrestein, Rita G. Sobral, Susana P. Gaudêncio, Estremadura Spur deep sea pockmarks, off Portugal coast: Actinomycete biodiversity, metabolomic profiling and antimicrobial potential, Submitted.

Juliana Oliveira, Rita G. Sobral, Pedro L Almeida, Nídia D Lourenço, Susana P. Gaudêncio, Maria AM Reis, Actinobacteria: bacteria that degrade plastics to produce bioplastics, In preparation.

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List of Abbreviations

ABS	Acrylonitrile butadiene styrene
EPS	Expanded polystyrene
FTIR-ATR	Fourier Transform Infrared spectroscopy with Attenuated Total Reflectance
GPC	Gel Permeation Chromatography
HDPE	High-density polyethylene
LDPE	Low-density polyethylene
NMR	Nuclear Magnetic Resonance spectroscopy
NSW	Natural seawater
PA	Polyamide
PC	Polycarbonate
PE	Polyethylene
PET	Polyethylene terephthalate
PHA	Polyhydroxyalkanoate
PLA	Polylactic acid
PMMA	Poly(methyl methacrylate)
PP	Polypropylene
PS	Polystyrene
PU	Polyurethane
PVC	Polyvinyl chloride
PVDF	Polyvinylidene fluoride
SDS	Sodium dodecyl sulphate
SEM	Scanning Electron Microscopy
SLS	Sodium lauryl sulphate

SSW	Synthetic seawater
UTS	Ultimate tensile strength
UV	Ultraviolet light

List of Symbols

ε_r	Elongation at break
σ	Stress (in Pascal)
Δl	Stroke (in millimetres)
A_0	Area (m^2) of the initial cross-section
l_0	The initial length between the claws
R_m	Tensile Strength
%T	Percentage of transmittance in FTIR-ATR spectroscopy
T_m	Melting temperature

1

Introduction

1.1. The Problem

Plastics have remarkable thermo-elastic and mechanical properties, so they are produced and used on a large scale. Plastics have a broad range of applications due to of their high stability and resistance, malleability, lightness, chemical inertness, durability, modelling capability, low water permeability, and, mainly, low cost (Thompson et al. 2009; Tokiwa et al. 2009; Huerta et al. 2018; Raddadi and Fava 2019).

Plastics are polymers, which means they are organic macromolecules formed by hundreds or thousands of segments linked together, forming a chain. The term "plastic" in the industry is defined as a class of synthetic organic polymers that pass through the plastic state, that is, a mouldable state between liquid and solid, at a temperature above room temperature (Gibbs 1977).

Nowadays, plastic is an integral part of our life, being present in an immeasurable number of objects (packaging, garbage bags, soft drink bottles, etc.), and its production and use in several activities and industrial sectors increased dramatically over the last few years (Figure 1.1.) (Gibbs 1977; Tokiwa et al. 2009). The use of plastic materials has many advantages and allowed the development of advances and benefits in society, for example, the general improvement of health, as disposable medical equipment is made with plastic materials (Hopewell, Dvorak, and Kosior 2009; Correia et al. 2020; Ügdüler et al. 2020).

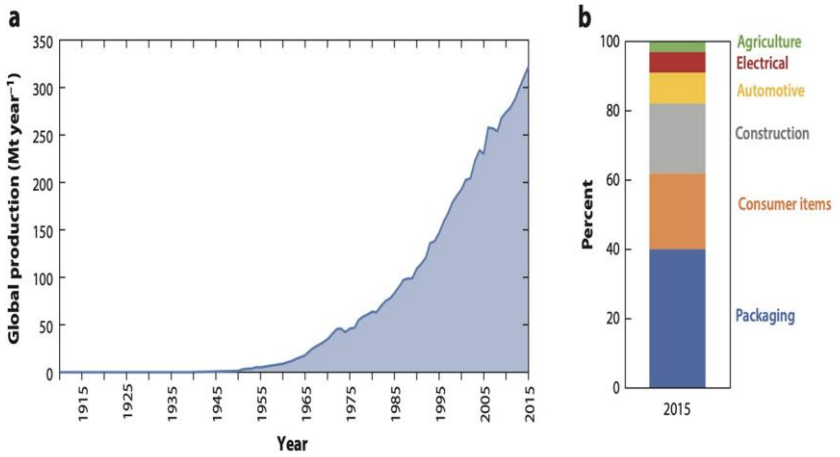

Figure 1.1.:Evolution of total plastics production worldwide: a) World production in million metric tons per year (Mt·year^{-1}); b) Plastics utilization distribution of plastics in 2015 (Source: Worm et al. 2017).

According to the statistics, between 1950 and 2015 the quantity of global plastic waste was estimated to be 6.3 billion tons (M. Zhang et al. 2019). Only 9% of plastic waste has been recycled, 12% incinerated, and 79% still ends up in landfills or the environment (Geyer, Jambeck, and Law 2017). Plastics can be recycled mechanically, chemically, by composting, and by dumping in landfills. However, their globally low recycling rate is mostly due to the costs of collecting, sorting and reprocessing, and due to the low market value of recycled plastics (OECD 2018; Scalenghe 2018).

In 2017 and 2018 the plastic production reached 350 and 359 million tons worldwide, respectively (Raddadi and Fava 2019). Europe is the third global producer, manufacturing 62 Mt of plastic in 2018, with a decrease of around 2.5 Mt compared to the previous years that reflects environmental concerns (Association of Plastics Manufacturers 2018, 2019). However, the high plastic production leads to the lack of proper plastic waste management, inducing the accumulation of over 250 thousand tons of plastic pieces floating in the ocean (Eriksen et al. 2014).

The adopted models and patterns of plastic production and consumption by today' society have dangerous impacts on the environment, as the generation of plastic waste is continuously increasing, creating a major environmental problem that is demanding attention to the search for solutions, especially concerning marine pollution (Savelli et al. 2017; Walker 2018; Wysocki and Billon 2019; J. Oliveira et al. 2020). In fact, each year billions of tons of garbage are thrown, intentionally or unintentionally, into the environment (Oelofse et al. 2004; Andrady 2011). These residues tend to accumulate in certain locations, mostly in marine and coastal environments, being found in remote areas and in middle of the oceans due to waves, currents, and winds (Gregory 2009; Dhage, Vijay, and Kelkar 2009; GESAMP 2015; Sebille et al. 2020). This causes marine pollution leading to intergenerational impacts and

problems that will persist and will continue to increase (Cheshire et al. 2009; X. Li et al. 2019; Lestari and Trihadiningrum 2019).

Marine litter is mainly constituted by plastics, which represent 90% of the solid waste found in the oceans. Thus, environmental scientists have pointed out that plastic materials represent one of the greatest threats to the environment (Williams 1999; Sheavly and Register 2007; Scalenghe 2018).

Due to its low degradation rate, plastics remain in the environment for long periods. Marine plastic pollution has negative ecological, social, and economical impacts, which demands solutions for their mitigation (Hopewell, Dvorak, and Kosior 2009; Raddadi and Fava 2019). It is estimated that 1 million seabirds and 100 thousand marine mammals die annually due to plastics present in open waters. Moreover, 44% of all sea birds, 86% of sea turtles, and 43% of all marine mammal species, and many fish have been affected by entanglement of marine litter or by its ingestion. Thus, plastics already started to enter the food chain, which can lead to human health problems. Furthermore, plastic debris can contain chemical pollutants, that leach to the environment, causing toxicity (Figure 1.2.) (Derraik 2002; GESAMP 2015; Urbanek et al. 2017; UNEP 2018).

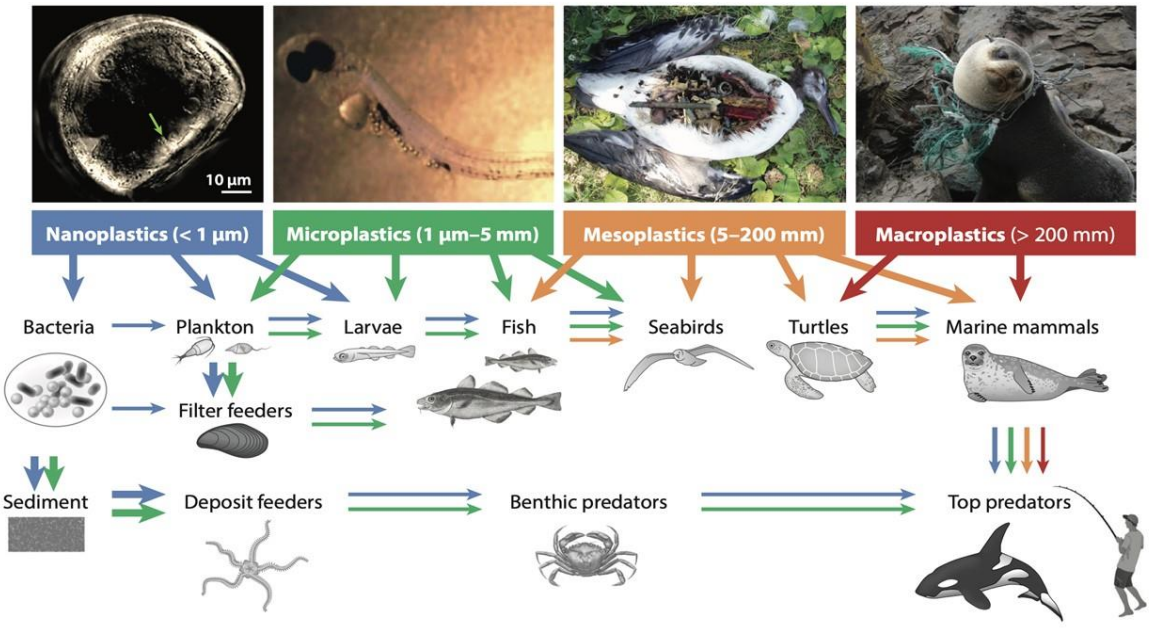

Figure 1.2.: Uptake and possible trophic transfer of plastic pollution in marine food webs. Plastic debris of different size classes has been shown to affect species directly by ingestion or entanglement (*thick arrows*) or indirectly via uptake with food sources (*thin arrows*). Fauna of different sizes and trophic positions will be exposed to particles of different sizes (*blue* to *red*) with some degree of bioaccumulation expected, for both particles themselves and associated chemical pollutants. Photographs depict (*left* to *right*) nanoplastic particles taken up by oyster larvae, microplastic beads ingested by European perch, dead albatross chick with micro- and mesoplastic debris in the stomach, sea lion entangled in macroplastic fishing gear (Source: Worm et al. 2017).

1.2. Plastic types

Currently, the most used polymers are synthesized from chemical monomers derived from petroleum, in the presence of a catalyst and an energy source, typically heat. Monomers are handled in the form of a fluid or a solution or emulsion, through two processes: condensation (step-growth) and addition/polymerization (chain-growth) (Jefferson 2019).

The conventional plastics that control the European Market are polypropylene (PP) 19.3%, low-density polyethylene (LDPE) 17.5%, high-density polyethylene (HDPE) 12.2%, polyvinyl chloride (PVC) 10%, polyurethane (PU) 7.9%, polyethylene terephthalate (PET) 7.7%, polystyrene (PS) 6.4% and other plastics, such as expanded polystyrene (EPS), acrylonitrile butadiene styrene (ABS), polyamide (PA), polycarbonate (PC), polyvinylidene fluoride (PVDF) and poly(methyl methacrylate) (PMMA) accounting for 19% (Association of Plastics Manufacturers 2019). The above-mentioned plastic types have different applications that are described in more detail in Table 1.1. For example, PE is usually used in packaging products that have a short useful lifetime. PET is typically used in bottles and cups. PP is used in auto parts, food containers, and industrial fibres. PVC is commonly used in building construction, as it has a much longer lifetime. PU is used in medical devices, packaging, coating, and building construction. PS is usually used in utensils, tableware, cafeteria trays, and containers. EPS, mainly known as Styrofoam, is widely used in packaging due to its good shock-absorbing properties. ABS is used in appliances, automotive, packaging, pipes and fittings, and business machines. PA is used in motor vehicles, industrial yarns, the textile industry, and packaging. PC has a range of applications, from architectural to automotive, digital recording, electrical, and electronic. PMMA is used in biomedical and pharmaceutical applications, such as drug delivery/release systems and cranioplasty, in chemistry, for molecular separation (in chromatography techniques), and in the optical field, in lenses, microscopes, and lasers. PVDF is used in industrial applications, it is also used in batteries, and powder coatings, among others (Adams et al. 1993; Noguchi et al. 1998; Inc 2009; Tokiwa et al. 2009; Bostick 2010; Ali, Karim, and Buang 2015; GESAMP 2015; Stéphane Jeol, Cumming 2015). Table 1.1. summarizes the main applications of the most used above-mentioned plastics.

Table 1.1.: Plastics types and their main applications (Gondal and Siddiqui 2007; Oliveira et al. 2020, adapted)**.**

Plastic-type	Application
LDPE	Dairy products and fruit packaging films; greenhouse films; organic fertilizer film; industrial packaging bags; waste containers; plastic crates; shopping bags.
HDPE	Packaging of milk, water and fruit juice, oil, and lubricating oil; industrial barrels; waste collection containers.
PP	Garden furniture; non-woven bags and non-woven industrial bags; packaging and food containers films; industrial barrels; laboratory clothes; auto parts.

PET	Water and soft drink packaging; carpet fibres; chewing gum; coffee spoons; drink cups; food packaging and wrapping; heat-sealed packaging; kitchen trains; plastic bags; squeeze packaging; toys.
PVC	Food packaging and wrapping; toilet tissue boxes; cosmetics; bumpers; floor tiles; pacifiers; shower curtains; toys; water barrels; garden hoses; car upholstery; inflatable pools.
PU	Refrigerator and freezer thermal insulation systems, body car production (interior "headline" ceiling sections, doors, windows); building and construction applications; electrical and electronics industries to encapsulate; seal and insulate; pressure-sensitive; microelectronic components; medical applications (catheter, general-purpose tubing, surgical drapes, injection-moulded devices).
PS	Food packaging of meat, fish, cheese, yogurt, appetizer, ham, and seafood; rigid and foam dishes; audio cassette and cd boxes; storage boxes; building insulation; ice buckets; wall tiles; trays; disposable cups for hot drinks.
ABS	Appliance (including kitchen appliance housings, vacuum cleaners, and power tools); automotive; packaging; pipes; and fittings (mainly of the drain, waste, and vent applications).
PA	Motor vehicles (alternative to metal in automotive under the hood parts); food packaging; sports and leisure (manufacture of Ski bindings, for example); furniture market (manufacture of stadium seats, for example); electrical and electronics applications.
PC	Appliance (refrigerators, air conditioners, coffee machines, food mixers, washing machines, etc.); automotive; building and construction (glazing application, facades, security windows, skylights); electrical and electronic and medical instruments (surgical instruments, drug delivery systems, haemodialysis membranes, blood reservoirs, blood filters, etc.).
PMMA	Biomedical applications (preparation of bone cement for drug delivery, cranioplasty, dental implants); molecular separation application (chromatographic techniques); optical applications (lenses, microscopes, lasers, etc.); sensor, solar, and nanotechnology applications.
PVDF	Electrical and electronic devices (wire insulation/cable jacketing in aircraft and electronics industry); filtration and separation equipment (filters, membranes, housings); industrial applications (pipes, fittings, valves, tubing); and medical applications.

In this dissertation, biodegradation studies were focused on four plastic types PE, PLA, PS, and PVDF.

1.2.1. Polyethylene (PE)

PE is considered the simplest basic structure of any polymer, it is a linear hydrocarbon polymer consisting of long chains of ethylene monomers $(C_2H_4)_n$ (Figure 1.3a) (Huerta et al. 2018). It is formed by opening the double chain of ethylene molecules and joining them in straight or branched chains. This structure gives PE a hydrophobic nature and a dimension too large to pass through microbial cell membranes (Arutchelvi et al. 2008; Bardají et al. 2020).

This polymer can be classified as low-density polyethylene (LDPE) and high-density polyethylene (HDPE), due to differences in resistance to chemicals and flexibility toughness. HDPE is characterized by its large density to strength ratio, which means that its intermolecular forces are strong and resistant

to many different solvents. On the other hand, LDPE is resistant to acids, alcohols, bases, and esters, but is unstable in the presence of strong oxidizing agents, aliphatic, and aromatic hydrocarbons (Scalenghe 2018). HDPE has mostly linear chains, while LDPE has a high degree of branching. Polyethylene can be easily processed for various products because it is made from petrochemical feedstock through an efficient catalytic polymerization process. These features make it a very versatile material with a wide range of applications (Arutchelvi et al. 2008). PE is considered a recalcitrant material, which contributed to its prevalence and over-accumulation in the environment. Despite this, PE biodegradation studies by microorganisms have had success in the past decade (Soni et al. 2008).

1.2.2. Polystyrene (PS)

PS is a homopolymer and results from the polymerization of a styrene monomer (Figure 1.3b). When in the pure solid-state it is a colourless and hard plastic with limited flexibility (Mckeen 2014). It is possible to find this polymer in three different forms - crystalline, high impact, and expended (EPS). It is the combination of low cost/easy production, transparency/easy colouring, and shiny surface that has led to the successful commercialization of this polymer (Abhijith, Ashok, and Rejeesh 2018). This plastic type is used in a wide range of products due to its resistance to biodegradation, stiffness, lightweight, and easy synthesis (Mor and Sivan 2008).

1.2.3. Polyvinylidene Fluoride (PVDF)

PVDF is constituted by repeated units of $(CH_2CF_2)_n$ and it is considered a semicrystalline polymer (Figure 1.3c). PVDF has good chemical resistance (having excellent resistance to corrosive acids), high mechanical strength, thermal and hydrolytic stability, good aging resistance, and good resistance to degradation under UV radiation (Kang and Cao 2014; Momenzadeh et al. 2020). Although PVDF is considered a non-biodegradable polymer, it shows high biocompatibility and bioneutrality (Houis et al. 2010).

1.2.4 Polylactic acid (PLA)

PLA is considered a biodegradable polyester that can be produced by a fermentative biotechnological process using agricultural products as feedstock (such as sugarcane, potato, corn, wheat) (Qi, Ren, and Wang 2017). PLA results from a chemical polymerization of the bio-based lactic acid monomers that are obtained by fermentation of the agricultural feedstock (Figure 1.3d). The monomers can be chemically polymerized either by ring-opening polymerization or direct polycondensation (Prathipa, Sivakumar, and Shanmugasundaram 2018). PLA presents optical, mechanical, and barrier properties comparable to conventional plastics and has a range of applications, from packaging to medical

applications (for example in surgical sutures, since the polymer is hydrolysable into soluble oligomers in the human body) (Jarerat, Tokiwa, and Tanaka 2003).

In spite of being considered a bioplastic, PLA is, in fact, only biodegradable under industrial composting conditions. However, the full potential of this polymer will only be attained with the development of adequate infrastructures for sorting, recycling, and composting PLA plastics (Tokiwa et al. 2009; Castro-Aguirre et al. 2016; Qi, Ren, and Wang 2017).

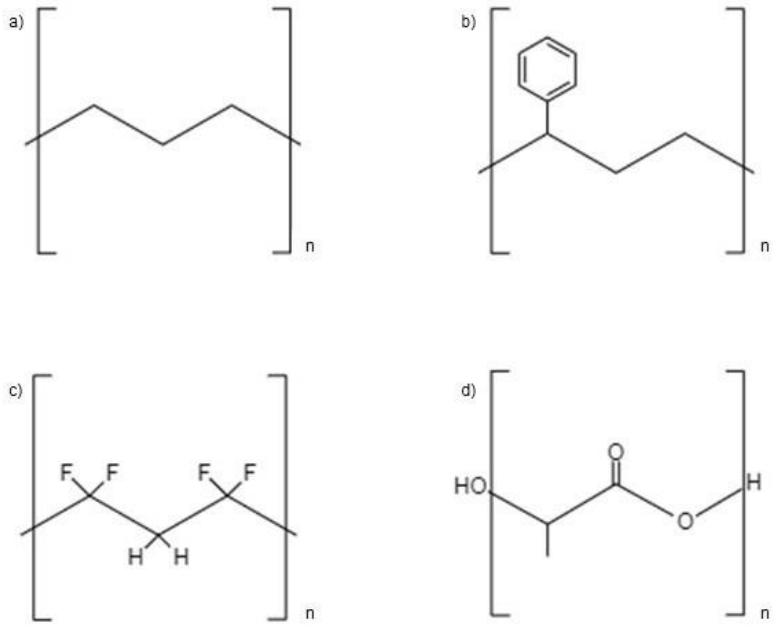

Figure 1.3.: Molecular structure of: a) polyethylene; b) polystyrene; c) polyvinylidene fluoride; d) polylactic acid (Chemical structures were drawn in ChemDraw).

1.3. Microplastics

Plastics are becoming a big environmental concern since they can be truly harmful, principally when they become microplastics (Scalenghe 2018). Microplastics are particles with dimensions between 1 μm and 5 mm and are considered as emerging pollutants, since they have high molecular weight, are hydrophobic, are highly recalcitrant against biodegradation and they are not commonly monitored or regulated in the environment with potentially known or suspected adverse ecological and human health effects. Besides, microplastics are present in several environments, including marine, freshwater, and terrestrial ecosystems (M. Zhang et al. 2019; Park and Kim 2019). Characteristics such as small size, composition, and persistence in the environment, contribute to their global dispersion through ocean currents (Desforges et al. 2014).

Microplastics can be classified as primary and secondary microplastics, according to their origin. When the plastic particles are manufactured in the microparticle range of sizes, they are considered primary microplastics, and include microbeads used in cosmetic formulations, plastic powders used in moulding, among others (Derraik 2002; Andrady 2011). Secondary microplastics result from the breakdown of larger plastic items and include fragments and fibres derived from textiles, tires, and paints. Secondary microplastics can also result from incorrect discards that released macroplastics (fragments larger than 5 mm) into the environment. In this way, plastics start to lose their mechanical integrity, first through physical and chemical environmental factors (such as waves, wind, UV radiation, etc.), and then by biological environmental factors (Figure 1.4.). The thermo and photo-oxidative processes in nature weaken the plastic integrity leading to the production of progressively smaller particles (<5 mm) (C. Moore 2007; C. J. Moore 2008; Huerta et al. 2018; GESAMP 2015; Correia et al. 2020).

Microplastics are associated with severe environmental contamination since they can be found even in remote locations and far from their anthropogenic sources of emissions (Park and Kim 2019). Several studies indicate the presence of microplastics in different sea and coastal areas, for example, in the waters of the Mediterranean Sea about 0.15 particles per m^3 have been determined (de Lucia et al. 2014). Microplastics have a severe negative impact on marine ecosystems and human health, either by ingestion, by adsorption of organic pollutants, or by leaching of toxic chemicals (M. Zhang et al. 2019). When microplastics settle in certain locations, for example on sandy beaches, they can change the heat transfer, resulting in slower heating and lower sediment maximum temperatures. These variations can cause damaging hormonal variations in animal reproduction and temperature-dependent sex determination of numerous animals, besides interfering with eggs hatching time (Carson et al. 2011; J. Oliveira et al. 2020).

It may seem that microplastics are still a small proportion of the total mass of plastics in the ocean, but microplastics will continue to exist and persist due to the constant degradation of macroplastics. The problem continues to increase at the rate that macroplastic waste in the ocean undergoes continuous degradation and will prevail for many years, even after plastic discharges stop (GESAMP 2015; Correia et al. 2020).

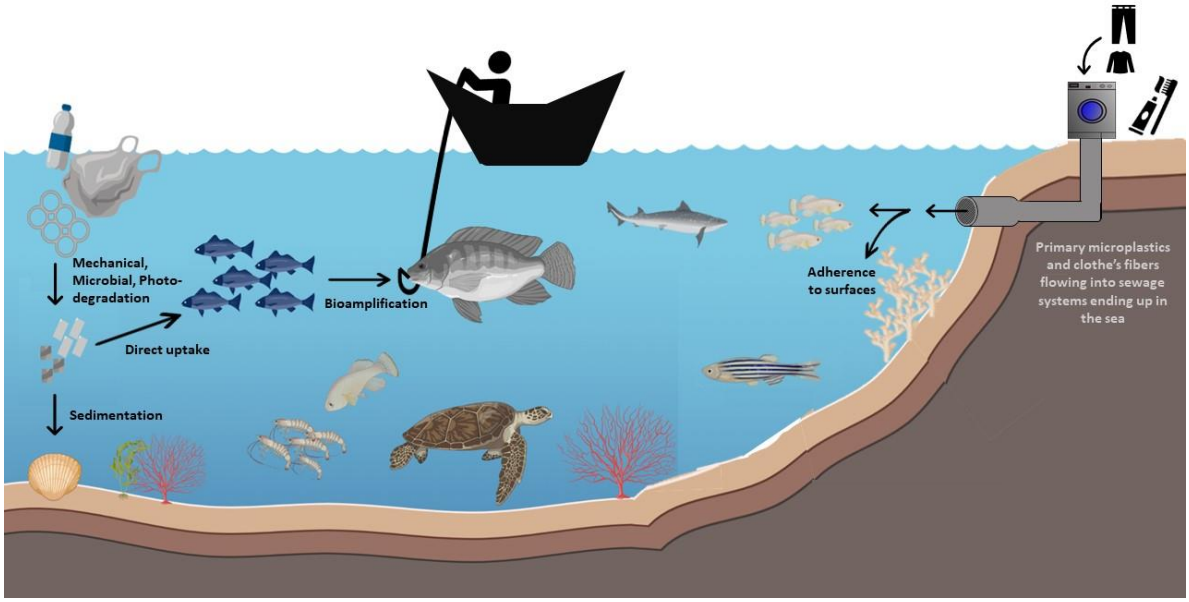

Figure 1.4.: Plastic pollution causes negative effects on the ecosystem and food chain, affecting the population's health, by the ingestion of food contaminated with microplastics. For example, fish can contain significant percentages of microplastics and when ingested by humans can cause several inflammatory diseases and even cancer. Microplastics appear in the environment when they are produced to be that size and cannot be contained by sewer networks or appear by the degradation of larger plastics (Figure created in Biorender and PowerPoint).

1.4. Degradation of (micro)plastics

Studies indicate that plastics may undergo degradation by various mechanisms, depending on their chemical nature, as well as on the conditions to which they are subjected (Raghavan 1995). Degradation is a chemical reaction that leads to the fission of polymer chains and can irreversibly modify the properties of polymeric materials (Santos et al. 2002).

When plastics enter the marine environment, the material density, compared with the seawater density, influences the material behaviour. The density will determine if the discarded material sinks or floats. Even when a plastic float, it will lose its integrity over time by degradation and can eventually settle in marine sediments. The polymer degradation depends on the nature of the polymer and on the conditions that it is subjected to, ranging from abiotic factors (sun, heat, humidity, etc.) to assimilation by microorganisms (Karamanlioglu, Preziosi, and Robson 2017). The polymer degradation can be classified as abiotic or biotic degradation.

While abiotic degradation is caused by environmental factors (as temperature, UV radiation, wind, waves, etc.), biotic degradation (or biodegradation) occurs by the action of microorganisms that modify and consume the polymer, changing its properties. Both degradation types occur simultaneously in nature (Restrepo-flórez, Bassi, and Thompson 2014).

Regarding the abiotic degradation of plastics, it can be classified as thermal, mechanical, and chemical (photodegradation, thermal, and photo-oxidation) degradation. UV radiation can be considered the central cause of plastic degradation in the environment, because the debris on the beaches, soils, or floating in the water are exposed to solar radiation, inducing their oxidative degradation (Sazanov, Florinsky, and Koton 1979; Scott 1995; Kamo et al. 2004; L. Li et al. 2004; Chen et al. 2018).

On the other hand, biotic degradation takes place by the action of enzymes produced by microorganisms that occur in the environment. During the biodegradation process, organic compounds are converted into simpler organic compounds. This process generates CO_2, methane, and microbial cellular components as sub-products (Chandra and Rustgi 1998; Banker et al. 2016). The factors affecting the biodegradability of plastics are the polymer and the microbial characteristics. The microbial characteristics include the microorganism type, distribution, growth conditions (temperature, pH, oxygen concentration, nutrients, etc.), and enzyme types (intracellular and/or extracellular enzymes, inducing exo- or endo-polymer cleavage). Polymer characteristics comprise the physical and chemical properties, such as surface conditions (surface area, hydrophobic and hydrophilic properties), first-order structures (chemical structure, molecular weight, and molecular distribution), and high order structures (glass transition temperature, melting temperature, modulus of elasticity, crystallinity and crystal structure) (Tokiwa et al. 2009).

1.4.1. Degradation of microplastics by microorganisms

Even though microplastics can persist for long periods in the environment, there are several studies and reports indicating the potential of microorganisms to degrade certain types of polymers in controlled conditions. Pure cultures of bacteria or fungi, bacterial biofilms, and bacterial consortia were used in reported degradation assays. These microorganisms were able to use polymers as carbon source in culture media with minimal nutrients, inducing changes in the polymer´s physical and morphological properties, and in their chemical structure (Janssen et al. 2002). The recent investigations and studies about plastics biodegradation are centred on the isolation of microorganisms from their natural terrestrial and marine environments since microorganisms can adapt to almost every environment through the adaption of their metabolisms. This increases the probability of finding polymer degrading microorganisms (Park and Kim 2019).

The use of pure cultures allows the discovery of the metabolic pathways to evaluate the effect of different environmental conditions during degradation. When using bacterial consortia it is possible to achieve a stable microbial community and eliminate the toxic effect of metabolites produced by some strains present in the consortia, improving biodegradation efficiency (Janssen et al. 2002; Yuan et al. 2020). On the other hand, microplastics are a good support for the colonization and growth of microorganisms. Therefore, the biofilm formation first requires the microorganisms' adherence to the surface of microplastics and the alteration of its properties. After this, microorganisms secrete enzymes that release additives and monomers out of the microplastics, increasing biodegradation. Thus, plastic starts to lose its mechanical properties and stability, becoming fragile from microbial attack. Finally, water and

microbial filaments start to penetrate the polymer resulting in decomposition and utilization of microplastics by biofilm microorganisms (Yuan et al. 2020).

In a laboratory environment, several conditions influence the final result of plastic biodegradation, since some pre-treatments (with UV radiation or temperature, for example), a combination of conventional plastics with a bioplastic, and optimization of experimental conditions can improve the biodegradation rate.

There are numerous examples with evidence of biodegradation. A *Bacillus* strain (*Bacillus sp. YT1*) was used on a PE biodegradation assay. In two months, *Bacillus sp. YT1* degraded about 10.7% of the initial PE weight, creating damage in the microplastic surface, including holes and pits, and introducing carbonyl groups during the biodegradation assays, which revealed high degrading capacity. This strain formed a biofilm on PE, and this enabled the microorganisms to utilize the non-soluble substrate efficiently (Yang et al. 2014). Bacterial consortia are also found as plastic degraders, which is the case of a mesophilic mixed bacterial culture (*Bacillus* sp. and *Paenibacillus* sp.) that reduced 19% of PE weight in 2 months (Park and Kim 2019). Another example is *Zalerion maritimum*, a marine fungus, that could degrade PE by decreasing 43% of the initial mass of the films, in minimum growth media (Paço et al. 2017). Also, the fungus *Aspergillus tubingensis* could reduce PU tensile strength by 90% in 0.75 months (Khan et al. 2017). Hence, there are several strains capable of degrading different types of polymers.

1.4.2. Actinobacteria

Actinobacteria is a very important phylum in this present work since they were used to perform the biodegradation assays of this thesis.

Actinobacteria, named actinomycetes, are Gram-positive bacteria. These are present in various ecological habitats, like soils, freshwater, and marine environments (Arasu et al. 2012). This phylum can grow in extreme climatic conditions, having high biotechnological applications. Thus, actinobacteria are studied by chemists, pharmaceutical companies, among others (Gohain et al. 2020). Nowadays, actinobacteria are used in the production of antibiotics, fungicides, and anticancer drugs. Some strains can digest resistant carbohydrates (such as cellulose and chitin), others are used in bioremediation due to their ability to degrade toxic materials or to play a role in the recycling of organic carbon. Recently, actinobacteria started to be used in the degradation of complex polymers, meaning that actinobacteria have numerous distinct metabolisms and can perform different functions in the environment (Sharma, Dangi, and Choudhary 2014).

Actinobacteria can produce a wide variety of hydrolytic enzymes and bioactive metabolites. Their ability to grow on different polymers comes from the production of these enzymes, which also facilitates the degradation of high molecular weight compounds to simpler ones (Amobonye et al. 2020).

Table 1.2. summarizes the main polymer degrader actinobacteria. Different genera and species can degrade different polymer types.

Table 1.2.: Plastics degraded by actinobacteria strains, used biodegradation assay conditions and detection methods. "•" indicates marine-derived actinobacteria. n.a. information not available. (J. Oliveira et al. 2020, adapted)**.**

Actinobacteria strain	*Plastic type*	*Biodegradation assay conditions (Media, time, rpm, T ºC)*	*Biodegradation detection method*	*Polymer reduction (%)*	*Reference*
Rhodococcus ruber strain C208	PE	Synthetic media, for 2 months, 150 rpm, at 30 ºC	Weight loss; SEM	7.5	(Sivan, Szanto, and Pavlov 2006)
Rhodococcus rhodochrous ATCC 29672	PE	Mineral salt media, for 6 months, at 27 °C and 85% humidity	FTIR; SEM; GPC	n.a.	(Bonhomme et al. 2003)
Micrococcus sp.	PE	Nutrient broth media, for 1 month	Weight loss	6.61	(Kathiresan 2003)
***Streptomyces* KU8**	PE	Mineral salt medium, for 6 months, 150 rpm, at 30 ºC	Clear zone method; weight loss	46.16	(Usha, Muthusamy, and Palaniswamy 2011)
Bacterial consortia Arthrobacter viscosus; Micrococcus lylae; M. luteus; Bacillus mycoides; B. cerus; B. pumilus; B. thuringiensis	PE	Films buried in soil for 7.5 months, at RT	Weight loss; FTIR; SEM; elongation at the break	17.0	(Nowak et al. 2011)
Microbacterium paraoxydans •	PE (pre-treated with nitric acid)	Minimal broth media, for 2 months, 180 rpm, at RT	Weight loss; FTIR	61.0	(H. Rajandas et al. 2012)
Rhodococcus ruber	PE (pre-treated with UV radiation)	Minimal synthetic medium, for 1 month, 150 rpm, at 30 °C	Quantitative estimation of bacterial biomass; weight loss; FTIR; SEM	8	(Orr, Hadar, and Sivan 2004)
Streptomyces badius	Starch-PE (10 d-heat-tread)	0.6% yeast extract media, for 0.75 months, 125 rpm, at 37 ºC	FTIR; tensile strength at brake; GPC	n.a.	(Pometto III, Lee, and Johnson 1992)
Streptomyces setonii	Starch-PE (10 d-heat-tread)	0.6% yeast extract media, for 0.75 months, 125 rpm, at 37 ºC	FTIR; tensile strength at brake; GPC	n.a.	(Pometto III, Lee, and Johnson 1992)
Streptomyces viridosporus	Starch-PE (10 d-heat-tread)	0.6% yeast extract media, for 0.75 months, 125 rpm, at 37 ºC	FTIR; tensile strength at brake; GPC	n.a.	(Pometto III, Lee, and Johnson 1992)

***Streptomyces* sp.**	PET	Mineral medium, for 0.6 months, 120 rpm, at 28 ºC	Weight loss; SEM; GC-MS analysis (to identify biodegradation products)	68.8	(Farzi, Dehnad, and Fotouhi 2019)
Rhodococcus ruber	PS	Synthetic media, for 2 months, 120 rpm, at 35 ºC	Weight loss; SEM	0.8	(Mor and Sivan 2008)
***Microbacterium* sp. NA23**	PS in form of EPS	Mineral salts media, for 2 months, 120 rpm, at 30˚C	FTIR; SEM; HPLC (to identify biodegradation products)	n.a.*	(Atiq et al. 2010)
Rhodococcus sp. strain 36 ●	PP	Bushnell Haas (BH) media, for 1.25 months, at 29 ºC	Weight loss; FTIR; SEM	6.4	(Auta et al. 2018)
Rhodococcus rhodochrous ATCC 29672	PP (pre-treated with photo and thermo-oxidation)	Mineral media, for 6 months, at 27 ºC	FTIR; ^{1}H NMR; ADP/ATP ratio	n.a.	(Fontanella et al. 2013)
Micrococcus sp. AF10	PU	Films buried in soil, for 6 months, at 30-35 ºC	Clear zone test; SEM; FTIR	n.a.	(Shah, Hasan, Akhter, et al. 2008b)
Arthrobacter sp. AF11	PU	Films buried in soil, for 6 months, at 30-35 ºC	Clear zone test; SEM; FTIR	n.a.	(Shah, Hasan, Akhter, et al. 2008b)
Amycolatopsis orientalis ● ***(enzyme production)***	PLA (production of purified enzyme)	Incubation for 8h, 140 rpm, at 30 ºC	FTIR, pH variation; enzyme degrading activity	100	(Jarerat, Tokiwa, and Tanaka 2006)
Saccharothrix waywayandensis ●	PLA	Basal media with 0.1% gelatine, for 0.25 months, 180 rpm, at 30 ºC	Weight loss; pH variation	95	(Jarerat and Tokiwa 2003)
Kibdelosporangium aridum ●	PLA	Basal media with 0.1% gelatine, for 0.5 months, 180 rpm, at 30 ºC	Weight loss; pH variation; SEM	97	(Jarerat, Tokiwa, and Tanaka 2003)
Actinomadura sp. T16-1 (enzyme production)	PLA (production of PLA-degrading enzyme)	Basal media with 0.2% gelatine, for 96h, 150 rpm, at 50 ºC	Enzyme activity	n.a.	(Sukkhum, Tokuyama, and Kitpreechavanich 2009)

*Only grew on EPS surface but did not use it as a sole carbon source.

In recent studies, actinobacteria revealed relatively good degradation capacities of different polymers. For example, *Rhodococcus ruber* C208 strain could, in two months, degraded about 7.5% of the initial weight of a PE film, using PE as a sole carbon source. *R. ruber* formed a biofilm on the PE surface after 12-15h incubation and after one day the biofilm structure had reached its final size and the signs of

biodegradation appeared after 16 days (Sivan, Szanto, and Pavlov 2006). In other research, the strain *Microbacterium paraoxydans* could reduce 61% of polyethylene. This was possible because PE went through a nitric acid treatment before the biodegradation assay, which facilitated and enhanced the microbial attack. This treatment reduced the bonds in the polymers, breaking them into smaller chains and allowing the incorporation of carbonyl groups in the backbone of the polymer, triggering biodegradation (H. Rajandas et al. 2012). There are still other reports showing that *Rhodococcus, Micrococcus, Streptomyces, Arthrobacter* species, and other actinobacteria are capable of degrading certain types of microplastics, such as PP, PLA, PU, and PE (Kathiresan 2003; Mor and Sivan 2008; Shah et al. 2008; Fontanella et al. 2013; Auta et al. 2018).

Several studies indicate that the Actinobacteria phylum demonstrates an efficient PLA degradation capacity. PLA degrading actinobacteria can degrade this biopolymer either under field trials or under laboratory conditions (Butbunchu and Pathom-aree 2019). Successfully PLA-degrading actinobacteria genera were *Actinomadura*, *Amycolatopsis*, *Kibdelosporangium*, *Micromonospora*, *Nonomuraea*, *Pseudonocardia*, *Saccharothrix*, *Streptoalloteichus*, *Streptomyces*, *Thermomonospora*, and *Thermopolyspora*. However, the most dominant PLA-degrading actinobacteria are members of the *Amycolatopsis* genus (Jarerat and Tokiwa 2003; Jarerat, Tokiwa, and Tanaka 2006; Butbunchu and Pathom-aree 2019).

1.5. Methods to detect microplastics biodegradation

Several techniques can be applied to detect and quantify biodegradation activity by microorganisms. The methods that can be used to assess microplastic biodegradation are the clear zone method, gravimetric determination of weight loss, Fourier Transform Infrared Spectroscopy with Attenuated Total Reflectance (FTIR-ATR), Scanning Electron Microscopy (SEM), Gel Permeation Chromatography (GPC), Tensile Strength (R_m) and Elongation at break (ε_r), Nuclear Magnetic Resonance (NMR) Spectroscopy, pH variation, ADP/ATP ratio, among others that are less common.

The most relevant methods for this dissertation will be described below.

The clear zone method serves as a previous screening of available microorganisms to find which ones have the potential to degrade certain types of polymers. Usually, agar plates containing emulsified polymers are used for microorganism inoculation. Therefore, this method provides access to the degradation potential of microorganisms towards a selected polymer. When a microorganism grows on an emulsified media and consumes the polymer, a clear zone around the colony appears, meaning that the degradation of the polymer occurs. This does not indicate necessarily the consumption of all compounds but reflects the breakdown of the polymer chains. When a microorganism can degrade the polymer in the medium, it excretes extracellular enzymes that diffuse through the agar and degrades the polymer into water-soluble materials (Augusta, Miiller, and Widdecke 1993; Mergaert and Swings 1996).

The gravimetric determination of weight loss is widely used for polymeric degradation evaluation. This method must be used and interpreted carefully because it can be affected by inaccuracy, since the biodegradation extent is limited and relatively slow, so it has to be associated with other techniques to prove that biodegradation occurred (Raddadi and Fava 2019). The weight loss will depend on the incubation time and conditions, and on the microorganisms and polymers used (Nowak et al. 2011).

Regarding FTIR spectroscopy, it is used to evaluate and study chemical modifications that the structure of polymeric films have undergone. This technique allows the monitoring of the changes that occur in the functional groups on the polymers surface, such as hydrogen bonding, end group detection, cross-linking behaviour of molecules, and copolymer composition in liquid or solid form. When analysing a FTIR spectrum, it is important to take into account that the chemical changes in the functional groups are due to the microbial activity, by the transformation of certain chemical groups (Azapagic, Emsley, and Hamerton 2003; Hadad, Geresh, and Sivan 2005; Singh and Sharma 2008).

It is also possible to assess the biodegradation extent through the modifications of the tensile strength (R_m) and elongation at break (ε_r). Different polymers have different values of tensile strength and elongation at break, as they are constituted by different monomers and the chains are oriented differently. Therefore, different polymers require different applied forces to break or deform them. After microbial attack, for example, in biodegradation assays, significant changes in the mechanical properties occur due to biochemical modifications of the polymers. The modifications can result from the disintegration of polymer chains or the formation of cross-linking bonds between monomers chains, consequently, R_m and ε_r values will differ from the original polymer (Nowak et al. 2011).

Moreover, searching for polyhydroxyalkanoates (PHA) inclusions in bacteria can also be an indirect way to assess carbon source biodegradation. Some microorganisms produce polyesters (PHAs is the general class of compounds), that are also susceptible to microbial attack by enzymes. PHAs are synthesized as a reserve material for carbon storage and energy reserve by several bacterial strains, and their production begins under growth-limiting conditions (Reis et al. 2011; Meereboer, Misra, and Mohanty 2020). PHA synthesis is important in biodegradation since the metabolic pathways are related to bio-assimilation. Usually, PHAs are directly synthesized of a carbon substrate via fermentation inside a microorganism. Different hydroxyalkanoic acids can be obtained, homo- or co-polyesters, depending on the microorganisms and the cultivation conditions (Fuessl, Yamamoto, and Schneller 2012; Meereboer, Misra, and Mohanty 2020). PHA can be produced through a three-enzyme pathway, containing β-ketothiolase, acetoacetyl reductase, and PHA synthase (Fuessl, Yamamoto, and Schneller 2012). In this way, PHA are biodegradable (bacteria that produce extracellular PHA depolymerase can easily biodegrade it) and biocompatible aliphatic polyesters with a microbial origin (Lambert and Wagner 2017). These can replace conventional/synthetic plastics in several applications, such as packaging and pharmaceutical and medical products (Reis et al. 2011; Cooper 2013).

1.6. Research objectives

A marine biotechnology research field is emerging, which focuses on the search for solutions to overcome marine pollution by plastics and microplastics.

We hypothesize that different actinobacteria strains are capable of degrading different types of (micro)plastics by using them as a carbon source, and then transformed them into bioplastics, through the production of PHA inclusions. In this way, this work aims at contributing to the search for solutions and sustainable methods to mitigate the described problem, by developing a new approach for (micro)plastic biodegradation, discovering new strains of biodegrading actinobacteria, and, simultaneously, evaluating their ability to produce bioplastics (PHAs).

For that, the plastic biodegradation ability of actinobacteria isolated from oceanic sediments of the *Estremadura Spur* will be evaluated. A screening of actinobacteria from the available collection will be performed in order to evaluate their potential to biodegrade different types of polymers, using them as a sole carbon source (polymer emulsified media).

Subsequently, the strains high plastic biodegradation potential selected by the screening test will be cultured in liquid media and will be used in plastic film biodegradation assays. Different culture media conditions, as well as the impact on biodegradation of plastic film pre-treatment with UV radiation will be evaluated. The extent of biodegradation will be monitored by weight loss validated through physical and structural changes, by mechanical assays and Fourier Transform Infrared spectroscopy. The possible presence of PHA inclusions in the actinobacteria with plastic biodegradation activity will be also analysed.

Overall, biodegradation assays will be performed to characterize the biodegradation response of different actinobacteria to different types of plastics, aiming at finding an effective method for plastics biodegradation.

2

Materials and Methods

2.1. Reagents and equipments

For this work, the following reagents were used: polyethylene terephthalate (PET) and polylactic acid (PLA), purchased from Goodfellow; polyvinylidene fluoride (PVDF); polyvinyl chloride (PVC); polystyrene (PS); high- and low-density polyethylene (HDPE and LDPE, respectively), acrylonitrile butadiene styrene (ABS), and sodium lauryl sulphate (SLS), were purchased from Sigma-Aldrich; plastic films were produced at Materials Department, CENIMAT, FCT NOVA. Agar-agar (article code: LB143-1000) and acetone (CH_3COCH_3, purity > 99%) were purchased from LabChem. Dichloromethane (CH_2Cl_2, HPLC grade), was purchased from VWR PROLABO. Yeast extract (ref.: 212750) was purchased from Becton, Dickinson and Company. Filtered natural seawater (NSW) – from Costa da Caparica, synthetic seawater (SSW), and distilled water. Ethanol (C_2H_5OH, 96%), purchased from Aga. Sodium dodecyl sulphate (SDS) (article code: CN 30.4) was purchased from Carl Roth. Nile blue stain was purchased from Sigma-Aldrich.

The following equipment was used: a sonicator (Emmi-D60 from EMAG Technologies), a scale (KERN EW 600-2M, d=0.01g), a magnetic stirrer hotplate (Stuart Scientific), an autoclave (Panasonic MLS-3781L), two different shakers – shaker at 25 ºC (Sartorius, CERTOMAT) and shaker at 30 ºC (Lab Company, SKC 6200), uniaxial tensile testing machine (Rheometric Scientific Minimat with a 100 N load cell, CENIMAT) for fibbers and thin films, a heated press (CENIMAT), a UV lamp (Elnorlux, 15 W and 50 Hz), a laboratory oven (Panasonic, MIR-254), a laminar flow hood (Faster, Bio 48, 2994/13), a scale (OHAUS, Analytical Plus 110S), an FTIR-ATR spectrophotometer (Perkin Elmer Spectrum Two), a thermostatic bath (Nahita, Model 601), and an epifluorescence microscope (Olympus BX51, Japan) at 1000X.

2.2. General Experimental Procedure

Emulsified plastic media were prepared for actinobacteria biodegradation screening. PVDF, PS, and PLA were the polymers used to prepare the emulsified media for the screening. These media preparation required dissolving the polymers in dichloromethane, adding SLS, and sonicating the mixtures. After sonication, the dichloromethane was evaporated, agar and a mixture of 75% NSW and 25% distilled water were added. The emulsified media were autoclaved before being transferred to Petri dishes. Different emulsified media were prepared with PVDF, PS, and PLA, following the same procedure described above, but with the addition of yeast extract (0.1% (m/v)), to compare the biodegradation potential and the presence and absence of this component in the culture media.

For the screening investigation, the 36 actinobacteria strains used in this work had been previously isolated from marine sediments collected in the *Estremadura Spur*, of *Continental Portugal* coast, between *Cabo Carvoeiro* and *Cabo da Roca*. From the 36 tested strains, 3 were selected to perform LDPE and PS biodegradation (PTE-001; PTE-025; PTE-056), and 1 was selected for PLA biodegradation (PTE-048).

Actinobacteria strains were cultured in liquid media before being transferred to the biodegradation assay. PTE-001 was developed in SSW liquid media containing PS emulsion, while PTE-025, PTE-056, and PTE-048 were grown in SSW liquid media with yeast extract.

LDPE, PS, and PLA films used in the biodegradation assays were produced according to the procedure described in section 2.4.2. and half of the films were pre-treated with UV radiation (photo-oxidation). After film sterilization, they were aseptically added to the biodegradation assays with the mentioned strains. Biodegradation was monitored by film weight loss, variations in the functional groups (FTIR-ATR) and in the mechanical properties analysed by tensile strength tests, and detection of PHA inclusions in the actinobacteria.

All these steps have been optimized and are described in detail in the following sections (Figure 2.1.).

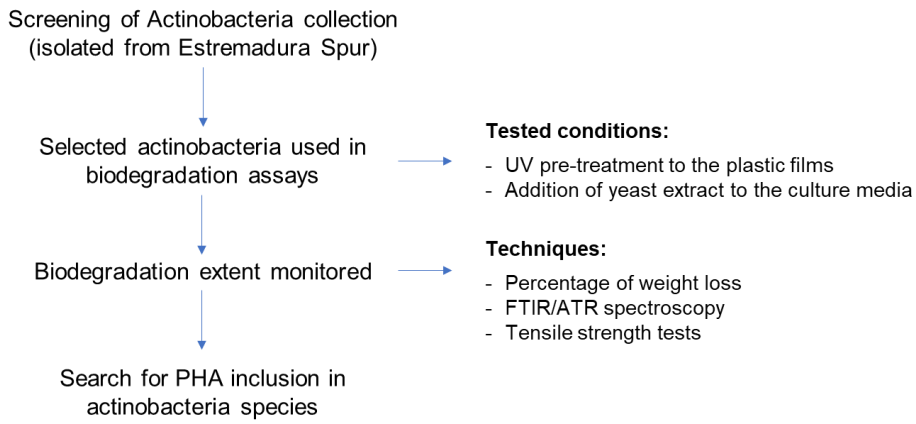

Figure 2.1.: Schematic representation of the general experimental procedure.

2.3. Screening of actinobacteria with plastic degradation potential

2.3.1. Preparation of polymer-emulsified media

Polymer emulsified media were prepared in different conditions, to optimize them for the screening of plastic degradation by actinobacteria. Nine polymers were tested in twelve different emulsifying conditions, as described in Table 2.1. The common medium (named NSW medium) was composed of 75% NSW, 25% distilled water, and 14 gL^{-1} agar. This medium was also used as a control.

In assay 1, the emulsified medium was prepared by adding NSW, distilled water, 0.05 g of polymer, and 18 gL^{-1} agar. After mixing, the medium was autoclaved (Tachibana et al. 2017).

In assay 2, the polymer (0.15% wt/vol) was dissolved in 1.5 mL of dichloromethane. The solution was added to the NSW medium (without agar) and then sonicated, for 15 min. After sonication, CH_2Cl_2 was evaporated in a magnetic stirrer hotplate at 60 ºC, for 6 min. Finally, 14 gL^{-1} of agar were added to and the medium was autoclaved (Tachibana et al. 2017).

For assay 3, 0.05 g of polymer were dissolved in 2 mL of dichloromethane followed by the addiction of 0.1 g of SLS surfactant and NSW medium. The solution was sonicated for 15 minutes and then heated on a magnetic stirrer hotplate for dichloromethane evaporation before being autoclaved (Urbanek et al. 2017).

In the case of assay 4, 0.07 g of the polymer were dissolved in 2.5 mL of dichloromethane. After that, 0.01 g of surfactant and the NSW medium before were added to the mixture. The solution was sonicated for 15 minutes and then heated for dichloromethane evaporation before being autoclaved (F. Li et al. 2012; Urbanek et al. 2017, adapted). The differences between this assay and assay 3 were the quantities of polymer, dichloromethane, and surfactant.

Assay 5 was similar to assay 3, but acetone was used as a solvent, instead of dichloromethane.

Assay 6 was equivalent to assay 4, but the solvent used to dissolve the polymers was acetone in alternative to dichloromethane.

Assay 7 was equal to assay 4, but with a 5-fold decrease in the surfactant concentration.

Assay 8 was performed only with the polymers that dissolved in dichloromethane. The Petri dishes were composed of two layers: the lower layer contained autoclaved NSW medium and the upper layer contained the polymer suspension, in which 0.1% (w/v) of the polymer was dissolved in 10mL of dichloromethane and then sonicated for 15 minutes before being autoclaved. The suspension was placed over the first layer and dichloromethane was allowed to evaporate (Augusta, Miiller, and Widdecke 1993).

Assay 9 was similar to assay 8, but instead of using only dichloromethane for polymer suspension, 0.01 g of surfactant were used for the upper layer.

Assay 10 was similar to assay 4, but after the evaporation of dichloromethane, 1 gL^{-1} of yeast extract was added into the medium (Yottakot and Leelavatcharamas 2019).

In assay 11 emulsified PLA and PS were prepared by dissolving, overnight, 0.07 g of polymer pellet in 3 mL of dichloromethane. After the dissolution, 0.01 g of surfactant and 6 mL of distilled water were added and sonicated using an ultrasonicator, at 50% power for 10 minutes. Simultaneously, the NSW medium was prepared (with half of the quantity of distilled water because the other half was used in the emulsion) and autoclaved. The emulsion and NSW medium were mixed and the dichloromethane present in the emulsion was evaporated on a hotplate, before pouring into Petri dishes.

Finally, assay 12 was similar to assay 11, with the addition of yeast extract (0.1% w/v) to the NSW media.

The autoclavation was performed at 120 ºC for 20 minutes. Table 2.1. summarizes the twelve assays and the different polymers used to obtain the polymer-emulsified media.

Table 2.1.: Polymer-emulsification media conditions.

Assay	**Polymer quantity (g)**								**Agar quantity (g)**	**Solvent used**	**Surfactant quantity (g)**	**Sonification (Yes/No)**	**Yeast Extract (g/L)**	**Medium volume (mL)**
	PET	PVDF	PVC	PS	LDPE	HDPE	ABS	PLA						
1	0.05	-	-	-	0.05	0.05	-	-	0.9	NSW + distilled water	-	No	-	50
2	0.075	0.075	0.075	0.075	0.075	-	-	-	0.7	dichloromethane	-	Yes	-	
3	0.05	0.05	0.05	0.05	0.05	0.05	0.05	-	0.7	dichloromethane	0.1	Yes	-	
4	0.07	0.07	0.07	0.07	0.07	0.07	0.07	-	0.7	dichloromethane	0.01	Yes	-	
5	0.05	0.05	0.05	0.05	0.05	0.05	0.05	-	0.7	acetone	0.1	Yes	-	
6	0.07	0.07	0.07	0.07	0.07	0.07	0.07	-	0.7	acetone	0.01	Yes	-	
7	0.05	0.05	0.05	0.05	-	-	0.05	-	0.7	dichloromethane	0.02	Yes	-	
8	0.01	0.01	0.01	0.01	-	-	0.01	-	-	dichloromethane	-	Yes	-	10
9	0.01	0.01	0.01	0.01	-	-	0.01	-	-	dichloromethane	0.01	Yes	-	
10	-	0.07	-	-	-	-	-	-	0.7	dichloromethane	0.01	Yes	0.005	50
11	-	-	-	0.07	-	-	-	0.07	0.7	dichloromethane	0.01	Yes	-	
12	-	-	-	0.07	-	-	-	0.07	0.7	dichloromethane	0.01	Yes	0.005	

- the component was not added to the emulsified media.

2.3.2. Selection of actinobacteria for the screening assay

Marine-derived actinobacteria strains, previously obtained, isolated, and grown, as mentioned in section 2.1., were evaluated in order to select different actinobacteria species, from the in-house, *Estremadura*

Spur, Blue Biotechnology and Biomedicine Lab, taxonomically characterized collection. Thus, 36 strains (Table 2.2.) were used in the screenings.

Table 2.2.: Code and actinobacteria strains used in the biodegradation screening assays.

PTE code	Actinobacteria species
PTE – 001	*Streptomyces gougerotii*
PTE – 002	*Micromonospora zamorensis*
PTE – 003	*Micromonospora mangrove*
PTE – 006	*Streptomyces coelicolor*
PTE – 008	*Actinopolymorpha cephalotaxi*
PTE-009	*Streptomyces aculeolatus*
PTE-011	*Micromonospora chokoriensis*
PTE – 013	*Micromonospora chokoriensis*
PTE – 015	*Micromonospora schwarzwaldensis*
PTE-016	*Micromonospora chalcea*
PTE – 017	*Verrucosispora sonchi*
PTE – 020	*Micromonospora terminaliae*
PTE – 023	*Streptomyces malachitospinus*
PTE – 024	*Micromonospora chalcea*
PTE – 025	*Micromonospora matsumotoense*
PTE – 026	*Streptomyces camponoticapitis*
PTE – 028	*Streptomyces malachitofuscus*
PTE – 029	*Micromonospora chaiyaphumensis*
PTE – 030	*Micromonospora vinacea*
PTE – 032	*Streptomyces intermedius*
PTE – 033	*Actinomadura geliboluensis*
PTE – 041	*Streptomyces flavogriseus*
PTE – 042	*Streptomyces aculeolatus*
PTE – 046	*Actinomadura sporangiiformans*
PTE – 048	*Nocardiopsis prasina*
PTE – 052	*Streptomyces griseolus*
PTE – 054	*Streptomyces ovatisporus*
PTE – 055	*Micromonospora echinospora*
PTE – 056	*Micromonospora terminaliae*
PTE – 058	*Streptomyces chumphonensis*
PTE – 064	*Streptomyces sampsonii*
PTE-067	*Saccharopolyspora gloriosae*

PTE – 068	*Micromonospora maritima*
PTE – 073	*Streptomyces xiamenensis*
PTE – 074	*Saccharomonospora xinjiangensis*
PTE – 075	*Saccharopolyspora gloriosae*

2.3.3. Screening assay

Actinobacteria with the potential to degrade polymers were identified by the clear zone test in agar plates with a medium containing the polymer as a carbon source (Augusta, Miiller, and Widdecke 1993; Pranamuda and Tokiwa 1999, adapted). The 36 actinobacteria strains (Table 2.2.) were inoculated onto agar plates, containing 0.1% (w/v) emulsified polymers, prepared according to the procedures previously described in section 2.3.1. corresponding to assays 4 and 10 for PVDF and to assays 11 and 12 for PLA and PS. Altogether, 216 screening assays were performed with the actinobacteria strains and the described emulsified media. Polymer degradation was investigated after 2 weeks of incubation at 25 ºC. The actinobacteria with the potential to degrade polymers were selected by their growth on the medium and by the formation of a clear halo zone around the colonies. The size of the clear halo zone was measured with a ruler (Yottakot and Leelavatcharamas 2019). The selected bacteria were considered as potential polymer-degrading strains and selected for the further biodegradation studies of this work.

2.4. Plastic film biodegradation with selected actinobacteria strains

2.4.1. Liquid media for actinobacteria culture

The strains that showed the highest potential to degrade plastics in the screening assay, assessed by their growth and appearance of a halo, were selected to be cultured in liquid media (Urbanek et al. 2017). Different media conditions were tested in order to optimize actinobacteria culture and to evaluate if bacterial growth was different in synthetic seawater and natural seawater, for cost-effectiveness purposes. All media were composed of 75% synthetic or natural seawater and 25% distilled water and autoclaved at 120 ºC for 20 minutes, before being transferred to an Erlenmeyer. Table 2.3. summarizes the different conditions tested.

Table 2.3.: Liquid media culture conditions used to optimize selected actinobacteria growth before being transferred to the biodegradation assays.

Assay	Natural Sea Water (mL)	Synthetic Sea Water (mL)	Yeast Extract (g/mL)	PVDF emulsion (g/mL polymer)	PS Emulsion (g/mL polymer)
1	18.75	-	-	-	-
2	-	18.75	-	-	-
3	-	18.75	0.0025	-	-

4	18.75	-	0.0025	-	-
5	18.75	-	-	1	-
6	18.75	-	0.0025	1	-
7	18.75	-	-	-	1
8	18.75	-	0.0025	-	1
9	-	18.75	-	1	-
10	-	18.75	0.0025	1	-
11	-	18.75	-	-	1
12	-	18.75	0.0025	-	1

- The component was not added to the medium.

After media optimization, the selected strains from the previous solid agar plates screening assay that were stored at -80 ºC in an ultra-freezer, were defrosted and inoculated directly from the vial to the prepared liquid media (25 mL), in a sterilized environment (Table 2.3.). The pre-inoculums grew for 2 weeks, at 25 ºC. The strains that had the best development under the used conditions, were used for the biodegradation assays, described in section 2.4.4.

2.4.2. Production of polymeric films on a heated press

For the production of polymeric films, PVDF was substituted by LDPE pellets, as PVDF was only available as a powder, making its moulding difficult in a heated press. On the other hand, LDPE was very hard to dissolve for the preparation of an emulsified medium.

PS, LDPE, and PLA pellets were transformed into films, using a heated hydraulic press. In order to obtain thin films with smooth surfaces, and also to produce more films in less time, aluminium plates with a clean surface were used between the heated press plates. In this way, we were able to remove the aluminium plates from the press after producing the film and allow it to cool down outside while another set of aluminium plates were used to produce another film. Before the production of each film, it was necessary to thoroughly clean the aluminium metal plates and heat the press to the desired temperature. The temperature was selected considering the melting temperature (T_m) of each polymer. For each film processing, it was required that the temperature of the press was higher than the T_m of each polymer. For LDPE, PS, and PLA the T_m is 135 ºC, 240 ºC, and 180 ºC, respectively (Sodergard and Stolt 2002; Scalenghe 2018). Thus, the temperature used on the heated press was around 150 ºC, 260 ºC, and 200 ºC, respectively (Table 2.4.). After weighing 1 g of LDPE and PS pellets, it was necessary to place the pellets on the bottom aluminium plate until the pellets melted, i.e., until becoming transparent. The other aluminium plate was then put on top, and the polymer was pressed, the working pressure was 10 - 20 bar. After waiting 5 minutes, the plates were removed from the press, carefully and with heated resistant gloves because of the high temperature. Finally, before separation, the aluminium plates cooled down.

After separation and when the polymer was fully cooled, the film was removed, and it was ready to use for the biodegradation assays. For PLA the process was similar, but 1.5 g of PLA pellets were used and instead of using aluminium metal plates, aluminium foils were used.

Table 2.4.: Polymer pellets, quantities, and temperatures used to produce polymeric films on a heat press.

Polymer Type	Polymer quantity (g)	Melting point (ºC)	Press temperature (ºC)	Time on heat press (min)
LDPE	1	135	150	10
PS	1	240	260	5
PLA	1.5	~150	200	4

2.4.3. Pre-treatment of polymeric films

Half of the LDPE, PS, and PLA films, prepared as described in section 2.4.2., were put in a holder in front of a UV light at an irradiance of 15 W, for two hours, at room temperature. The holders were put at a distance of 15 cm from the lamp during the UV treatment (J. Zhang et al. 2018).

After the UV treatment, the polymer films were cut into small rectangles (5 x 20 mm) and identified with small cuts that allowed their ordination. The same type of identification was used for regular films that did not undergo UV treatment.

All the films were disinfected with 2% SDS, for 30 minutes, rinsed with distilled water, and then allowed to dry at 50 ºC in a laboratory oven overnight. After drying, the films were placed in 70% (v/v) ethanol for 30 minutes and allowed to air dry, in a controlled environment, in a laminar flow hood (J. Zhang et al. 2018; Yottakot and Leelavatcharamas 2019). The films were aseptically added to a sterilized Erlenmeyer.

2.4.4. Biodegradation assays

The biodegradation assays for the 3 polymer types were performed using several conditions to evaluate the degradation capacity of the selected actinobacteria strains. For each strain, biodegradation was tested with regular films (LDPE, PS, and PLA films) and with films pre-treated with UV radiation (LDPE UV, PS UV, and PLA UV), as described in Table 2.5. Furthermore, another condition was tested by adding yeast extract (0.1% (m/v)) to the culture media in 3 biodegradation assays with polystyrene and polylactic acid, named PS – II, PS UV- II films, and PLA UV-II. The culture media of the biodegradation assays were composed of 75% SSW and 25% distilled water, named SSW medium.

Studies on the biodegradation of LDPE, LDPE UV, PS, and PS UV were carried out in triplicate using 250 mL Erlenmeyer flasks containing 100 mL of SSW medium, and 10 sterilized rectangles of polymeric films. Biodegradation assays of PS-II and PS UV-II followed the conditions mentioned for LDPE, LDPE UV, PS, and PS UV, with the addition of yeast extract to the SSW medium.

The degradation studies of PLA and PLA UV were carried out in triplicate using 150 mL Erlenmeyer flasks containing 75 mL of SSW media, and 3 sterilized polymeric films. Biodegradation of PLA UV-II was carried out following the conditions for PLA, PLA UV, with the addition of yeast extract to the SSW medium.

All the biodegradation assays were inoculated with one of the selected strains selected for this study at 10% (v/v) into the medium (Table 2.5.). The strains were grown in different conditions, as described in section 2.4.1, for 2 weeks. The strains used for LDPE and PLA biodegradation assays were grown in SSW media with 0.1% (m/v) of yeast extract. The strains used for PS biodegradation were grown in PS emulsified culture media with yeast extract (0.1% (m/v)).

The control experiments were carried out using sterilized films and SSW medium that were not inoculated with actinobacteria. The biodegradation assays were carried out in Erlenmeyer flasks incubated at 30 ºC and 100 rpm.

The monitoring of LDPE UV and PS UV polymeric films inoculated with *Streptomyces gougerotti* and *Micromonospora terminaliae* was performed every 30 days until 210 days of incubation. The same monitoring was followed for LDPE, PS, and PS-II films until 180 days of incubation. LDPE UV and PS UV films inoculated with *Micromonospora matsumotoense* and PS UV-II films inoculated with *Streptomyces gougerotti* were also monitored every 30 days until 150 days of incubation. PLA, PLA UV, and PLA UV-II films were monitored after 30 and 60 days of incubation (Table 2.5.) (Jarerat and Tokiwa 2001, 2003). All the experiments were carried out in a laminar flow hood for a sterilized environment.

Table 2.5.: Biodegradation assay conditions, polymers, and strains used.

Strain	Polymer type used	Assay conditions
Streptomyces gougerotti	LDPE	SSW medium, for 180 days, 100 rpm, at 30 ºC
	LDPE pre-treated with UV	SSW medium, for 210 days, 100 rpm, at 30 ºC
	PS	SSW medium, for 180 days, 100 rpm, at 30 ºC
	PS-II	SSW medium with yeast extract, for 180 days, 100 rpm, at 30 ºC
	PS pre-treated with UV	SSW medium, for 210 days, 100 rpm, at 30 ºC

	PS pre-treated with UV-II	SSW medium with yeast extract, for 90 days, 100 rpm, at 30 ºC
Micromonospora matsumotoense	LDPE	SSW medium, for 180 days, 100 rpm, at 30 ºC
	LDPE pre-treated with UV	SSW medium, for 90 days, 100 rpm, at 30 ºC
	PS	SSW medium, for 180 days, 100 rpm, at 30 ºC
	PS pre-treated with UV	SSW medium, for 90 days, 100 rpm, at 30 ºC
Micromonospora terminaliae	LDPE pre-treated with UV	SSW medium, for 210 days, 100 rpm, at 30 ºC
	PS pre-treated with UV	SSW medium, for 210 days, 100 rpm, at 30 ºC
Nocardiopsis prasina	PLA	SSW medium, for 60 days, 100 rpm, at 30 ºC
	PLA pre-treated with UV	SSW medium, for 60 days, 100 rpm, at 30 ºC
	PLA pre-treated with UV-II	SSW medium with yeast extract, for 60 days, 100 rpm, at 30 ºC

2.4.5. Monitoring of plastic film biodegradation

For biodegradation monitoring, the plastic films were periodically removed with sterile forceps from the biodegradation assays. The formed bacterial biofilm was washed off with 2% (w/v) SDS and shaking for 5h, then washed with distilled water. The films were placed on filter paper and dried overnight in a laboratory oven at 50 ºC (Sivan, Szanto, and Pavlov 2006; J. Zhang et al. 2018).

The degradation of LDPE, PS, and PLA by the selected plastic degrading actinobacteria was analysed by the plastic film weight loss, structure changes by FTIR-ATR, mechanical assays to detect changes in mechanical properties, and detection of PHA vesicles in the actinobacteria cells. Regular removal and monitoring of the plastic films were made during the first 12 weeks (90 days). After that, and due to unforeseen pandemic COVID-19 events, the films were only removed and analysed in the 24th week (after 150 days).

2.4.5.1. Plastic film weight loss

The polymeric films were weighted on an analytical balance accurate to 0.0001 g, after been dried in the oven. The films were weighted, both before incubation and after being removed from the biodegradation assay and cleaned. The weight loss of the plastic films was calculated according to the equation [1] (Vey et al. 2007; Yottakot and Leelavatcharamas 2019).

$$\%\, Weight\ loss\ = \frac{m_{ini}\ -\ m_{dry}}{m_{ini}} \times\ 100\% \qquad [1]$$

Where m_{ini} is the initial weight of the polymeric film before the degradation assay and m_{dry} is the dry weight of the polymeric film after the biodegradation assay. The weight loss was expressed as a percentage (%).

2.4.5.2. Fourier Transform Infrared spectra of plastic films

FTIR spectra of the LDPE, PS, and PLA film samples were recorded in the 400-4000 cm^{-1} range using a Perkin Elmer Spectrum Two Fourier Transform Infrared Spectrophotometer with attenuated total reflectance (FTIR-ATR). This method was used to analyse the chemical variations in the structure of the plastic films used in the biodegradation assay, in comparison with the original polymers and the corresponding controls (Janczak et al. 2018).

2.4.5.3. Plastic Film Tensile Test

To test the differences in the mechanical properties of the films, before and after the biodegradation assays, tensile tests were conducted on the films (5 x 20 mm), at room temperature, using an uniaxial tensile testing machine from Rheometric Scientific (Minimat Firmware 3.1) equipped with a load cell of 100 N. Film thicknesses (e) was measured with a digital micrometre with 1 µm resolution. Mechanical assays were performed by fixing the film ends in the claws of the tensile test machine, the film was then pulled at a constant speed of 0.5 mm/min in opposite directions while the load was continuously registered. The load (N) and stroke (mm) values were obtained for each test and were treated using Excel. The load (N) and stroke (mm) were converted using the following equations to Stress (σ) (equation [2]) and Strain (ε) (equation [3]), respectively, for normalization of the tests.

$$\sigma = \frac{\boldsymbol{F}}{\boldsymbol{A_0}} \quad \text{[Pa]} \qquad [2]$$

$$\varepsilon = \frac{\boldsymbol{\Delta l}}{\boldsymbol{l_0}} \quad \text{[adimentional]} \qquad [3]$$

Where F is the load in *Newtons*, A_0 is the initial value of the area (m^2) of the cross-section of each sample, Δl is the stroke in *millimetres* and l_0 is the initial length (*mm*) between claws *i.e.* the initial length of the sample. Based on these parameters, the Young Modulus (MPa), the Yield Strength (MPa), and the Ultimate Tensile Strength (UTS) (MPa) were determined.

A typical graphical representation of the stress variation with the extension, presented in Figure 2.1, allows obtaining the characteristic curve for a polymeric material.

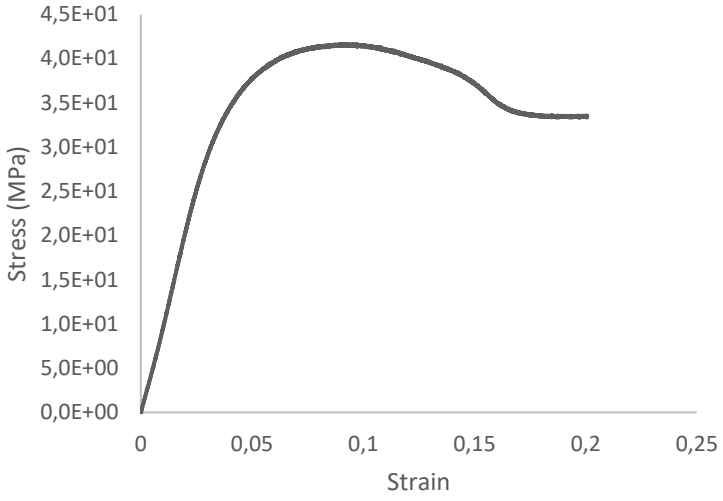

Figure 2.2.: Characteristic curve of stress vs extension of a polymeric material. In this case, the LDPE characteristic curve.

2.4.5.4. PHA Inclusions in the actinobacteria cells

To evaluate the production of PHA by the selected actinobacteria strains, a method developed by Ostle and Holt and adapted from Serafim et al. was applied to observe PHA inclusions. A drop of Nile blue stain was added to ~1 mL of bacterial sample, which was then placed in a thermostatic bath at 55 ºC for 15 min. After this incubation period, the sample was examined under an Olympus BX51 epifluorescence microscope (Japan) at 1000X. With this method, intracellular PHA granules were observed as bright orange fluorescence (Ostle and Holt 1982; Serafim et al. 2002).

2.4.5.5. Statistical analysis

The program used for statistical analysis was GraphPad Prim 5. The statistical significance was calculated was used to test the effect of UV radiation pre-treatment and the addiction of yeast extract to the culture media, using two-tailed paired t-test with 95% confidence interval (J. Zhang et al. 2018; Aly et al. 2015).

3

Results and Discussion

3.1. Polymer emulsified medium

From the 12 assays performed to prepare polymer emulsified media to use in the actinobacteria biodegradation screening, 7 were successful (Table 3.1.). However, the biodegradation assays were performed using 4 of these polymer emulsions, specifically the ones prepared with the conditions of the assays 4, 10, 11, and 12.

Table 3.1.: Successful polymer emulsified media achieved with different assay conditions.

Assay number	Polymer(s)	Solvent	Surfactant (g/L)	Yeast Extract (g/L)
3	PVDF	dichloromethane	0.1	-
4	PVDF		0.01	-
5	PVDF	acetone	0.1	-
6	PVDF		0.01	-
10	PVDF	dichloromethane		0.005
11	PS; PLA			-
12	PS; PLA			0.005

- yeast extract was not used in these assays

PVDF, PS, and PLA emulsified media, with and without yeast extract were used for the clear zone test with all the 36 strains presented in Table 3.1.

The 12 assays performed to prepare the polymers emulsions are discussed below.

The premise used for assay 1 was not correct. It was thought that by almost reaching the melting point of the polymers during autoclavation, they would dissolve in the media. However, after the

autoclavation, the polymer particles did not dissolve properly. The main reason for this was the melting temperature: PET can be mould at 163 ºC, but only melts at 200 ºC, while LDPE can be mould at 63 ºC, but only melts at 135 ºC (Scalenghe 2018), however the autoclave in the laboratory only reaches a temperature of 120 ºC.

For assay 2, LDPE and HDPE did not dissolve in dichloromethane. Considering the polymers that dissolve in this solvent and were autoclaved, PVDF and PET particles were not efficiently distributed in the media, PCV particles sank, and PS pellets turned into a film. In this assay, surfactant was not used. The purpose of this component is to decrease the surface tension between a microplastic particle and the liquid media, which helps to stabilize the emulsion since the surfactant alters the interfacial properties by absorbing to the boundary between two immiscible phases (Yamashita, Miyahara, and Sakamoto 2017), which explains why the emulsion media were not successful.

In assay 3 the surfactant SLS was added to the emulsions and it was possible to transfer PET and PVDF emulsified media into Petri dishes. The PVDF medium looked homogeneous on the Petri dish. However, the PET medium was not totally homogenous, since it was possible to observe microplastic particles and the absence of their regular distribution. An emulsion is a dispersed mixture of small droplets of different fluids that simply do not mix if added together. So, to achieve the finest emulsion it is necessary to add a component that can facilitate this process and to mix the solution intensively. This mixing is the industrial conventional process for making emulsions, which consists of mechanical shear to break up droplets to promote the mixture. However, this intensive mixture is not totally efficient, since only 0.1% of the mixing energy goes on creating smaller droplets, and the rest of the energy simply heats the mixture (Johnston 2017). This can be a possible explanation of why emulsions of most polymers were not well achieved in assay 3.

PVDF emulsified media was well accomplished on assay 4, PVDF powder was well dissolved and homogeneous. This one was made several times (Figure 3.1.), for later performing the biodegradation screening. Successful emulsified media were not achieved with the other polymers using the conditions of this assay.

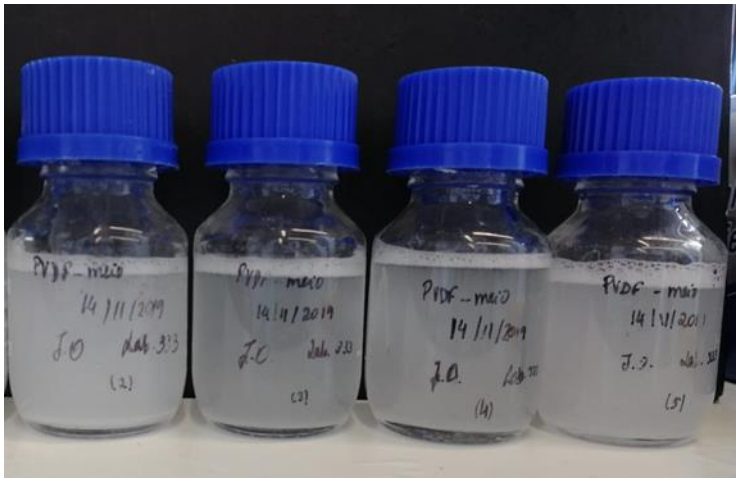

Figure 3.1.: Several PVDF-emulsified media prepared as described in assay 4.

With assay 5, PVDF, PVC, and PET were dissolved in acetone, and the emulsified media appeared homogeneous. However, after the autoclave, only the emulsion media containing PVDF was successful, looked homogeneous and with the particles consistently distributed. The PET particles did not dissolve completely, and the PVC particles sank.

The results for assay 6 were very similar to those obtained for assay 4, but by observing the final polymer emulsified media, it was determined that dichloromethane was the best solvent for the used polymers. Assay 7 was not efficient for any polymer.

In this way, PVDF polymer was successfully emulsified in assays 4, 5, and 6. However, comparing the PVDF emulsions, assay 4 was more effective than the others.

The main goal for the emulsified media preparation for the screening assays was to use low boiling solvents, which could be easily evaporated before autoclave and be used for polymers' dissolution. The boiling point of dichloromethane is about 39.8 ºC (available at https://pubchem.ncbi.nlm.nih.gov/compound/6344, accessed at 06/11/2020) and the boiling point of acetone is 56 ºC (available at https://www.sigmaaldrich.com/chemistry/solvents/acetone-center.html, accessed at 06/11/2020). Despite all the attempts, PE pellets (LDPE and HDPE), PET, PVC, and ABS pellets did not dissolved in these solvents. The LDPE and HDPE are highly resistant to chemicals and several solvents, and PET has high resistance to alcohols and solvents. Thus, unless superacids were used these polymers did not dissolve (Crawford and Quinn 2017; Scalenghe 2018). On the other hand, ABS and PVC dissolved in dichloromethane and acetone. However, after autoclave, the emulsified media with these polymers failed to homogenize. Several factors can affect the chemical resistance of the polymers, such as the temperature, the solvent quantity, polymers additives, among others. In this way, when the solvent was evaporated and the temperature was rising by the autoclave, the polymer particles reorganized

themselves by grouping. Thus, the reactions between the solvent, polymer, and the media were not chemical favourable, and unable achieving an emulsion with these polymers.

In assays 8 and 9 a "coat" was created on the top of the solidified NSW media. This coat was extremely fragile, it fell apart as soon as the plates were scratched. Therefore, these conditions were not used in further assays.

From all the prepared media, it was possible to determine that PVDF was the most effective and usable polymer for emulsions. PVDF emulsified media was prepared in a 50 mL flask, using the conditions determined and optimized in assay 4. When the PVDF media was successfully carried out, one more component was added to the media, as described in assay 10. The yeast extract was successfully added to the screening media. This allowed obtaining two different types of media with PVDF.

Regarding assay 11, emulsified media performed with PLA and PS polymers was well accomplished. This method proved to be the most suitable to emulsify polymers into a medium. PLA and PS emulsified media could be plated into Petri dishes and used for the actinobacteria biodegradation screening. After optimizing the conditions of this assay, yeast extract was also added as a new component, assay 12, which was also well accomplished.

3.2. Screening of actinobacteria with plastic degradation potential – Clear zone test

Plastic degradation screening assays performed with the selected actinobacteria strains, described in Table 3.2., for PVDF, PS and PLA emulsified media with and without yeast extract allowed to identify which strains had the potential to degrade these plastics. From the 36 available strains, and the 216 performed screening assays, 14 presented a positive result, *i.e.* grew in the emulsified media (Table 3.2.). The actinobacteria that grew and formed a halo around their colonies were chosen for the liquid media biodegradation assays.

Table 3.2.: Actinobacteria strains that grew using plastic (PVDF, PS, PLA) as carbon source.

Emulsified media	Actinobacteria strains that grew	PTE code	Clear halo (mm)	Time (days)
PVDF	*Micromonospora matsumotoense*	PTE-025	-	17
PVDF and yeast extract	*Micromonospora matsumotoense*	PTE-025	1	14
	Micromonospora terminaliae	PTE-056	-	14
	Micromonospora chalcea	PTE-024	-	14
PS	*Streptomyces gougerotti*	PTE-001	4	17
	Streptomyces xiamenensis	PTE-073	-	17
	Streptomyces intermedius	PTE-032	-	17
PS and yeast extract	*Streptomyces gougerotti*	PTE-001	-	14
	Micromonospora matsuense	PTE-025	-	14

	Streptomyces xiamenensis	PTE-073	-	14
	Streptomyces intermedius	PTE-032	-	14
PLA	*Nocardiopsis prasina*	PTE-048	1	7
PLA and yeast extract	*Nocardiopsis prasina*	PTE-048	-	7
	Micromonospora maritima	PTE-048	-	14

- absence of clear halo zone.

The incubation time for these actinobacteria strains was mainly for two weeks. One strain *Micromonospora matsumotoense* (code name PTE-025) grew on PVDF emulsified media. On PVDF emulsified media supplemented with yeast extract three strains were able to grow: *Micromonospora chalcea* (code name PTE-024), *Micromonospora terminaliae* (code name PTE-056), and PTE-025. PTE-025 formed a halo of 1 mm.

In PS emulsified media the only strain that grew extensively and formed a clear halo was *Streptomyces gougerotti* (code name PTE-001). The halo around the colonies measured 4 mm. *Streptomyces xiamenensis* (code name PTE-073) and *Streptomyces intermedius* (code name PTE-032), were observed in the media containing PS, but their growth was not as significant as of PTE-001. PS emulsified media containing yeast extract promoted the growth of PTE-001, PTE-073, PTE-032, and additionally to emulsified media without yeast extract, the growth of PTE-025.

Regarding PLA emulsified media, the strain *Nocardiopsis prasina* (code name PTE-048) grew extensively in the media after only 7 days, forming a clear halo of 1 mm in half the time of the previous strains. PLA emulsified media containing yeast extract promoted the growth of PTE-048 and *Micromonospora maritima* (code name PTE-068).

Figure 3.2. shows the photographs, taken under the microscope, of the actinobacteria strains that revealed the highest growth.

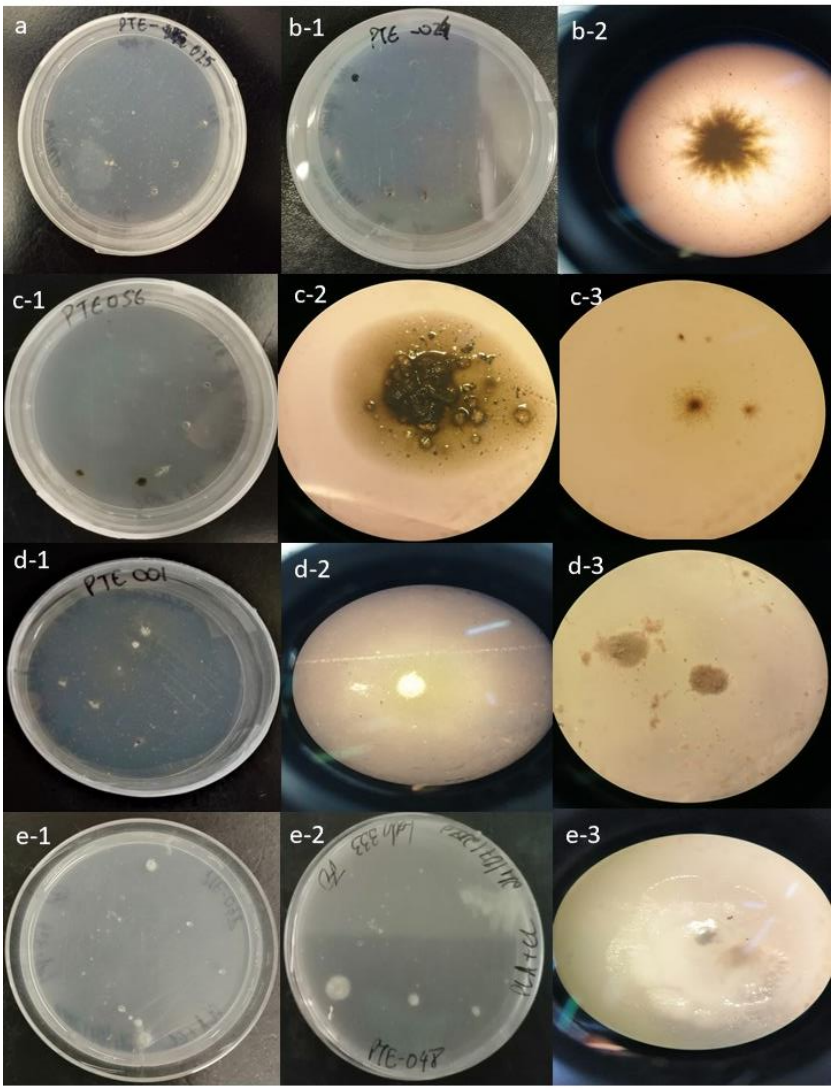

Figure 3.2.: Actinobacteria strains that grew in the PVDF, PS, and PLA emulsified media: a) *Micromonospora matsumotoense* grew in PVDF emulsified medium on a Petri plate; b-1) *Micromonospora chalcea* grew in PVDF emulsified medium with yeast extract on a Petri plate; b-2) *Micromonospora chalcea* grew in PVDF emulsified medium with yeast extract observed at the microscope; c-1) *Micromonospora terminaliae* grew in PVDF emulsified with yeast extract on a Petri plate; c-2) *Micromonospora terminaliae* in PVDF emulsified medium with yeast extract observed at the microscope; c-3) *Micromonospora terminaliae* in PVDF emulsified medium with yeast extract observed at the microscope; d-1) *Streptomyces gougerotti* in PS emulsified medium on a Petri plate; d-2) *Streptomyces gougerotti* in PS emulsified medium observed at the microscope; d-3) *Streptomyces xiamenensis* in PS emulsified medium observed at the microscope; e-1) *Nocardiopsis prasina* in PLA emulsified medium on a Petri plate; e-2) *Nocardiopsis prasina* in PLA emulsified medium on a Petri plate; e-3) *Nocardiopsis prasina* in PLA emulsified medium observed at the microscope.

Actinobacteria strains took ~ 2 weeks to grow, these are slow-growing microorganisms, it is regular to experience such periods of time for their incubation. The addition of yeast extract promoted the growth

of several strains in these emulsified plastic media, noticeable by the growth of some strains that only grew in the media where this component was added. The strains selected for the plastic film biodegradation assays were PTE-001, PTE-025, PTE-056, and PTE-048.

Although *S. gougerotti* was the only strain that showed a significant halo formation, the strains that grew in an emulsified media without yeast extract grew in a media with the plastic polymers as the sole carbon source and no other nutrients source. This indicates the plastic biodegradation potential of the actinobacteria strains. However, PTE-001 PTE-025 and PTE- 048 were more efficient to biodegrade PS, PVDF, and PLA, respectively, since these showed an apparent clear halo zone (Table 3.2.).

PTE-001 and PTE-025 were the strains chosen to perform the biodegradation assay of LDPE and PS films since these were the ones that demonstrated the greatest potential to degrade plastics. PTE-056 strain was used in the biodegradation assays of LDPE and PS films treated with UV radiation. The strain *Nocardiopsis prasina* (PTE-048) was chosen to perform the biodegradation assays of PLA films.

Moreover, it was possible to observe, by the clear zone test, that *Streptomyces* and *Micromonospora* were the most dominant genera, regarding (micro)plastic biodegradation in this work. Several species belonging to *Streptomyces* genera that are different from the ones used in this study, *Streptomyces* KU8, *S. badius, S. setonii,* and *S. viridosporus* (mentioned in Table 1.2.) have been described in the literature as capable of biodegrading different polymer types, especially PE (Pometto III, Lee, and Johnson 1992; Usha, Muthusamy, and Palaniswamy 2011; Abraham et al. 2017). *Micromonospora* genera have never been reported in the literature for plastic biodegradation. Moreover, there are no studies reporting PLA degrading *Nocardiopsis* genera (Ghosh, Pal, and Ray 2013; J. Oliveira et al. 2020).

3.3. Culture of selected Actinobacteria strains in liquid media

The screening assays were first performed with PVDF and PS polymers since PLA was not available, due to order delays. Thus, pre-inoculum optimization was only performed for the PVDF and PS polymers to evaluate bacterial development.

PTE-001, PTE-025, and PTE-056 were cultured for 14 days. The goal of this process was to evaluate the differences between the use of synthetic seawater (SSW) and natural seawater (NSW) and the addition of yeast extract in the media. This section served as a qualitative method, to verify by the naked eye which strains appeared to have their regular aspect when cultured.

The strains cultured on NSW developed faster and in higher amount than the ones cultured in SSW. Usually, natural seawater contains other nutrients and minerals that the synthetic one does not have, which promotes growth. However, NSW can have contaminants that can act as a secondary carbon, which we wanted to avoid.

Despite this, for the consequent biodegradation assays, it was decided to opt for actinobacteria cultures in synthetic seawater, for not introducing unknown variables. As an example, Figure 3.3. shows the growth of PTE-001 in liquid media composed of PS emulsion in NSW (Figure 3.3a), in SSW (Figure 3.3b), in NSW with yeast extract (Figure 3.3c), and SSW with yeast extract (Figure 3.3d).

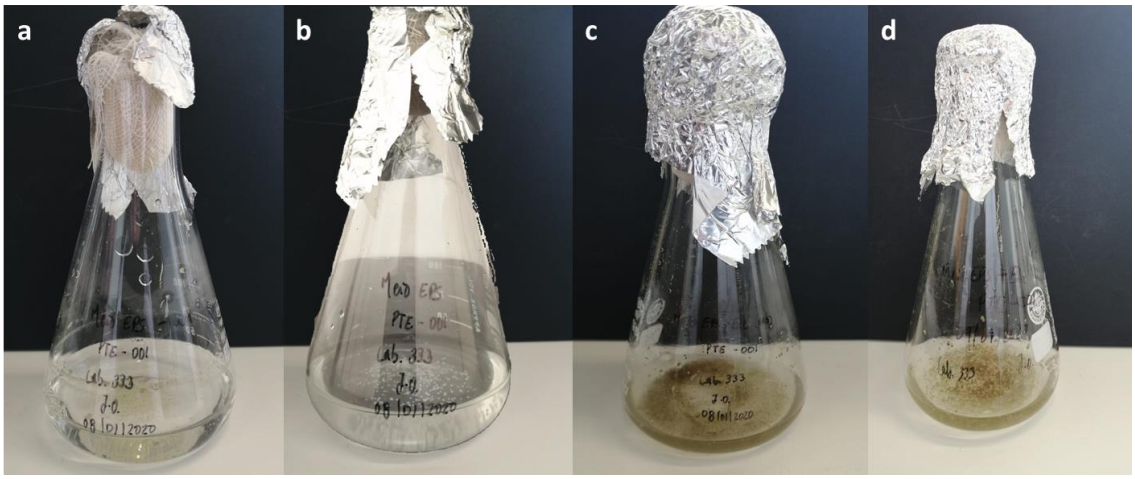

Figure 3.3.: Actinobacteria strain *Streptomyces gougerotti* pre-inoculums, in different conditions: a) *Streptomyces gougerotti* cultured in NSW liquid media containing emulsified PS; b) *Streptomyces gougerotti* cultured in SSW liquid media containing emulsified PS; c) *S. gougerotti* in NSW liquid media with yeast extract and emulsified PS; d) *S. gougerotti* in SSW liquid media with yeast extract and emulsified PS.

The addition of yeast extract in the culture media revealed notable differences in the bacterial growth (Figure 3.3.), which increased *S. gougerotti* development. Natural seawater also influenced bacterial growth, since a higher amount of bacterial biomass was observed in these cases (stronger culture color and higher number of bacterial colonies). Synthetic seawater was chosen to perform all the biodegradation assays, as mentioned before, since the major differences were due to the addition of yeast extract to the culture media and to guarantee that there were no other carbon sources available besides the polymers.

To obtain faster results, PTE-048 and PTE-56 were cultured in SSW liquid media containing yeast extract since this media ingredient promotes a faster development.

3.4. Plastic film biodegradation assays with selected actinobacteria strains

Biodegradation was analysed and quantified by weight loss, FTIR-ATR spectra, mechanical assays, and PHA vesicles production.

3.4.1. Plastic films used in the biodegradation assays

Biodegradation assays were performed with LDPE, PS, and PLA polymeric films. For the production of polymeric films, PVDF was substituted by LDPE pellets, as PVDF was only available as a powder, which difficult its moulding in a heated press, and LDPE was very hard to dissolve for the preparation of an emulsified medium. The PVDF backbone contains alternate C–F and C–H bonds, while polyethylene only contains C–H bonds. Generally, polymers lose small molecular fragments of side chains and polymeric backbone by bond scission. In this case, during degradation PVDF typically loses fluorine and hydrogen fluoride, forming new bonds, mainly C–C double bonds. During PE degradation, PE oligomers with C–C double bonds are formed, as well as carbonyl groups (Hossain, Muench, and Ensinger 2014; Ghatge et al. 2020). Besides, PE is one of the most used plastics in packaging and single-use objects, so its accumulation is massive in the environment (Orhan and Büyükgüngör 2000). Considering this, using LDPE to perform biodegradation assays seemed more advantageous.

3.4.1. Weight loss of plastic films

The analyse the weight reduction of the films used in the assays provided the first insights about the potential of the inoculated strains to degrade LDPE, PS, and PLA plastic films. Measurements of weight loss are frequently used when the polymer is exposed to selected microorganisms in culture media, being the polymer's sole carbon source (J. Zhang et al. 2018). This method is standardized for in-field and simulation biodegradability assays (B. T. Ho, Roberts, and Lucas 2018).

The film weight loss for each biodegradation assay with the inoculated strains PTE-001, PTE-025, PTE-056, and PTE-048 followed for 60 to 210 days, is presented in Table 3.3. Weight loss was calculated and compared with the corresponding controls considering the same period of time. The control films are currently being repeated as these were not performed in triplicate.

Table 3.3.: Plastic film weight loss at the end of the biodegradation assay, for each polymer type, selected strain and culture medium (*i.e.* culture media with and without yeast extract).

Polymer	Actinobacteria Strain	Time (days)	Weight loss (mg)	Weight loss (%)
LDPE	Control	180	0	0
	Streptomyces gougerotti		1.5 ± 0.26	0.5
	Micromonospora matsumotoense		0.6 ± 0.05	0.31
LDPE UV	Control	90 / 210	0.1 / 0.4	0.32 / 0.41
	Streptomyces gougerotti	210	1.83 ± 0.42	0.56
	Micromonospora matsumotoense	90	0.4 ± 0.02	0.22
	Micromonospora terminaliae	210	0	0
PS	Control	180	0.28	0.13

	Streptomyces gougerotti		0.89 ± 0.06	0.17
	Micromonospora matsumotoense		0.13 ± 0.05	0.04
PS (yeast extract in culture media)	Control	180	0.28	0.1
	Streptomyces gougerotti		1.03 ± 0.05	0.67
PS UV	Control	90 / 210	0 / 0.2	0 / 0.11
	Streptomyces gougerotti	210	1.92 ± 0.69	0.30
	Micromonospora matsumotoense	90	0.96 ± 0.27	0.17
	Micromonospora terminaliae	210	0.33 ± 0.17	0.17
PS UV (yeast extract in culture media)	Control	90	0	0
	Streptomyces gougerotti		0.4 ± 0.09	0.19
PLA	Control	60	0.1	0.39
	Nocardiopsis prasina		1 ± 0.26	0.84
PLA UV	Control		0.3	0.64
	Nocardiopsis prasina		0.7 ± 0.05	0.97
PLA UV (yeast extract in culture media)	Control		0.3	0.64
	Nocardiopsis prasina		1.1 ± 0.17	1.27

3.4.1.2. LDPE films and LDPE UV films

One of the major sources of environmental pollution is LDPE since huge amounts of this polymer are getting accumulated in the environment. As it takes hundreds of years for its efficient degradation, it is necessary to find solutions to this problem, such as the discovery of microorganisms to degrade this polymer (Usha, Muthusamy, and Palaniswamy 2011).

It is important to highlight that studies about LDPE degradation by actinobacteria are still rare. In biodegradation assays of LDPE films inoculated with *Streptomyces gougerotti*, PTE-001, and *Micromonospora matsumotoense*, PTE-025, the percentage of weight loss was 0.5% and 0.31%, respectively (Table 3.3.). The corresponding control did not lose any weight during the assay. Thus, the percentage of weight loss of the inoculated films with the mentioned strains is identical to the control films weight loss.

For the biodegradation assays of LDPE UV films an additional strain was used, *Micromonospora terminaliae*, PTE-056, along with the strains used in the previous assay (PTE-001 and PTE-025). The biodegradation assays with LDPE films pre-treated with UV radiation were carried out for different periods of time. Thereby, the assays with *S. gougerotti* and *M. terminaliae* were followed for 210 days and the assay with *M. matsumotoense* was followed for 90 days. These strains revealed a weight loss of 0.56%, 0% and, 0.22%, respectively. Comparing the weight loss percentages promoted by the different strains with the control, for the period of time, significant differences were not observed. The control films lost 0.32% of weight for 90 days and 0.41% weight in 210 days (Table 3.3.).

When analysing the results for LDPE UV biodegradation it was possible to verify that *M. terminaliae* and *M. matsumotoense* do not have the ability to degrade this polymer, since no statistically significant loss of weight was observed. The extension of polymeric biodegradation by *S. gougerotti* was not significant, as well, when comparing to the weight loss of control films in the same conditions.

The results for LDPE UV were not in agreement with the previous results for LDPE films. It was expected that films treated with UV radiation deteriorated to a greater extension than non-treated films. Generally, the use of UV radiation is applied to obtain lower molecular weight molecules, since UV radiation works as an initiator of PE oxidation, producing carbonyl groups that promote the microorganisms' attack (Sowmya et al. 2014). The polymeric carbon bonds are broken during this process which leads to a photo-induced degradation (Kumar Tiwari, Gautam, and Maurya 2018). In this way, a combination of photo-oxidation by UV radiation exposure and the use of microorganisms to perform plastic biodegradation should create a synergistic effect to improve plastic degradation (Montazer et al. 2018), which was not verified. A possible explanation for these results could be that the used pre-treatment was not efficient, *i.e.*, the time of exposure or the intensity of UV radiation may not have been enough to promote photolysis.

Considering studies already carried out with PE incubated with other actinobacteria strains, the weight loss percentage for this study should be higher. When this polymer was in contact with *Rhodococcus ruber* a 7.5% weight loss has been achieved, in 60 days. In this study, the initial degradation evidence was observed 16 days after the beginning of the assay, as mentioned in Table 1.2. (Sivan, Szanto, and Pavlov 2006). Using this same polymer treated with nitric acid and inoculated with *Microbacterium paraoxydans* PE reduction reached up to 61%. This pre-treatment enhances the biodegradation rate of this polymer, as it facilitates the microbial attack by reducing the native bonds in the polymer by breaking it into smaller chains (Heera Rajandas et al. 2012). This type of pre-treatment can be a useful alternative to UV radiation. *Streptomyces albogriseolus* LBX-2, *Micrococcus* sp., and bacterial consortia containing *Arthrobacter viscosus*, *M. lylae*, *M. luteus,* and *Bacillus* sp. enabled significant percentages of weight loss of 17.27%, 6.61%, and 17%, respectively (Kathiresan 2003; Nowak et al. 2011; Shao et al. 2019; Soleimani et al. 2020).

There are several biodegradation studies regarding *Streptomyces* genera since some strains of these genera demonstrate the potential to degrade this polymer. For example, *Streptomyces* KU8 could degrade about 46.16% of polyethylene in 6 months (Table 1.2.) (Usha, Muthusamy, and Palaniswamy 2011). On the other hand, *Streptomyces* sp. strain AJ1 showed a weight loss of 5.2% in 3 months (Abraham et al. 2017). Other studies demonstrated that *Streptomyces* genera could degrade low-density polyethylene, such as *S. coelicoflavus* NBRC 15399, *S. iakyrus*, *S. werraensis*, among others (Abraham et al. 2017). Furthermore, actinobacteria have been reported as capable of degrading PE when UV radiation is used

as a pre-treatment, which is the case of *Rhodococcus ruber* (C208) that degraded 8% of the initial dry weight of this polymer after 30 days (Table 1.2.) (Orr, Hadar, and Sivan 2004).

Nevertheless, there are no reports in the literature for LDPE and general plastics degradation ability of *Micromonospora* sp., meaning that this genus needs to be more study regarding (micro)plastic degradation.

The differences in results of this study and the literature, as well as the lower percentages of weight loss, differences in the culture media, pre-treatments performed to the plastic films, actinobacteria sources, different enzymes can be present in different strains of the same genera, acclimatization of the strain to the polymer, among others.

3.4.1.3. PS films and PS UV films

Polystyrene is considered a non-biodegradable polymer and it is not easily recycled (Chaudhary and Vijayakumar 2020). PS is one of the most used plastics, as it is mainly used for manufacturing single-use products such as disposable cups and packaging foams. Thus, a large portion of PS items ends up in landfills and in the ocean, remaining for hundreds of years due to their persistent nature to degradation (Syranidou et al. 2017).

Regarding biodegradation assays of PS films, *Streptomyces gougerotti* and *Micromonospora matsumotoense* were monitored for 210 days. During this time PS films lose about 0.17% and 0.04% weight, respectively, and the control films lost about 0.13% of weight (Table 3.3.). This polymer is extremely resistant to degradation, noticeable by the obtained results, which were not significant.

The biodegradation assays for PS UV films were followed for different periods, considering the strain used in each assay. *Streptomyces gougerotti* and *Micromonospora terminaliae* were monitored for 210 days, and *Micromonospora matsumotoense* was monitored for 90 days. These strains could reduce polymeric film's weight by 0.30%, 0.17%, and 0.17%, respectively (Table 3.3.).

To understand if the results were promising it is necessary to compare them with the control for the period of time. PS UV control films did not lose any weight for 90 days (0%), but at the end of the assay (210 days), control films lose about 0.11% weight (Table 3.3.). *S. gougerotti* showed a higher potential to degrade PS, which had already been verified by the clear zone test in section 3.2, than *M. matsumotoense*, even for different periods of time. That is, *Streptomyces gougerotti* performed better, losing ~2% more weight than the control films. The PS UV films incubated with *Micromonospora matsumotoense* and *Micromonospora terminaliae* lost 0.17% and 0.5% more weight than the control, respectively.

There was a slight difference between the weight loss of PS treated with UV radiation and non-treated. In this case, the UV radiation treatment had some effects on biodegradation, resulting in higher weight losses, which is in agreement with prior investigations (Sikandar Shah, Ahmad, and Ishaq 2017).

The reported studies performed with actinobacteria for PS biodegradation were not as successful as LDPE biodegradation assays. For example, *Rhodococcus ruber* only reduced the dry weight of this polymer by 0.8% in 60 days, while in the same period, *Rhodococcus ruber* could degrade LDPE dry weight by 7.1%, as mentioned in Table 1.2. (Sivan, Szanto, and Pavlov 2006; Mor and Sivan 2008).

However, there were some studies with higher percentages of PS weight reduction using other filo, as was the case of the research conducted by Evdokia Syranidou et al. (2017), which used a bacteria consortium to perform PS degradation, attaining 4.7% weight loss of PS after 6 months of incubation (Syranidou et al. 2017). *Bacillus cereus* is another strain with the potential to degrade PS since in 38 days it could reduce polymer weight by 7.4% (Auta, Emenike, and Fauziah 2017). *Pseudomonas sp*., isolated from super worms gut, also showed a capacity to degrade PS through several assays such as FTIR, NMR, and RT-qPCR (to measure the gene expression level of enzymes) (Kim et al. 2019). Therefore, the use of microorganisms with the potential to degrade PS may be a viable solution for reduce the accumulation of this polymer in the environment, being that further studies are needed regarding the actinobacteria potential to degrade PS.

3.4.1.4. PS films and PS UV films in media containing yeast extract

In the clear zone test, *Streptomyces gougerotti* exhibited the higher potential to degrade PS. Thus, in order to obtain a significant weight loss, more assays were performed with this strain and using yeast extract in the culture media.

As previously mentioned, it was described in the literature that the addition of this component improves biodegradation. Yeast extract is an excellent organic source of nitrogen, containing a high content of proteins and amino acids, vitamins, minerals, and growth factors that influences the growth of *Streptomyces* and their products, increasing mycelium biosynthesis (Suutari et al. 2002; Yottakot and Leelavatcharamas 2019).

PS films incubated with *S. gougerotti* in SSW and yeast extract lose about 0.67% of weight in 180 days. While PS UV films lost about 0.19% of weight in 90 days. In the control films, there was no variation in weight during the first 90 days, but after 180 days of incubation, the control films showed a percentage of weight loss of around 0.1% (Table 3.3.).

Comparing the results above with the ones in section 3.4.1.3 it was possible to verify that UV radiation and the addition of yeast extract increases the percentages of weight loss. Therefore, when comparing

the incubated PS films with and without yeast extract in culture media, weight losses increased from 0.17% to 0.67%, respectively, being that the films incubated with yeast extract were analysed for 180 days, and without the addiction were analysed after 210 days. On the other hand, when PS UV films were incubated without yeast extract for 90 days, *S. gougerotti* only degraded 0.09%, but when the strain was incubated with yeast extract for the same period, it degraded 0.19% of the polymer weight, that is twice the percentage of weight loss.

Studies using *Streptomyces* genera in biodegradation assays with yeast extract in the culture media demonstrated evidence to support a reduction in the integrity of polymers and sustenance to the benefit addition of this component to the culture media (Lee et al. 1991). Other research that also used yeast extract in culture media reported that it was a very important co-factor for different bacteria, improving the degradation activities, making PS degrading ability more efficient (Tang, Kuo, and Liu 2017).

It is important to find the optimal yeast extract concentration and use it in the culture media. Previously investigations with other actinobacteria strains set an ideal concentration of yeast extract between 1.30% and 3.26% in culture media (Yottakot and Leelavatcharamas 2019). This can be a possible explanation of why the PS biodegradation did not increase as much as expected after the addition of this component, as the used concentration of this component in our assays was 0.1% (m/v). Nevertheless, assays with yeast extract in higher concentration (3%) are currently being performed.

3.4.1.5. PLA films, PLA UV films, and PLA UV films in media containing yeast extract

Polylactic acid is a biocompatible thermoplastic and biodegradable aliphatic polyester, which can be produced from renewable resources (Jarerat and Tokiwa 2003). However, PLA is not readily biodegradable, unless under industrial composting conditions (Qi, Ren, and Wang 2017).

Three different PLA biodegradation assays were performed with *Nocardiopsis prasina* and monitored for 60 days. *N. prasina* could reduce the weight of PLA, PLA UV, and PLA UV-II films by 0.84%, 0.97%, and 1.27%, respectively. The corresponding controls lost about 0.39%, 0.64%, and 0.64% weight, respectively.

These results are in accordance with the results obtained in the previous sections, *i.e.* films without any treatment have lower percentages of weight loss when compared with films treated with UV radiation and where supplements (yeast extract) were added to the culture media, for the same assay time. However, PLA films lose a more significant weight percentage than PLA UV and PLA UV-II films, when comparing with the weight losses with the correspondent control. That is, PLA films lose 1.15% more weight than the control films, while PLA UV and PLA UV-II films lost 0.52% and 0.98% more weight than the control, respectively. This can be related to the photo-oxidative pre-treatment since the treated

films could be more prone to hydrolysis reactions by becoming more hydrophilic as a result of UV exposure (Jeon and Kim 2013).

When comparing the results obtained for PLA with LDPE and PS biodegradation assays in this work, for 60 days, it was noticeable that PLA is more biodegradable than LDPE and PS.

According to Jarerat et al. (2002), PLA-degrading actinobacteria belong to the *Pseudonocardiaceae* family and related genera, which includes *Amycolatopsis*, *Saccharothrix*, *Lentzea*, *Kibdelosporangium*, and *Streptoalloteichus* (Jarerat, Pranamuda, and Tokiwa 2002). The members of the actinobacteria phylum proved to be efficient in PLA degradation (J. Oliveira et al. 2020). For example, *Amycolatopsis orientalis* showed evident degrading activity, as the extracellular PLA-degrading enzyme produced by this strain degraded completely this polymer within 8h (Table 1.2.) (Jarerat, Tokiwa, and Tanaka 2006). Also, *Saccharothrix waywayandensis* strain could degrade 95% of PLA in 7 days, as mentioned in Table 1.2.

Researches proved that optimal conditions (polymeric film pre-treatment, yeast extract addition, etc.) for the biodegradation assays largely enhance percentages of weight loss (Yottakot and Leelavatcharamas 2019), which can be seen in the results obtained in this work.

Nocardiopsis genus exhibit a vast metabolic versatility and are biotechnologically important, as many species of this genus can mediate the breakdown of complex polymers that occur naturally (Bennur et al. 2015), which makes *N. prasina* a good target for conventional polymer biodegradation investigation.

3.4.2. FTIR-ATR Spectra of plastic films

FTIR is a very useful technique to analyse the effect of chemical variations in the plastic film's surface, due to biodegradation. This technique measures the absorbed light of the sample at each wavelength in the infrared region. The changes in the chemical properties of the films under study are used to compare to the corresponding control. This technique was commonly used in earlier biodegradation assays for different types of polymers described in the literature (Sangale, Shahnawaz, and Ade 2012; T. B. Ho 2018).

In this work, FTIR was used to analyse the chemical changes in the films after the incubation with the actinobacteria. Table 3.4. presents the most relevant differences in the spectra of each film, at the end of the biodegradation assay, inoculated with the selected strains.

Table 3.4.: Differences in absorption bands of FTIR spectra after the biodegradation assays and the meaning of those differences.

Strain	Polymer used in the biodegradation assay	Bands differences	Meaning
Streptomyces gougerotti	LDPE	Bands appeared at 1739, 1367, and 1217 cm^{-1}	Formation of carbonyl groups; primary and secondary alcohols
	LDPE UV	Bands disappeared at 1700 cm^{-1}, and peak appearance at 1096 cm^{-1}	Carbonyl groups oxidation; oxidized functional groups
	PS	Bands appeared at 3600, and 1735 cm^{-1}	Alcohols generation; formation of carbonyl groups
	PS (with yeast extract in culture media)	New bands at 1740 cm^{-1}, and 1216 and 1367 cm^{-1}, a small band at 3600 cm^{-1}	Formation of carbonyl groups; formation of carboxylic acids, esters, and alcohols; generation of alcohol
	PS UV	New bands at 1727 cm^{-1}; intensity increased at 1050 cm^{-1}; intensity decreased at 1026, 748, and 694 cm^{-1}	Indication of oxidation; an indication of a monosubstituted benzene ring in PS
	PS UV (with yeast extract in culture media)	The intensity of the bands increased, especially at ~1050cm^{-1}; the band at 1700 cm^{-1} disappeared	Indication of carboxylic acids, esters, and alcohols; evidence of PS film oxidation
Micromonospora matsumotoense	LDPE	The band appeared more evident at 1739 cm^{-1}	Formation of carbonyl groups
	LDPE UV	Intensity decreased at 1739 cm^{-1}, and a band appeared at 1070 cm^{-1}	Carbonyl groups hydrolysed; oxidized fraction
	PS	The intensity of the bands decreased over time and the appearance of a peak at 1735 cm^{-1}	Indication of slightly transformation of PS chemical structure; and formation of carbonyl groups
	PS UV	The intensity of the bands increased	Biodegradation of polymer chains
Micromonospora terminaliae	LDPE UV	Increase intensity at 1739 cm^{-1}	Carbonyl groups formation
	PS UV	No differences	No differences
Nocardiopsis prasina	PLA	The intensity of the bands' decrease	Biodegradation of polymer chains
	PLA UV	Increased bands intensity; new band appeared	Chain oxidation

		at 1382, 1127, and 704 cm^{-1}	
	PLA UV (with yeast extract in culture media)	Increase in bands intensity; new peaks at 1382, 1266, 1128, and 700 cm^{-1}	Formation of vinyl unsaturated groups, and chain oxidation

3.4.2.1. LDPE films and LDPE UV films

The characteristic LDPE absorption bands were identified: 2915 cm^{-1} (CH_2 asymmetric stretching), 2847 cm^{-1} (CH_2 symmetric stretching), 1472 cm^{-1} and 1461 cm^{-1} (bending deforming), 730 cm^{-1} and 718 cm^{-1} (rocking deforming) (Coates 2006; Pretsch, Buhlmann, and Badertscher 2009; J. Zhang et al. 2018). These bands assigned the characteristics of absorption bands of LDPE (Figure 3.4a).

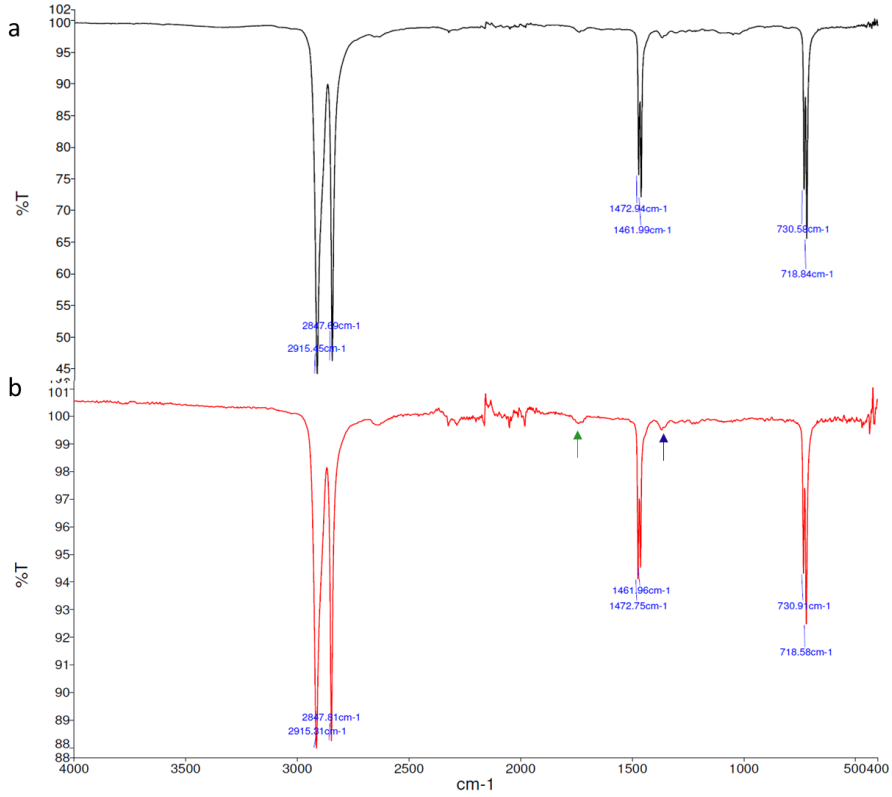

Figure 3.4.: FTIR spectra of control films: a) LDPE film; b) LDPE UV film – the green arrow represents a small band at 1700 cm^{-1} and the blue arrow represents a small band at 1300 cm^{-1}.

After the incubation of LDPE films with *Streptomyces gougerotti* new absorption bands appeared at 1739 cm^{-1} (C=O stretching), 1367 cm^{-1} and 1217 cm^{-1} (C–H) along with 1468 cm^{-1} band representing isopropyl groups, which indicates a decrease in the degree of polymerization (Pretsch, Buhlmann, and Badertscher 2009; J. Zhang et al. 2018) (Table 3.4.) (Figure 3.5.).

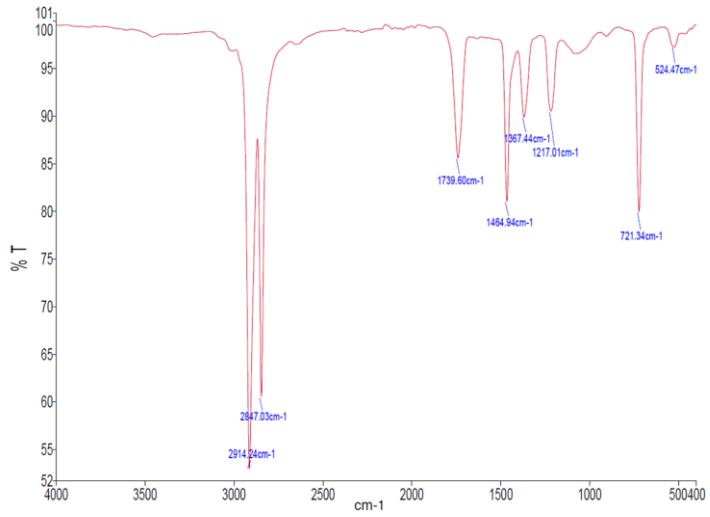

Figure 3.5.: FTIR spectrum of LDPE film after the incubation period with *Streptomyces gougerotti*.

Differences in the chemical structure of polyethylene were observed between the spectra of the control and after the biodegradation assay, indicating some degradation activity. The band at 1739 cm^{-1} is indicative of the formation of aldehydes, ketones, and carbonyl bands (–C=O–), intermediate products of biodegradation of this polymer. The appearance of this band is an indicator of the occurrence of biodegradation, already reported in the literature, demonstrating that the strains were capable of attacking or oxidizing polyethylene structures (Yang et al. 2014). The band appearing at 1217 cm^{-1} of the spectrum is correlated with primary and secondary alcohols (Muhonja et al. 2018). According to the literature, the bands at 1200-1300 cm^{-1} are indicative of various types of oxidation products formed during LDPE biodegradation, which represents the oxidized groups, such as moieties containing –OH groups that resulted from the biodegradation by the selected microorganisms (Hakkarainen and Albertsson 2004).

After LDPE films were incubated with *Micromonospora matsumotoense* new small absorption bands appeared. The most evident was at 1739 cm^{-1}, and the least intense were at 1367cm^{-1} and 1217 cm^{-1} (Table 3.4.). As describe above, these bands indicate the occurrence of biodegradation, even though the intensity is not as evident as in the case of the films incubated with *S. gougerotti* (Figure 3.6.).

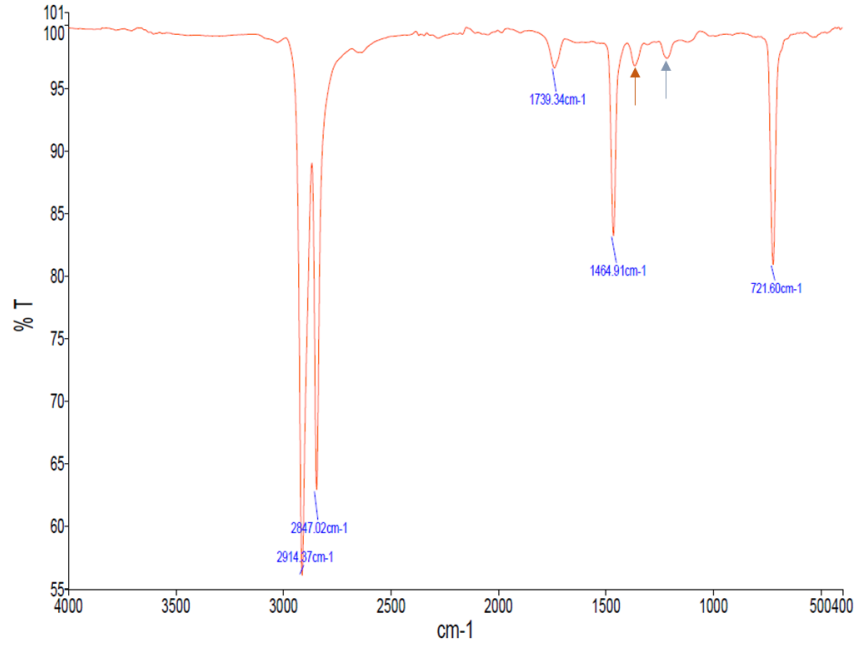

Figure 3.6.: FTIR spectrum of LDPE film after the biodegradation assay with *Micromonospora matsumotoense*. The orange arrow represents a small band at 1300 cm^{-1} and the grey arrow represents a small band at 1200 cm^{-1}.

Undoubtedly, PE biodegradation is a slow process, since the initiation of polyethylene chain breakage is a long and difficult step. Carbonyl groups are produced through the degradation stages, which enhances the biodegradation process. In this way, only the oxidised and short LDPE chains are biologically used by the microorganisms. Usually, after a long incubation time and advanced oxidation, methylene units are cutting-off by intracellular enzymes in β-oxidation and are completely degraded via the citric acid cycle resulting in the formation of CO_2 and H_2O (Hakkarainen and Albertsson 2004; Nowak et al. 2011; Esmaeili et al. 2013). Taking this into account, it was possible to determine that the strains used in this study were able to oxidise LDPE films in a small extension.

Considering the results obtained for the LDPE films, it was possible to corroborate the hypothesis that *Streptomyces gougerotti* and *Micromonospora matsumotoense* are capable of degrading polyethylene on a small scale.

To determine the extent of microbial degradation in LDPE UV films, it was important first to determine the effect of UV radiation exposure on the structural integrity of these films, by using FTIR spectroscopy. Regarding LDPE UV films no substantial differences were registered in the spectrum when compared with LDPE films, as the absorption bands at 2915 cm^{-1}, 2847 cm^{-1}, 1472 cm^{-1}, 1461 cm^{-1}, 730 cm^{-1}, and 718 cm^{-1} were observed. However, in LDPE UV films low-intensity peaks appear around 1700 cm^{-1} that corresponded to carbonyl groups formed by exposure to UV radiation (abiotic degradation)

and around 1300 cm^{-1} representing C–H stretching that along with 1468 cm^{-1} band represent isopropyl groups, which indicates a decrease in the degree of polymerization (Figure 3.4b). Moreover, a group of bands appeared between 2400 cm^{-1} and 2000 cm^{-1}, these bands can be related to the formation of intermediate products during photodegradation, representing carbon dioxide (O=C=O), ketene (C=O=C), alkyne (C≡C), and allene (C=C=C) stretching (available at https://www.sigmaaldrich.com/technical-documents/articles/biology/ir-spectrum-table.html, accessed at 06/12/2020). However, these bands are not predicted in the literature regarding LDPE photo-oxidation by UV radiation (Gardette et al. 2013).

Theoretically, after the UV treatment to LDPE films, two new bands were expected to appear at ~1640 cm^{-1} corresponding to –C=C–, groups induced by UV radiation, and at ~1713 cm^{-1} corresponding to –C=O groups, a carbonyl group formed by photo-oxidation after exposure to UV radiation (Montazer et al. 2018). Therefore, LDPE films should have been exposed to UV radiation for a longer period of time to promote photo-oxidation to a higher extent, before being incubated with the different strains in the biodegradation assays.

LDPE UV films were inoculated with *Streptomyces gougerotti* for 210 days. During this period two bands of pronounced intensity disappeared after the biodegradation assay, 730 cm^{-1}, and 1472 cm^{-1}, but these bands also disappeared in the non-treated LDPE films, when comparing to the control. The low intensity at 1700 cm^{-1} also disappeared (Table 3.4.). According to the literature, the eliminated band at 1700 cm^{-1} is an indication that some of the double carbon bonds of the LDPE might have been hydrolysed by bacteria, due to oxidation of carbonyl groups by enzymatic hydrolysis. Albertsson et al. proposed that the carbonyl groups were formed during exposure to UV radiation, and PE chains are metabolized via β-oxidation by bacteria (Albertsson, Andersson, and Karlsson 1987). Moreover, a new absorption band was observable at 1096 cm^{-1}, indicating the presence of oxidized groups by microorganisms' action (Table 3.4.) (Figure 3.7.) (Yamada-Onodera, Mukumoto, and Katsuyaya 2001; Abrusci et al. 2011; Montazer et al. 2018). It was possible to observe these differences, but the extension of biodegradation was not very significant. However, if this strain was capable of degrading polyethylene on a small scale when the polymer did not undergo any pre-treatment, it should be capable of degrading it when the polymer is photo-oxidated before the biodegradation assay.

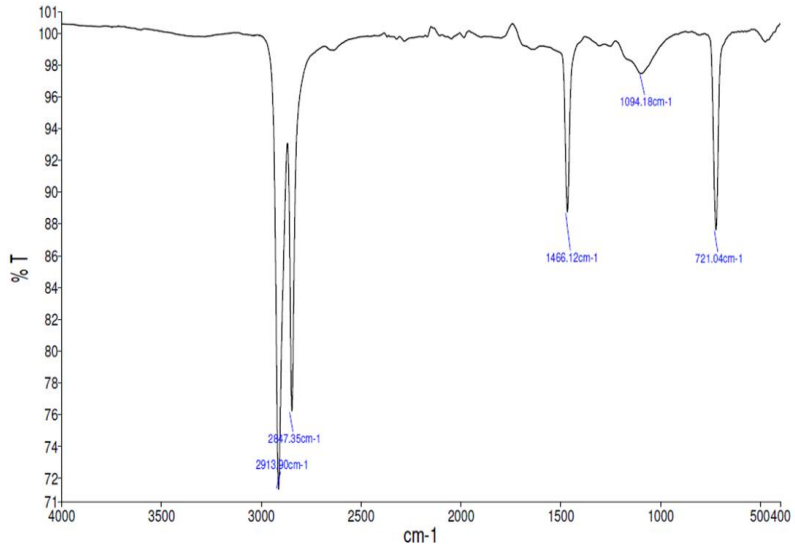

Figure 3.7.: FTIR spectrum of LDPE UV film after the biodegradation assay with *Streptomyces gougerotti.*

Micromonospora terminaliae was also inoculated for 210 days with LDPE UV films. After the biodegradation assay, the intensity of the band at ~1700 cm^{-1} increased in the spectrum. This means that carbonyl groups were not assimilated by this strain. Nevertheless, low-intensity absorption bands between 1000-1700 cm^{-1} (at ~1200 and ~1300 cm^{-1}) were observed, which is attributed to the oxidized groups by the action of the microorganisms (Table 3.4.) (Figure 3.8.).

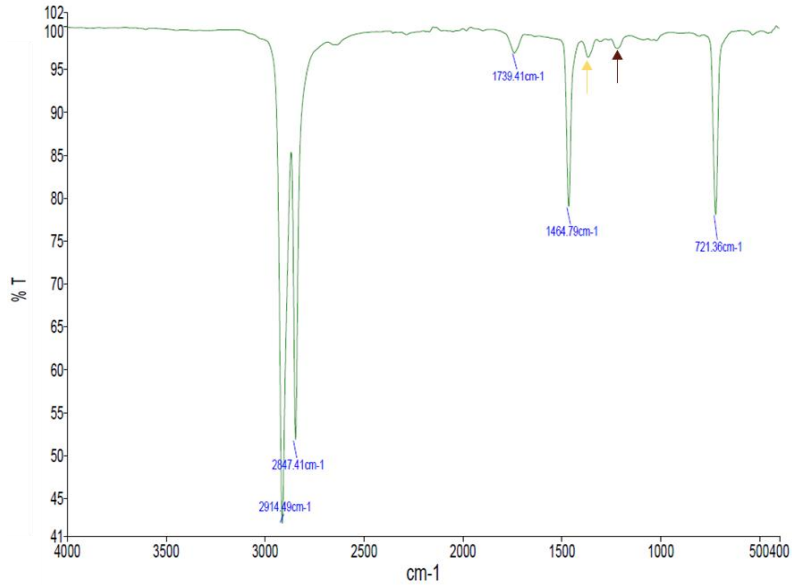

Figure 3.8.: FTIR spectrum of LDPE UV film after the biodegradation assay with *Micromonospora terminaliae.* The yellow arrow represents a low-intensity band at 1300 cm^{-1} and the brown arrow represents a low-intensity band at 1200 cm^{-1}.

Micromonospora matsumotoense was also inoculated for 120 days with LDPE UV films. During this period, the absorption band at 1700 cm^{-1} decreased, but the absorption bands at ~1070 cm^{-1}, in the range of 1000-1700 cm^{-1}, increased when compared to the corresponding control (Table 3.4.) (Figure 3.9.). The results are aligned with the biodegradation assumptions, this means that carbonyl groups were hydrolysed by this strain, and the absorption band at 1070 cm^{-1} was correlated with the oxidized groups, such as moieties containing –OH groups resulting from the action of the microorganisms.

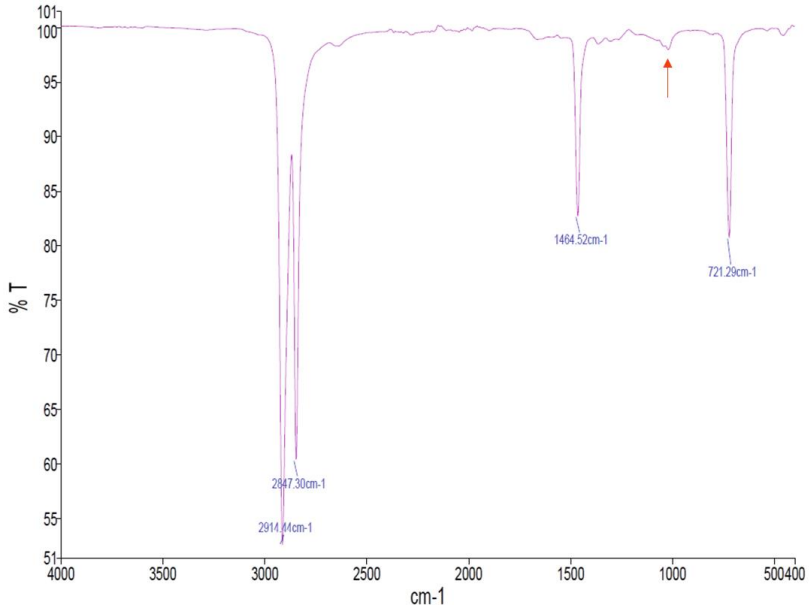

Figure 3.9.: FTIR spectrum of LDPE UV film after the biodegradation assay with *Micromonospora matsumotoense.* The red arrow represents a low-intensity band at 1070 cm^{-1}.

Considering the spectra results, *S. gougerotti* and *M. matsumotoense* revealed to have the potential to degrade both LDPE with and without UV treatment. On the other hand, *M. terminaliae* did not showed potential for polyethylene biodegradation, even when the polymer went through UV treatment. The results for LDPE films weight loss are in agreement with the results obtained in the FTIR-ATR spectra. Nevertheless, the weight loss results were not as significant as the results of FTIR spectra for LDPE UV films. In this case, polymers did not lose weight, but it was possible to determine small chemical changes on the surface of the films. Therefore, *S. gougerotti* and *M. matsumotoense* probably have the potential to also degrade LDPE after UV treatment.

However, there are experiments in which the microorganisms could only assimilate the products of chemically or physically pre-aged polyethylene, that is, products of pre-oxidized polyethylene and not

virgin polyethylene (Hasan et al. 2007; Sudhakar et al. 2008). This is the opposite of what happened in this study, where the actinobacteria strains assimilated better regular LDPE than LDPE pre-treated with UV radiation.

In other investigations with *Rhodococcus ruber*, an actinobacteria, the photo-oxidation (with UV radiation) treatment to polyethylene proved to be efficient and played an important role in the initiation of the biodegradation by the strain, because of the formation of carbonyl residues on the surface of the photo-oxidized polymer (Table 1.2.) (Orr, Hadar, and Sivan 2004).

3.4.2.2. PS films and PS UV films

Regarding polystyrene spectra, the observed bands were at 3059 cm^{-1} and 3025 cm^{-1} (aromatic C-H stretching vibration absorption), 2921 cm^{-1} and 2849 cm^{-1} (corresponding to the existence of methylene $–CH_2$ asymmetric and symmetric stretching), 1600 cm^{-1}, 1492 cm^{-1}, 1449 cm^{-1}, and 1027 cm^{-1} (correspond to deformational vibrations of both $–CH_2$ and aromatic C=C stretching vibration absorption, indicating the existence of benzene rings), 906 cm^{-1} (corresponding to vinyl C–H out-of-plane bend), 751 cm^{-1} (corresponding to C–H out-of-plane bending vibration of the aromatic ring and indicate that there is only one substituent in benzene ring) and 695 cm^{-1} (corresponding to ring-bending vibration) and 538 cm^{-1} (corresponding to alcohol OH out-of-plane bend) (Figure 3.10a) (Fang, Xuan, and Li 2010; Syranidou et al. 2017).

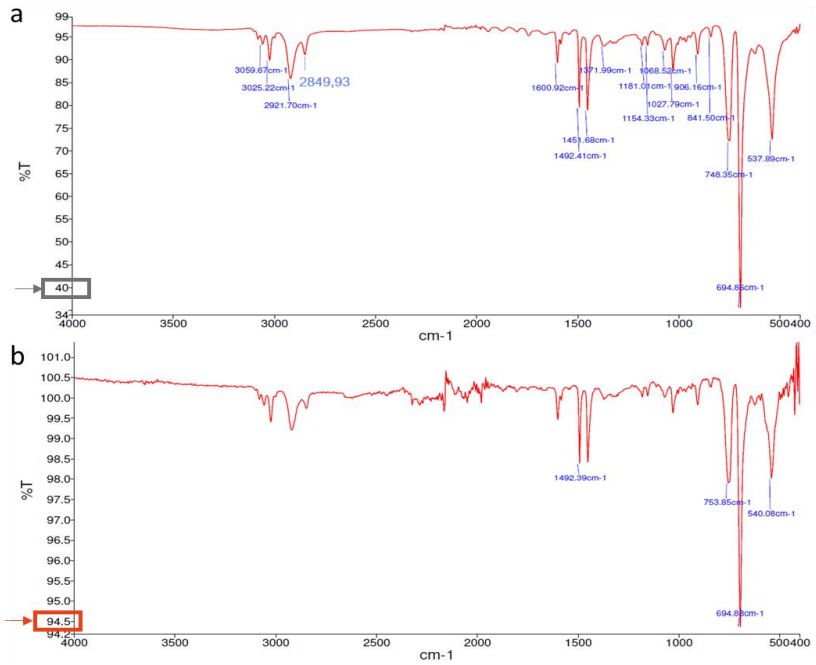

Figure 3.10.: FTIR spectra of control films: a) PS film; b) PS UV film. The grey arrow and rectangle represent the intensity band at 694 cm^{-1} in PS film. The orange arrow and rectangle represent the intensity band at 694 cm^{-1} in the PS UV film.

PS films were inoculated with *Streptomyces gougerotti* and after the biodegradation assay it was possible to notice the appearance of a new band at 1735 cm $^{-1}$, which indicates the formation of carbonyl (–C=O) during PS degradation. Interestingly, after completion of the biodegradation assay, another low-intensity band was detected at 3600 cm^{-1}, which represents O-H stretching (Table 3.4.) (Figure 3.11.). These suggest that alcohols (C–OH) can be generated as an intermediate stage on the way to the carbonyl, during PS degradation (Yang et al. 2014; Kim et al. 2019). Another noticeable information about the bands was observed, that was some of these bands shifted to lower intensities after the assay, which suggests that the bonds became weaker in the PS films (Pushpadass et al. 2010). Absorption bands between 1000-1200 cm^{-1} represent carboxylic acids, esters, and alcohols. Modifications on this region with the appearance of other oxidation bands at 1740 cm^{-1} and 3600 cm^{-1} supported the evidence of the oxidation process during biodegradation. Also, differences in the absorption bands at 756 cm^{-1} and 698 cm^{-1} indicated a monosubstituted benzene ring in the polymer (Atiq 2011). These changes in chemical structure evidenced polystyrene biodegradation by *S. gougerotti*.

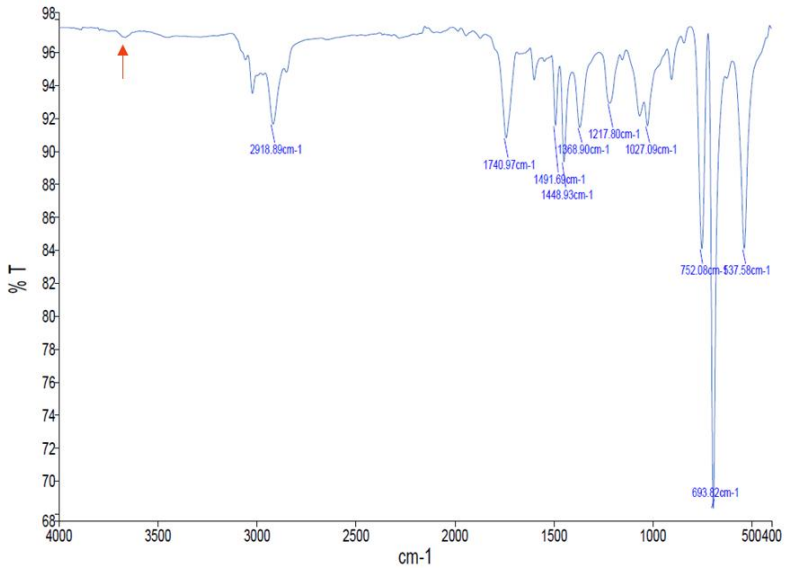

Figure 3.11.: FTIR spectrum of PS film after the biodegradation assay with *Streptomyces gougerotti.* The red arrow represents a low-intensity band at 3600 cm^{-1}.

Regarding the strain, *M. matsumotoense* inoculated with PS films it was observable that the intensity of the bands decreased over time. When the intensity of the bands decreases it can be an indication of degradation of the polymers by the secretion of enzymes from the microorganisms, which leads to polymeric disintegration. The changes in the intensity of the bands at 695 cm^{-1} and 755 cm^{-1} indicated that the chemical structure of PS was slightly transformed after the biodegradation assay, which was due to depolymerization (Shimpi et al. 2012; T. B. Ho 2018). Furthermore, a new band at ~1735 cm^{-1} increased

its intensity when compared to the corresponding control (Table 3.4.) (Figure 3.12). This absorption band indicated the formation of carbonyl groups (–C=O) during the degradation of the studied polymer.

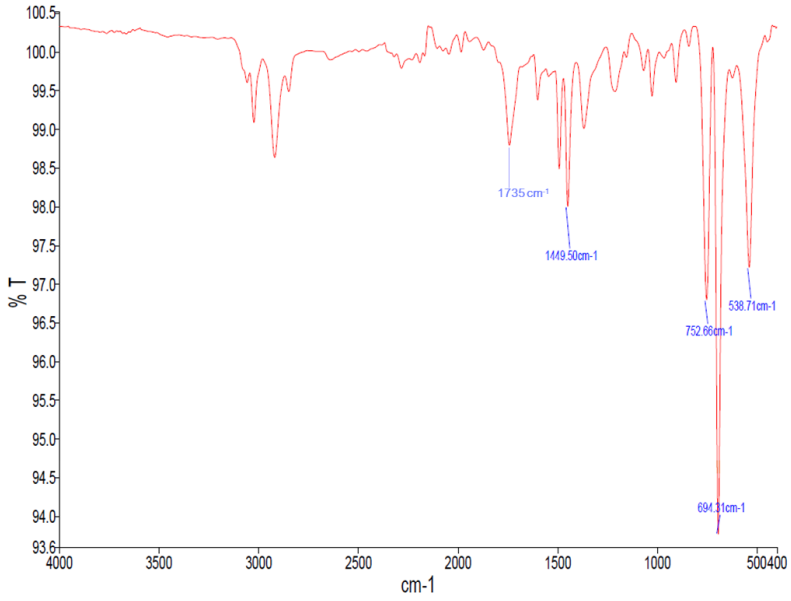

Figure 3.12.: FTIR spectrum of PS film after the biodegradation assay with *Micromonospora matsumotoense.*

Polystyrene is constituted by styrene monomers and styrene itself can be used as a carbon source by some microorganisms for growth. For example, *Rhodococcus ruber* has shown the potential to degrade PS by forming a biofilm on its surface (Mor and Sivan 2008). A novel biofilter with an agitator, developed by Jae Woong Hwang et al. (2008), was used for the treatment of gaseous styrene using *Brevibacillus* sp. The strain could remove 3 kg of styrene in one day. Yet, the biodegradation rate depends on the molecular weight and thickness of the plastic material (Hwang et al. 2008). Moreover, *Pseudomonas fluorescens* and *Curvularia* species also demonstrated the potential to degrade PS in other studies (Baggi et al. 1983; Motta et al. 2009). Thus, other actinobacteria species and other microorganisms are capable of degrading and using PS as a carbon source.

To determine the differences between PS and PS UV films, it was important to determine the effect of UV radiation exposure on the chemical structure of these films, by comparing their FTIR spectra. After the UV treatment, the absorption bands were practically the same, but the intensity increase. The most intense absorption band decreased, at 695 cm^{-1}, from values of transmittance around 92% to 40% (Figure 3.10b). This band corresponded to the ring bending vibration, evidencing a monosubstituted benzene ring in the polymer or PS depolymerization (Atiq 2011). Besides, a group of bands between 2400-2000 cm^{-1} appeared after UV exposure. As mentioned above, these bands are characteristic of the stretching of the following groups' carbon dioxide (O=C=O), ketene (C=O=C), alkyne (C≡C), and allene (C=C=C)

stretching (available at https://www.sigmaaldrich.com/technical-documents/articles/biology/ir-spectrum-table.html, accessed at 06/12/2020). These groups formation are not described in the literature for PS photo-oxidation by UV exposure (Yousif and Haddad 2013).

Polystyrene films treated with UV radiation were incubated, in independent assays, with *S. gougerotti*, *M. matsumotoense*, and *M. terminaliae*.

In the case of PS UV films incubated with *S. gougerotti*, a new band emerged at 1739 cm^{-1} after the biodegradation and an increase of intensity of the absorption band at ~1050 cm^{-1} was observed. At the same time, a decrease in the intensity of the band was evident at 1026 cm^{-1}, 748 cm^{-1}, and 694 cm^{-1} when compared to the control (Table 3.4.) (Figure 3.13.).

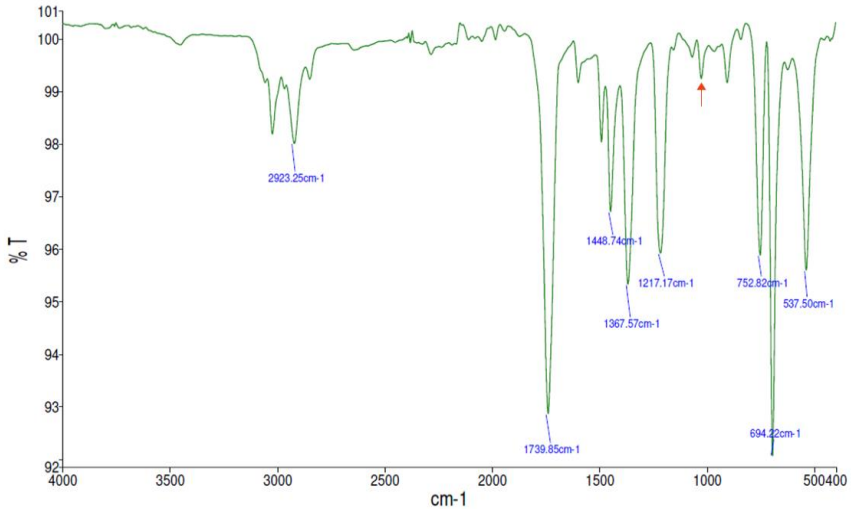

Figure 3.13.: FTIR spectrum of PS UV film after the biodegradation assay with *Streptomyces gougerotti.* The red arrow represents a low-intensity band at 1050 cm^{-1}.

The PS UV films incubated with *M. matsumotoense* and *M. terminaliae* did not presented significant differences to the corresponding control, *i.e.*, none of the absorption bands disappeared or new bands appeared. The main difference was noticed in the films incubated with *M. matsumotoense* in the intensity of the absorption bands that increased over time in the biodegradation assay.

The obtained results showed the potential of *S. gougerotti* and *M. matsumotoense* to alter PS surface and initiate the first steps of biodegradation. On the other hand, the strain *M. terminaliae* did not show any result in terms of polymers biodegradation.

Generally, the irradiation with UV light promotes the degradation of PS macromolecules, leading to the reduction of molecular weight and acceleration of the biodegradation rate (Sikandar Shah, Ahmad, and Ishaq 2017). The use of UV irradiation treatment for biodegradation was particularly notorious with the

use of *Rhizopus oryzae, Aspergillus terreus*, and *Phanerochaete chrysosporium* strains, which enhanced the effect on biodegradation by the isolates. In this case, samples showed increase in the intensities at 536 cm^{-1}, 748 cm^{-1}, 1026 cm^{-1}, 1361 cm^{-1}, 1450 cm^{-1} (C=C stretching vibration of aromatic compounds), 1735 cm^{-1}, 3313 cm^{-1} in only 60 days (Atiq 2011).

3.4.2.3. PS films and PS UV films in media containing yeast extract

As described above, yeast extract increases mycelium biosynthesis of *Streptomyces*, the genus of the strain that was used in these experiments (Yottakot and Leelavatcharamas 2019).

In the case of PS films inoculated with the strain and with yeast extract in the culture media, it was possible to visualize a sharp increasing of the absorption band at 1740 cm^{-1}, indicating the formation of carbonyl groups (–C=O). This band revealed a higher intensity than the PS film inoculated without yeast extract. A small band at ~3600 cm^{-1} was observable after the biodegradation assay, which corresponds to O–H stretching, suggesting the generation of alcohol as an intermediate stage on the carbonyl formation. Additionally, absorption bands at 1216 cm^{-1} and 1367 cm^{-1} appeared in the spectrum, which represents carboxylic acids, esters, and alcohols (Table 3.4.) (Figure 3.14). All this information supported the evidence of the oxidation process during PS biodegradation.

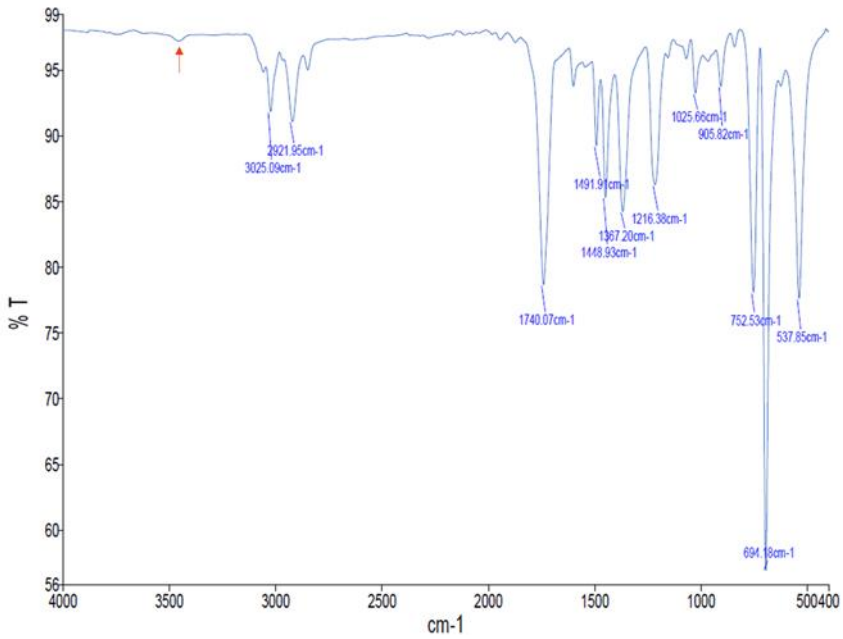

Figure 3.14.: FTIR spectrum of PS film after inoculation with *Streptomyces gougerotti* and with yeast extract in the culture media. The red arrow represents a low-intensity band at 3600 cm^{-1}.

Regarding PS UV films incubated with the same strain and yeast extract in culture media, the intensity of the bands increased in the FTIR spectra and it is observable an increase of intensity of the absorption

bands at 1360 cm^{-1} and ~1050 cm^{-1}, which represents carboxylic acids, esters, and alcohols (Figure 3.15). Also, the absorption band at 1700 cm^{-1} disappeared, which may be evidence of PS UV film oxidation.

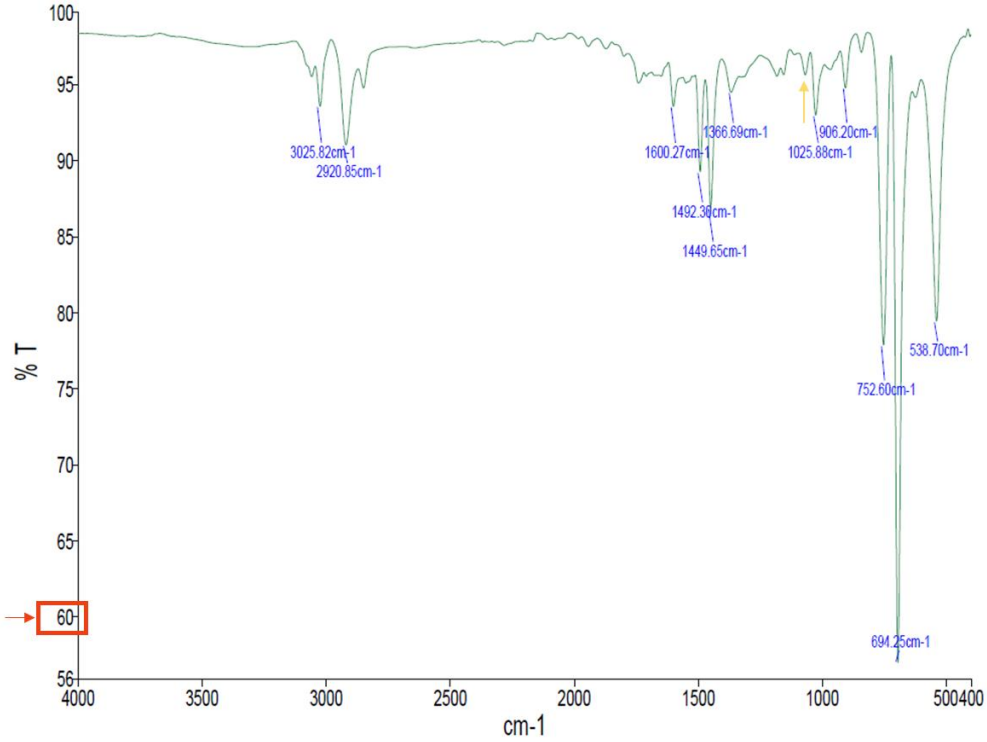

Figure 3.15.: FTIR spectrum of PS UV film after inoculation with *Streptomyces gougerotti* and with yeast extract in the culture media. The red arrow and rectangle evidence the intensity of the absorption bands and the yellow arrow represents the low-intensity band at ~1050 cm^{-1}.

The obtained results evidenced *S. gougerotti* potential to degrade PS in a small extension with the addition of yeast extract in the medium. Thus, when the strains were cultured close to the optimal conditions, plastic degradation was enhanced.

Both assays of PS and PS UV films with the addition of yeast extract in the culture media ran in different periods of time, being that the assay using PS UV films ran for a shorter time. The small biodegradation extension in PS UV films can be due to the assay time. Thus, the actinobacteria could have not enough time to promote biodegradation.

FTIR-ATR results revealed that PS biodegradation differs from microorganisms and microbial communities, highlighting the complex process beyond biodegradation. The variations in the intensities of the functional groups at the polymers' surface are due to their consumption or formation (Restrepo-flórez, Bassi, and Thompson 2014).

3.4.2.4. PLA films and PLA UV films

The characteristic absorption bands of PLA spectra were at 2996 cm^{-1} (C–H asymmetric stretching); 1749 cm^{-1} (stretching vibration of C=O group); 1453 cm^{-1} and 1365 cm^{-1} (asymmetric and symmetric vibrations of CH_3 group, respectively); 1180 cm^{-1}, 1125 cm^{-1}, 1082 cm^{-1}, and 1044 cm^{-1} (stretching vibration of O–C–C); 867 cm^{-1} (vibration of the O–CH_2–CH_3 group); and at 753 cm^{-1} and 699 cm^{-1} (deforming vibration of the αCH_3) (Janczak et al. 2018).

The main differences observed between PLA and PLA UV FTIR spectra were the intensity of the absorption bands, which decreased the intensity with the photo-oxidation treatment.

Biodegradation assays with PLA films were performed with *Nocardiopsis prasina*.

After the PLA films biodegradation assay, the intensity of the absorption bands decreased when compared to the corresponding control, being ~1750 cm^{-1}, ~1185 cm^{-1}, and 1090 cm^{-1} the three absorption bands that are more related to biodegradation (M. Oliveira et al. 2016). Besides this, no other differences were observed, which was not enough evidence to verify the polymer's biodegradation.

On the other hand, the absorption bands of PLA UV films increased, and new bands appear at 1382 cm^{-1}, 1127 cm^{-1}, and 704 cm^{-1} when compared to the corresponding control. These results indicate chain oxidation during biodegradation, which induced the cleavage of the long chains into shorter ones. Furthermore, the band at ~700 cm^{-1} corresponds to C–O double bonds (Table 3.4.) (Figure 3.16).

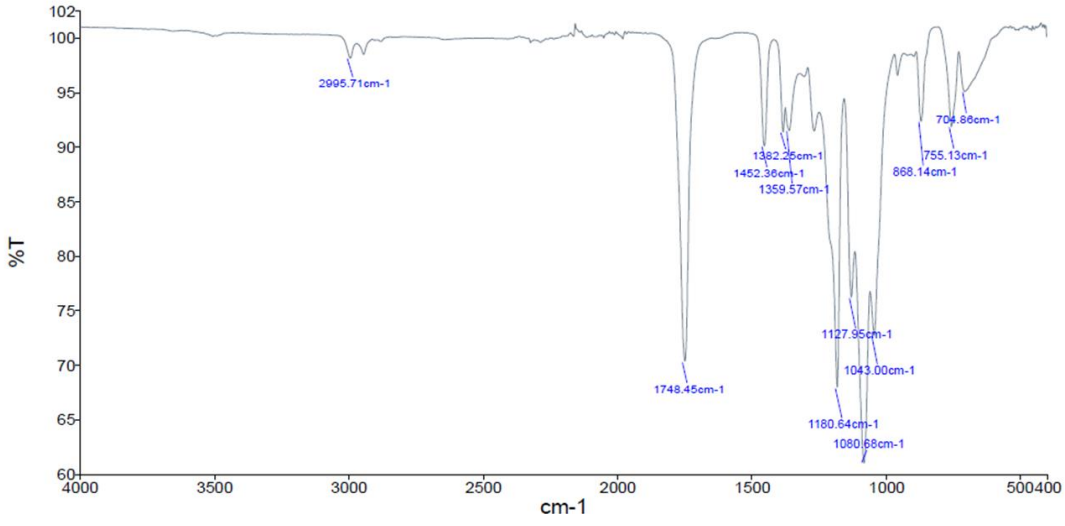

Figure 3.16.: FTIR spectrum of PLA UV film after inoculation with *Nocardiopsis prasina*.

Generally, all the absorption bands of PLA UV films inoculated with *N. prasina* and yeast extract in culture media increased intensity when compared to the correspondent control. This increase was higher

than that of PLA UV films without the yeast extract addition. Moreover, new bands appear at 1382 cm^{-1}, 1266 cm^{-1}, 1128 cm^{-1}, 956 cm^{-1}, and ~700 cm^{-1}. The bands at ~950 cm^{-1} are characteristic of vinyl unsaturated groups and the increase around 700 cm^{-1} corresponds to C–O double bond which is related to biodegradation occurrence (Table 3.4.) (Figure 3.17.). These changes in the chemical surface evidenced *Nocardiopsis prasina* capacity to degrade PLA.

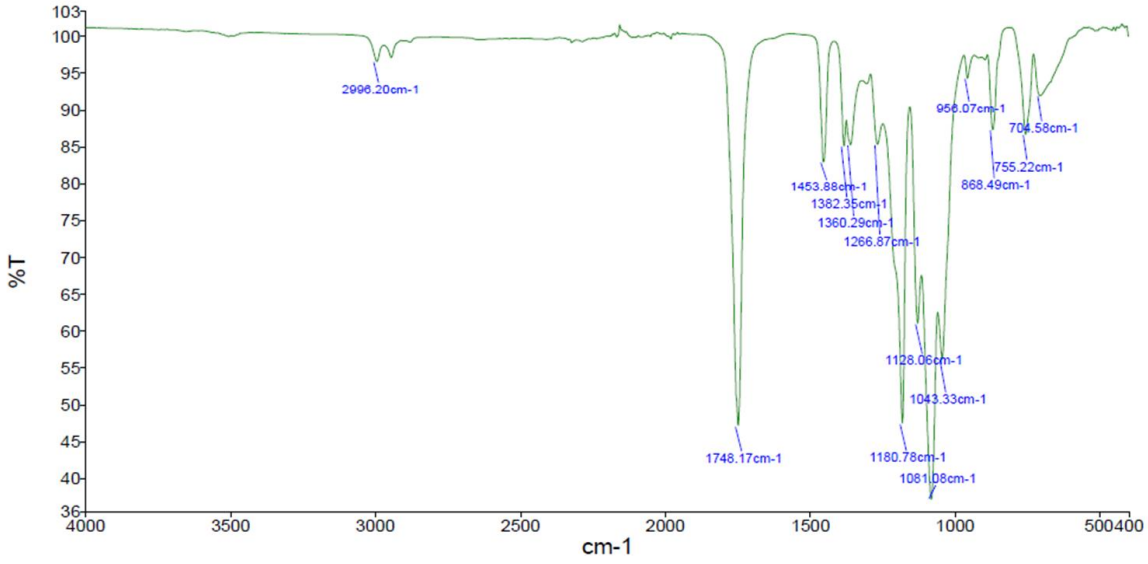

Figure 3.17.: FTIR spectrum of PLA UV film after inoculation with *Nocardiopsis prasina* with the addition of yeast extract to the culture media.

These results evidence the occurrence of biodegradation on PLA films treated with UV radiation, and that the addition of yeast extract to the culture media enhances this process. There are no reports in the literature regarding PLA degradation by *Nocardiopsis* genus. In this way, *N. prasina* can be considered a novel bacterial strain with the potential to degrade this polymer.

An effective PLA biodegradation is normally reported in literature when using optimal/composting conditions for this polymer degradation. Several studies reported microorganisms' ability to degrade PLA, specially actinobacteria phylum (Janczak et al. 2018; J. Oliveira et al. 2020). In Table 1.2. several actinobacteria strains capable of degrading PLA and use it as a sole carbon source are mentioned, overall this polymer biodegradation ranges from 95% to 100% (Jarerat, Tokiwa, and Tanaka 2003; Jarerat and Tokiwa 2003; Jarerat, Tokiwa, and Tanaka 2006). Biodegradation was confirmed in this and other studies by FTIR spectra analysis, observed by the changes in the chemical structure of PLA noticed by new vibrations, which indicate polymer biodegradation (Janczak et al. 2018).

3.4.3. Mechanical properties of the films

Tensile strength assays were used to determine the mechanical properties of the various polymers studied in this thesis. Tensile strength tests provide a measurement of the ability of a material to resist forces that tend to deform it, determining to what extent the material can be deformed before breaking (Dilara and Briassoulis 1998). The degree of degradation of the polymers under study was determined by measuring the tensile strength of each film, analysing different parameters, such as Young Modulus (ε), Yield Strength, and Ultimate Tensile Strength (UTS). The loss of tensile strength can be a characteristic indication of biodegradation (Khan et al. 2017), as the cleavage of the polymer's backbone during degradation weakens the plastic, so the tensile strength is proportional to extent of degradation (Cosgrove et al. 2007). In this way, polymer deterioration can be observed based on the mechanical properties that are sensitive to changes in molar mass (Janczak et al. 2018). Although the results obtained with this method are related to biodegradability, they needed to be used with other methods to conclude that biodegradation effectively occurred (Rosario and Dell 2010).

Young Modulus is a mechanical property that measures the tensile stiffness of solid material, and it quantifies the relation between tensile stress (force per unit area) and axial strain (proportional deformation) in the linear elastic region of a material. Yield strength is defined as the stress at which a predetermined amount of permanent deformation occurs, *i.e.* until this point the material can get back to its original dimensions. UTS is the maximum stress that a material can withstand while being stretched or pulled before breaking. In fragile materials, UTS is close to the yield point, while in ductile materials UTS can be higher (Engineers Edge 2020b).

It is important to remember that the films used in the biodegradation assays were prepared with a heat press and cut in the laboratory. Thus, the polymeric chains are not aligned along the uniaxial stress imposed, having different directions.

3.4.3.1. LDPE films and LDPE UV films

Generally, the standard Young Modulus for LDPE is between 110-450 MPa. The Young Modulus obtained for “standard” LDPE films and LDPE UV films made in the laboratory was about 831 MPa and 617.79 MPa, respectively. There are no standard values for Yield Strength and UTS for this polymer (Engineers Edge 2020a).

It was possible to observe a difference in the standard values and the ones obtained in the laboratory before running any assay. The tensile stress, which is the force per area unit acting on a plane transverse to the applied load, an essential measure of the internal forces within the material (Roylance 2008). Polymeric film production could lead to the tensile strength application imparted to the molecular

orientation of the polymer. This is because at the molecular level tensile properties are higher in the direction of the covalent bonds C–C chain than in the transverse direction, which is dominated by much weaker Van der Waals bonds. In this way, depending on molecular orientation, the load applied in the machine direction yield higher values of tensile strength than the load applied perpendicular to that direction (Dilara and Briassoulis 1998). Thus, the load applied to the polymeric film can explain the different standard values and the values obtained in the laboratory.

Between the films without and with UV treatment, the Young Modulus decreased, so this can be evidence of the effect of UV light in the polymers since UV irradiation induces the production of free radicals by oxidation. The free radicals cause the breakdown of polymer chains (Kiatkamjornwong et al. 1999).

LDPE films inoculated with *Streptomyces gougerotti* lose 19% of its Young Modulus, but during the incubation time the maximum value registered was 1259 MPa at 90 days of incubation and the minimum value was 686 MPa at the end of the biodegradation assay (180 days). The values of yield strength and UTS decreased by 31% and 19%, respectively (Figure 3.18.).

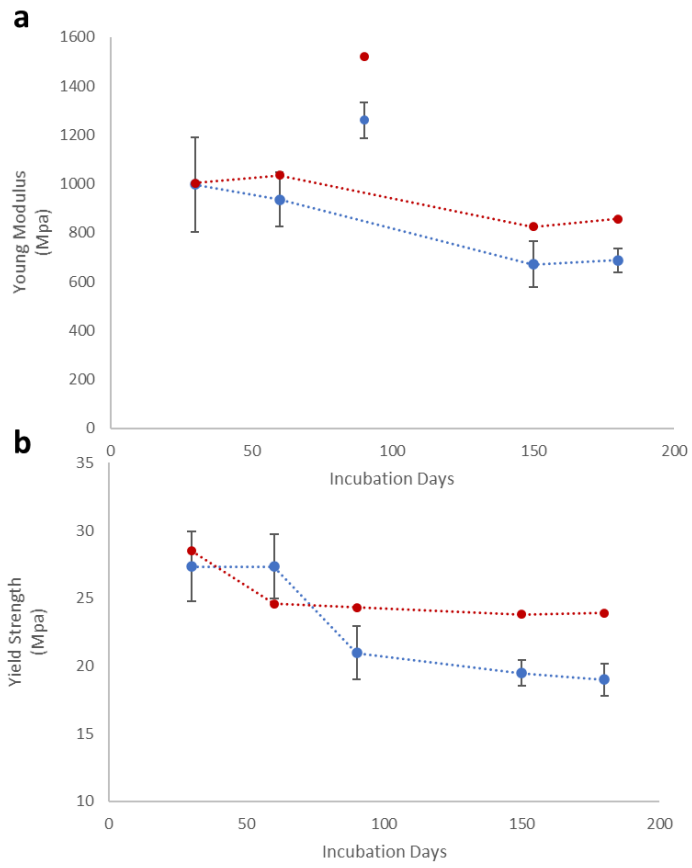

Figure 3.18.: Values obtained for the tensile tests performed with LDPE films. LDPE films were inoculated with *Streptomyces gougerotti*: a) Values obtained for Young modulus during the biodegradation assay; b) Values

obtained for Yield Strength during the biodegradation assay. The blue points represent the values obtained for *S. gougerotti* and the red point represent the control values.

It is observable in the graphics present in Figure 3.18. a general decreasing in the values over time. When comparing these values with the corresponding control, significance decreasing was found, except for UTS values. In this last case, the biggest decrease, in the beginning, was "compensated" with a slight increase at the end of the assay. However, these results indicated polyethylene biodegradation caused by *S. gougerotti*, since during this process the polymer backbone is cleaved by enzymes and the microplastic gets weaker, leading to a decrease in the tested values.

Additionally, LDPE films were also inoculated with *Micromonospora matsumotoense*. During the biodegradation assay Young Modulus, yield strength, and UTS decreased by 41%, 18%, and 9.4%, respectively (Figure 3.19.).

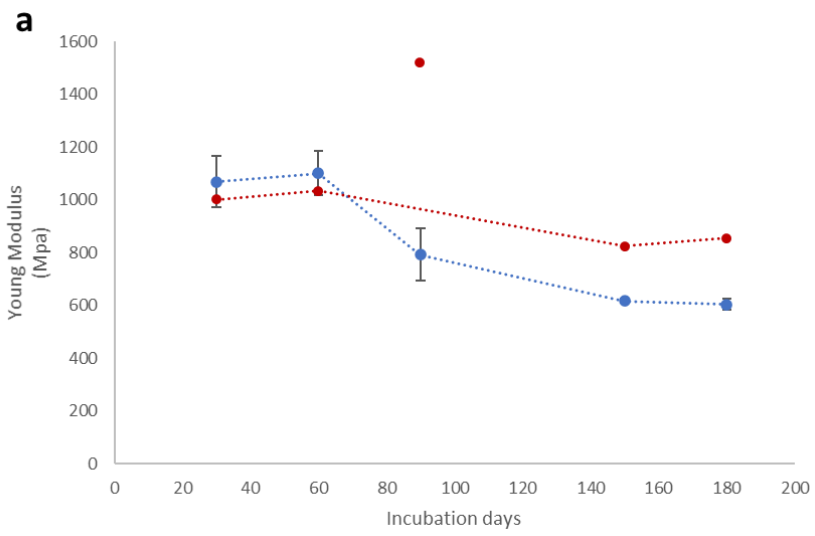

Figure 3.19.: Values obtained for the tensile tests performed with LDPE films. LDPE films were inoculated with *Micromonospora matsumotoense:* a) Values obtained for Young modulus during the biodegradation assay. The blue points represent the values obtained for *M. matsumotoense* and the red point represent the control values.

The Young modulus was the parameter that revealed the highest decrease, which measures the necessary load to induce a given deformation in the material. As the Young modulus decreases over time, the load needed to induce deformation also decreased and this can be a biodegradation indicator.

Comparing these values with the corresponding control, the Young modulus in the two different biodegradation assays presented significant differences because this parameter in the control films did not

suffered variations between the beginning and the end of the assay. However, yield strength and UTS decreased in the control of about 16% and 18%, respectively.

LDPE is a long-branched polymer, so when during biodegradation polymer chains are breakdown, most probably the branches are degraded first. This can explain the higher decreases in Young modulus and not such as a decrease in the other two tested parameters. Therefore, the differences in Young modulus can be used as evidence for LDPE biodegradation by both strains.

LDPE films treated with UV radiation were inoculated with *Streptomyces gougerotti, Micromonospora matsumotoense,* and *Micromonospora terminaliae*.

The LDPE UV films inoculated with *S. gougerotti* decreased all the tested parameters when compared to the corresponding control. In this case, Young modulus, yield strength, and UTS decreased by 43%, 43.3%, and 22.6%, respectively (Figure 3.20.).

In the ones inoculated with *M. matsumotoense* the decrease in the values of Young modulus, yield strength, and UTS were 12.4%, 26%, and 20.7%, respectively. It was only possible to perform the assay with this strain for 90 days and this can explain the lower decreases when compared to the LDPE UV films inoculated with *S. gougerotti*. Although, this strain showed some decrease in the parameter values compared to the invariability in the control, suggesting biodegradation of LDPE by *M. matsumotoense*.

Finally, regarding the assays with *M. terminaliae*, there were no significant differences in the percentage of Young modulus, as well as in yield strength percentage, when compared to the correspondent control. However, in this assay, UTS values of LDPE UV films decreased between the beginning and the end of the biodegradation.

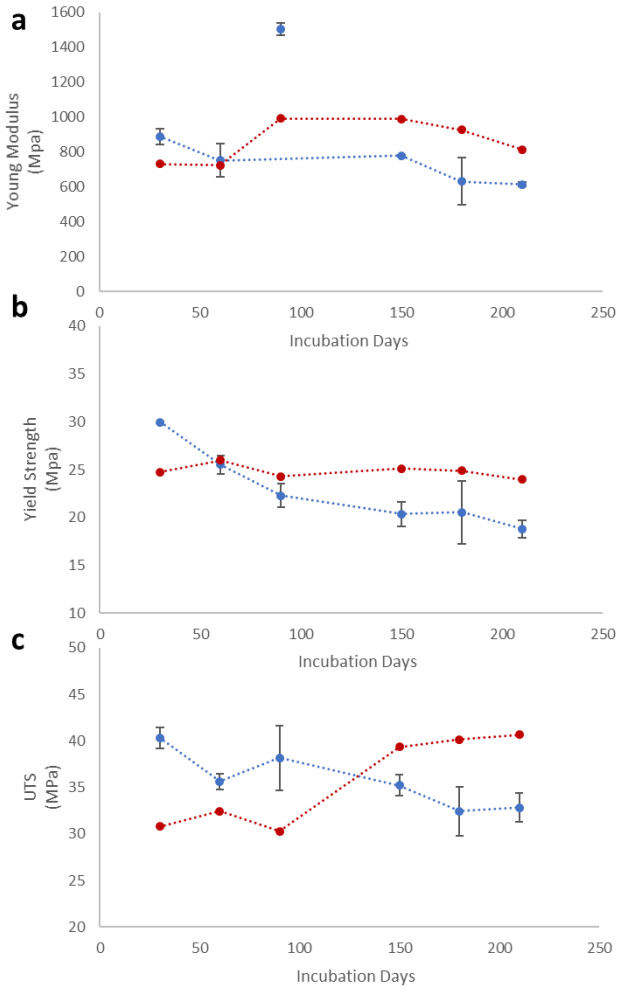

Figure 3.20.: Values obtained for the tensile tests performed with LDPE UV films: a) Values obtained for Young modulus during the biodegradation assay with *S. gougerotti*; b) Values obtained for Yield Strength during the biodegradation assay with *S. gougerotti*; c) Values obtained for UTS during the biodegradation assay with *S. gougerotti*. The blue points represent the values obtained for *S. gougerotti* and the red point represent the control values.

A reduction in these parameters' values supported the evidence of biodegradation by *Streptomyces gougerotti* and *Micromonospora matsumotoense*. The polymer chains were likely broken by the action of enzymes secreted by the strains. Thus the films became fragile, which is an indication of the preliminary stages of microbial decomposition (Esmaeili et al. 2013).

Other studies reported the reduction in the percentage of other parameters in mechanical assays after the biodegradation process (Jakubowicz, Yarahmadi, and Petersen 2006; Suresh et al. 2011; Nowak et al. 2011; Esmaeili et al. 2013). A report also showed a reduction in the percentage of elongation at break when LDPE was inoculated with a *Streptomyces* species (Lee et al. 1991).

3.4.3.2. PS films and PS UV films

The standard values of Young modulus and UTS for polystyrene are 3000-3500 MPa and 30-100 MPa, respectively (Engineers Edge 2020a).

PS films produced in this study, before any assay, presented Young modulus, yield strength, and UTS values of 1467.84 MPa, 12.6 MPa, and 21.13 MPa, respectively. While PS UV presented Young modulus, yield strength, and UTS values of 818.57 MPa, 11.93 MPa, and 12.29 MPa, respectively. Differences between the non-treated and treated films are visible in the Young modulus value, which can validate the success of UV irradiation to the films as a treatment to improve biodegradation.

When looking for the results of mechanical tests of the PS films inoculated with *Streptomyces gougerotti* it was not possible to observe a decrease in the parameters, contrariwise the values showed a tendency to increase through the biodegradation assay. On the other way, PS films inoculated with *Micromonospora matsumotoense* showed a 32% reduction of Young Modulus values when compared with a 13% reduction of the Young modulus in the control. During the assay, yield strength and UTS values increased.

Considering the results obtained for PS biodegradation it was noticeable that the films inoculated with *M. matsumotoense* showed significant variations in their mechanical properties. In this way, there is evidence for *M. matsumotoense* potential to degrade polystyrene microplastics. On the other hand, PS films incubated with *S. gougerotti* did not demonstrated changes when comparing with the control.

Additionally, PS UV films were inoculated with the strains mentioned above and with *Micromonospora terminaliae*. For these assays, there was not a decrease in the values of Young modulus, yield strength, and UTS for the three strains, except for the Young modulus of PS UV films inoculated with *M. terminaliae*. For the films inoculated with *S. gougerotti*, there was no evidence of biodegradation by microorganisms, because the variations were not significant. However, for the films incubated with *Micronomospora* species, even without significant variations in the tested parameters, the values were below the control, which can represent a biodegradation potential of polystyrene microplastics when these are treated with UV radiation.

Results for the polystyrene biodegradation did not show great biodegradation capacity by the used microorganisms. Despite that, when the films were treated with UV radiation it was observable an improvement of the results. With the performed mechanical assays it was not possible to state biodegradation of polystyrene microplastics by the actinobacteria strains used in these assays, except for Micromonospora *matsumotoense*, that could positively alter parameters values during the biodegradation assay of PS films. In this way, this polymer was not easily biodegradable.

As with other polymers, irradiation with ultraviolet light causes photo-oxidative degradation that leads to the breakage of the polymeric chain, producing radicals and reducing the molecular weight. This causes deterioration of mechanical properties. It was proved that polystyrene losses its mechanical properties due to the effect of UV light, since UV light induces the production of radicals through oxidation, leading to chain breakdown (Atiq 2011; Yousif and Haddad 2013). Considering these statements, it was possible to observe the effect of radiation between the PS and PS UV films. Notwithstanding, both polystyrene films did not present significant differences during the biodegradation, which did not allow the determination of the biodegradation extent of this polymer. Therefore, it will take longer time, with the used conditions in this study, for the degradation of PS to take place.

In other researches about PS, the higher variations in mechanical properties occurred when the polymer was blended with bioplastics since this type of inclusions accelerate molecular structural changes (Jasso G. et al. 1998; Kiatkamjornwong et al. 1999). A blend can be a possible solution for future production of products made of polystyrene, as it enhances biodegradation and will be beneficial when they become litter after disposal (Kiatkamjornwong et al. 1999).

3.4.3.3. PS films and PS UV films in media containing yeast extract

Both assays were carried out with *Streptomyces gougerotti*. In the case of PS films, there was significant variation in the Young modulus, this parameter decreased about 58% between the beginning and the end of the assay. Yield strength and UTS did not vary, but the values were lower than the values of the corresponding control.

Regarding PS UV films, the Young modulus decreased its value about 56% during the assay. On the other hand, the values of yield strength and UTS did not have a significant decrease.

As mentioned before the addition of yeast extract in the culture media supports the reduction in the integrity of polymers, because it improves biodegradation and the degradation ability for more efficient polystyrene degradation (Lee et al. 1991; Tang, Kuo, and Liu 2017). Thus, it should lead to a weakening of the mechanical properties of polystyrene and a decrease in correlated mechanical values. The obtained results for Young modulus evidenced the potential of *S. gougerotti* to degrade polystyrene. However, the obtained data for yield strength and UTS values only slight differences were observed. As polystyrene films were made in the laboratory, the polymeric chains could be dispersed and not be orientated. Throughout the assays, this generated entropy may have decreased and "created" a more oriented polymer, "complicating" the tensile strength tests.

In statistical analyses significance was found when yeast extract was added to the culture media for yield strength and UTS values (p-value < 0.05). This indicates that the addition of yeast extract enhances

the biodegradation of plastic films, since these two parameters are associated with the degradation of polymeric chains.

3.4.3.4. PLA films and PLA UV films

The standard Young modulus, Yield strength, and UTS values for PLA are 2300 MPa, 35.9 MPa, and 26.4 MPa, respectively (SD3D 2017). The values obtained for the same parameters of PLA films made in the laboratory were 2554 MPa, 61 MPa, and 66.4 MPa. For PLA UV films the values were 864 MPa, 28 MPa, and 29 MPa, respectively. The obtained Young modulus values of regular PLA are close to the standard. Generally, all the parameter values decreased after the UV treatment, which can be an indication of the success of this film's irradiation.

Regarding PLA films inoculated with *Nocardiopsis prasina* it was possible to observe a significant decrease in Young modulus when compared to the correspondent control. This parameter decreased by 18% during the assay (Figure 3.22.). However, Yield strength and UTS did not vary throughout the biodegradation assay.

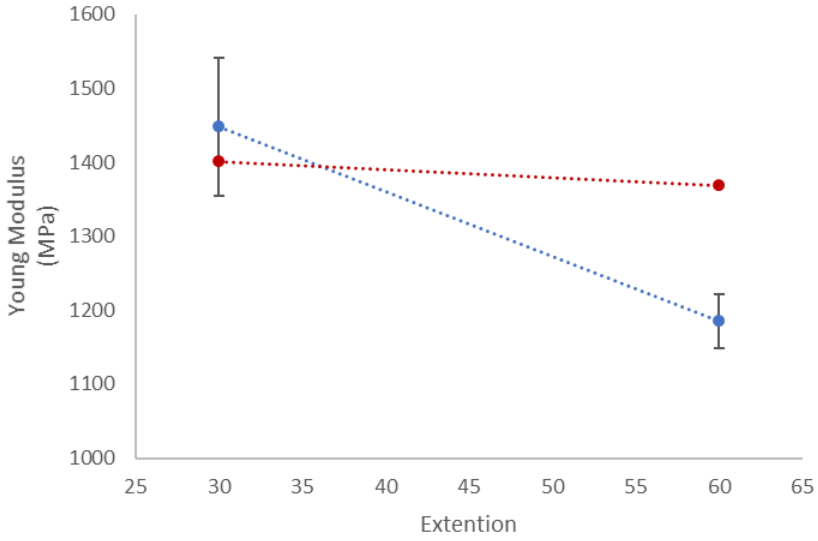

Figure 3.21.: Young modulus values during the biodegradation assay of PLA films inoculated with *Nocardiopsis prasina.* The blue points represent the values obtained for *N. prasina* and the red point represent the control values.

Young modulus of PLA UV films inoculated with and without yeast extract (in the culture media) also decrease during the assays by 29% and 2%, respectively. Nevertheless, Yield strength and UTS for both PLA UV assays increased during the 60 days assay.

In statistical analysis, significance was found when the PLA films were treated with UV radiation, when compared with the regular films (p-value < 0.05), which indicates that the UV treatment was effective, and it increased the biodegradation effects.

Young modulus decreased was more pronounced in the PLA UV films inoculated with yeast extract in the cultural media (Figure 3.23.). In this case, the conditions were close to the optimal conditions, which enhance biodegradation, explaining the obtained results.

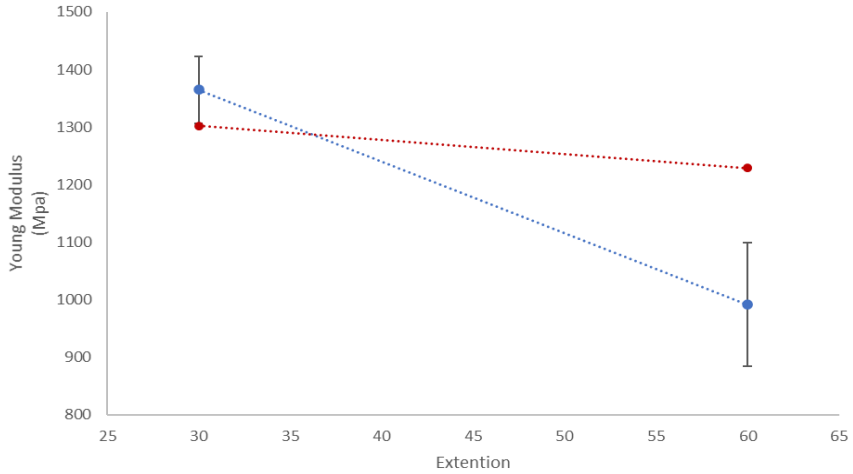

Figure 3.22.: Young modulus values during the biodegradation assay of PLA UV films inoculated with *Nocardiopsis prasina* and with the addition of yeast extract to the cultural media.

Yield strength and UTS values in the biodegradation assays of PLA films treated and non-treated with UV radiation showed an absence of positive variations. Comparing these results with FTIR spectra, it was determined that during biodegradation alterations occur in PLA film's surface, by the formation of C–O double bonds and unsaturated vinyl groups. Thus, the polymer could become more resistant to deformational forces and stretching, which could explain the absence of variation in these parameters.

The results obtained in this study indicated that polylactic acid was more sensitive to biodegradation in laboratory conditions than low-density polyethylene and polystyrene.

It was possible to find evidence about the biodegradation of polylactic acid in several environments, particularly in composting. Thus, to improve the biodegradation of this polymer, alternative environments in optimal conditions can be created (Walczak et al. 2015). There are several studies on the literature indicating the capacity of polymer degrading actinobacteria, for example, *Amycolatopsis* genera (Butbunchu and Pathom-aree 2019). Nevertheless, *Nocardiopsis* genera are not reported in PLA biodegradation studies, which can be an opportunity to discover new polymer degrading actinobacteria.

In reported studies the decreases in PLA strength are used to determine biodegradation, when the films are inoculated with microorganisms (Sedničková et al. 2018; Janczak et al. 2020).

3.4.4. PHA inclusions in actinobacteria cells

Members of different microbial genera synthesise polymeric lipids such as polyhydroxyalkanoates and about 300 PHA-producing bacterial strains have been identified. Synthetised PHA is produced by several bacteria species as internal carbon storage and energy reserve, under different conditions (Reis et al. 2011). When essential nutrients, like nitrogen and phosphate sources, are limited and the carbon source is in excess, bacteria strains with the potential to produce PHA, accumulated these biopolymers as intracellular inclusions (Matias et al. 2009). The plastic film biodegradation assays with actinobacteria were performed with was an excess of carbon source and a limitation of other nutrients. The presence of PHA granules in the actinobacteria cells was assessed in order to evaluate the PHA production potential of actinobacteria, by using plastics as carbon source. Thus, in the current study the detection of PHA inclusions in the actinobacteria cells was used as an indirect method to assess plastic biodegradation.

All the strains used in the different biodegradation assays were stained and visualised at the epifluorescence microscope to search for PHA inclusions. The strain *Streptomyces gougerotti* was inoculated with an excess carbon source and limited other essential nutrients, in the biodegradation assays with LDPE, LDPE UV, PS, and PS UV films. When *S. gougerotti* was incubated with LDPE films some fluorescence was observed in the epifluorescence microscope. However, it was due to the sample's own fluorescence and not to the presence of PHA vesicles. Nevertheless, observing this strain when incubated with PS films (Figure 3.24.), LDPE and PS films treated with UV radiation (Figure 3.25.), some PHA vesicles were observed although with little fluorescence intensity. Regarding PS and PS UV films inoculated with this strain and containing yeast extract in the culture media, no PHA vesicles were detected. This can be explained by the presence of other nutrients, like nitrogen by the addition of yeast extract in the culture media.

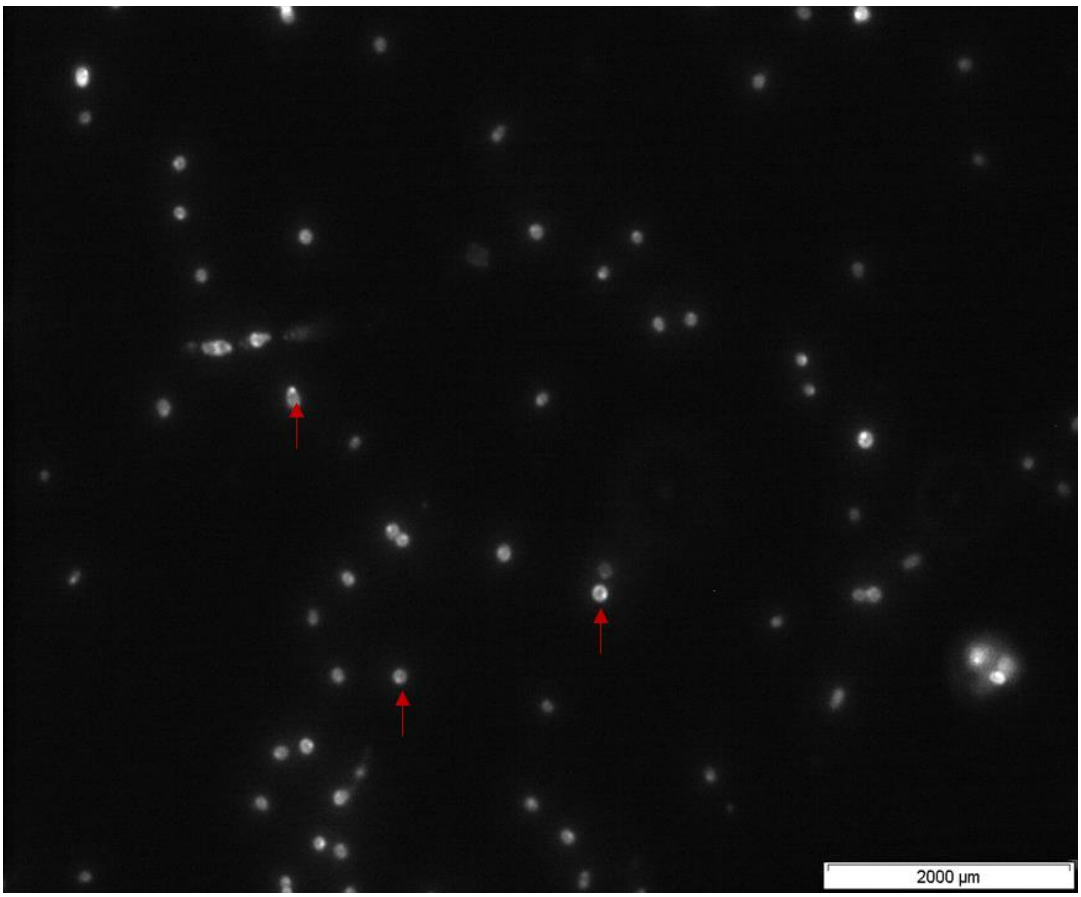

Figure 3.23.: Visualization of *Streptomyces gougerotti* in an epifluorescence microscope to search for PHA vesicles produced during the biodegradation assay with PS films. The red arrows point to the PHA inclusions.

Studies have been made to evaluate the potential of PHA production by genotypic and phenotypic characterizations, made by PCR detection and sequencing analysis of the *pha*C genes encoding the PHA synthase and PHA production experiments (Inoue et al. 2016). Some *Streptomyces* strains have been described as having the *pha*C genes (Valappil et al. 2007), although *S. gougerotti* still needs further investigation. The most relevant actinobacteria genera in terms of PHA production, are *Streptomyces*, *Rhodococcus*, *Nocardia*, and *Corynebacterium* (Valappil et al. 2007; Matias et al. 2009).

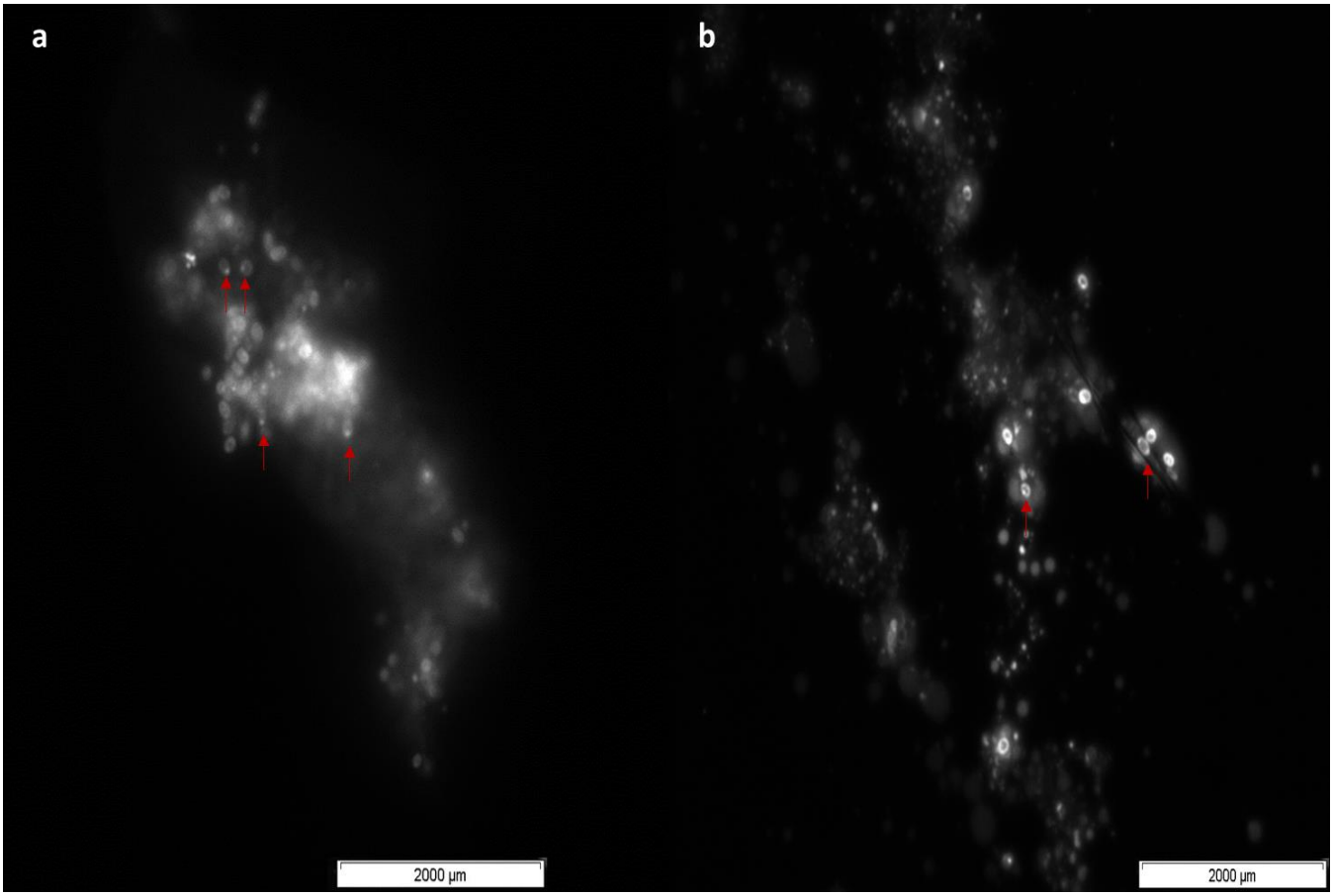

Figure 3.24.: Visualization of *Streptomyces gougerotti* in an epifluorescence microscope to search for PHA vesicles produced during the biodegradation assay: a) *Streptomyces gougerotti* strain after being inoculated with LDPE UV films; b) *Streptomyces gougerotti* strain after being inoculated with PS UV films. The red arrows point to the PHA inclusions.

The strain *Micromonospora matsumotoense* was also inoculated with an excess carbon source and limited essential nutrients in the biodegradation assays with LDPE, LDPE UV, PS, and PS UV films. It was possible to clearly observe PHA accumulation in *M. matsumotoense* when inoculated with PS films treated with UV radiation (Figure 3.26.). Otherwise, PHA vesicles were not visible when the strain was inoculated with LDPE or LDPE UV, or PS films. These results can indicate the potential of *M. matsumotoense* of using pre-treated PS as a sole carbon source since the UV irradiation facilitates the microbial attack and the beginning of biodegradation. LDPE used as a carbon source may not have triggered a response to the activation of the genes responsible for the production of PHA.

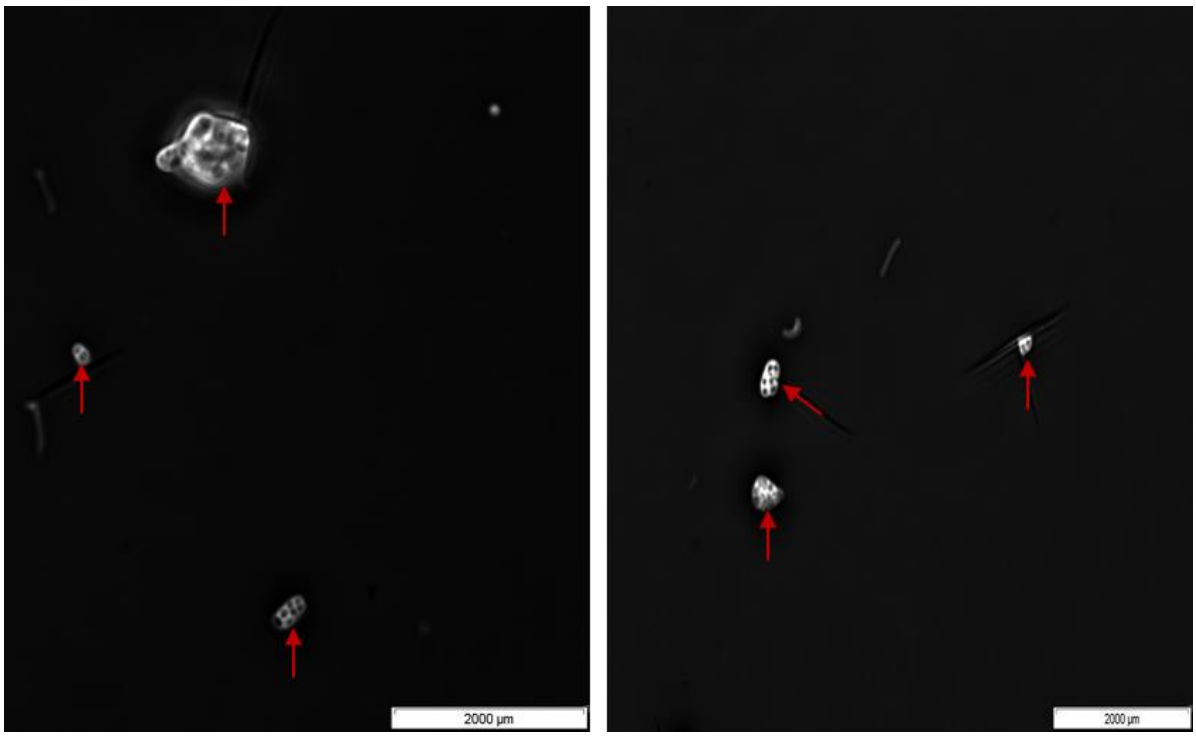

Figure 3.25.: Visualization of *Micromonospora matsumotoense* in an epifluorescence microscope to search for PHA vesicles produced during the biodegradation assay of PS UV films: a) and b) represent the different location of the strain in the coverslips. The red arrows point to the PHA inclusions.

The strain *Nocardiopsis prasina* was inoculated with PLA and PLA UV films with an excess carbon source and limited essential nutrients. The polymer used in this case is considered biodegradable under certain conditions. *N. prasina* was used in this biodegradation assay and observed in the epifluorescence microscope in order to search for PHA vesicles. In both cases, *N. prasina* presented autofluorescence, so PHA inclusions were not identified.

It is important to notice that Nile blue, the stain used to identify PHA vesicles, binds to lipids.

An interesting hypothesis can be placed, actinobacteria used in this study can use conventional plastics to produce PHA, a biodegradable bioplastic. Further studies to produce PHA in higher amount to enable structural and physico-chemical characterization would be of primordial importance.

Micromonospora terminaliae did not present PHA vesicles when inoculated with LDPE and PS films treated with UV radiation by observation at the epifluorescence microscope. Considering these results and the previous ones for *M. terminaliae* it is possible to determine that this strain has no potential to degrade LDPE and PS polymers.

In the literature there is no information about the presence of the *pha*C gene, which is responsible for the production of PHA, in *Micromonospora* genera, but there is evidence for the accumulation of

triacylglycerols (Wältermann and Steinbüchel 2005). Considering this information some strains of *Micromonospora* genera may have the capacity to produce PHA, such as *Micromonospora matsumotoense* (used in this study), depending on culture conditions optimization, it will be possible to discover new actinobacteria strains capable of producing polymeric lipids (PHA in this case) and improve future bioplastics production.

4

Conclusion

In the present study, LDPE, PS, and PLA degradation by different strains of actinobacteria was achieved. Increasing solvent dissolution time, adding a surfactant, and sonication were the keys to achieving the desired polymer emulsions to prepare the media for the biodegradation screening. From the 36 used actinobacteria, 8 revealed the capacity for growing in media using plastic as the sole carbon source. Of these, three, *Streptomyces gougerotti*, *Micromonospora matsumotoense*, and *Nocardiopsis prasina* showed better results in the clear zone test by producing a halo of 1-4 mm. These strains have never been previously reported in the literature for plastic degradation.

Strains *Streptomyces gougerotti*, *Micromonospora matsumotoense*, and *Nocardiopsis prasina*, were able to biodegrade LDPE, PS, and PLA plastics. These results were corroborated by FTIR analysis and tensile strength tests. *S. gougerotti*, *M. matsumotoense*, and *N. prasina* altered the chemical surface of the polymers and caused variations in mechanical properties by chain scission. The chemical changes in the polymer's surface were more evident by the appearance or disappearance of certain IR bands, being the carbonyl group (at 1740 cm^{-1}) more correlated with the occurrence of biodegradation. Moreover, the Young modulus values, one of the parameters related to polymers mechanical properties, varied for low-density polyethylene from 12.4% to 43%, the films inoculated with *S. gougerotti* had the highest decrease and the films inoculated with *M. matsumotoense* had the smallest decrease in this parameter value. Considering polystyrene films, the maximum decrease in Young modulus was 58% when PS films were inoculated with *S. gougerotti*, and the smallest decreased was 3.51% when PS films were inoculated with *M. matsumotoense*. Finally, this parameter varied from 2.59% to 29% in PLA films inoculated with *N. prasina*.

Biodegradation becomes more evident as the assays approached the optimum conditions, by performing a UV pre-treatment to the plastic films and by the addition of yeast extract to the culture media.

S. gougerotti produced PHA inclusions when inoculated with LDPE and PS with a previous UV radiation treatment, proof of assimilation of the polymers as a carbon source. *M. matsumotoense* produced PHA inclusions when inoculated with PS treated with UV radiation.

This study serves as an alert to environmental pollution caused by the accumulation of plastics since plastics and microplastics have negative impacts on the environment, health, society, and the economy. This study joins others in contributing to the search for solutions to mitigate this problem. The use of microorganisms for plastic biodegradation has the potential of being used efficiently without causing major negative impacts. The described biodegradation processes are not feasible for direct application into the ocean and landfills, as the use of microorganisms could create serious ecological imbalances. Industrial approaches using these methods would imply, as any other recycling method, the collection of plastic waste from both land and the ocean. It should always address the plastic pollution problem before it reaches these environments. Thus, the search for alternative biodegradable bioplastics is of utmost importance. In this way, more sustainable methods and solutions to decrease the pollution caused by plastics will be discovered in the years to come.

In addition, it is important to avoid single-use plastics in our everyday life and to opt for more eco-friendly alternatives. It is also important to educate the population about the microplastics problem since they are considered a persistent pollutant. With this education, the use of plastics on a large scale may decrease. Small gestures make a difference.

5

Future Work

This study intended to discover new bacterial strains for plastic biodegradation and for possible bioplastic production (PHA).

As pure bacterial cultures were used it would be interesting to study the biodegradation mechanism of each one of the strains with this capacity and identifying the plastic degradation by-products, as a way of improving and enhancing biodegradation in future researches about (micro)plastics as pollutants.

Knowing the mechanisms of plastic biodegradation, the enzymes involved in this process could be isolated and applied as a more sustainable method to mitigate (micro)plastics pollution.

Moreover, the detailed study of genes involved in biodegradation could be an advantage to understand which genes trigger this process and possible correlations between all the discovered species capable of degrading certain types of microplastics.

In order to improve the biodegradation process, more effective pre-treatments for plastics could be attempted before the biodegradation assay, and different yeast extract concentration could be used to optimise and enhance the microorganism's biodegradation. Furthermore, it is important to optimise PHA production by the selected actinobacteria.

6

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