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**Plastic degrading microorganisms:
towards microbial biorecycling
factories**

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Resumo Microrganismos degradadores de plástico: rumo a fábricas microbianas biorecicladoras

Os polímeros de plástico são muito resistentes, têm baixa necessidade de manutenção e um baixo custo, o que os torna preferíveis a outros materiais tais como vidro, madeira e metais. Hoje em dia, os plásticos são essenciais e estão amplamente difundidos na sociedade moderna, tendo aplicações variadas que vão desde o embalamento, construção civil, indústria automível, eletrônica, habitação, desporto, agricultura, entre outros. O aumento da utilização e produção de plásticos nos últimos anos, aliado à sua elevada estabilidade e baixa biodegradabilidade, faz com que milhões de toneladas de plástico se acumulem anualmente, ameaçando tanto os ecossistemas como a saúde pública.

Os resíduos de plástico são tratados principalmente por 3 métodos: aterro sanitário, incineração e reciclagem. No entanto, estes métodos têm custos enormes, são insustentáveis e têm várias consequências negativas, pelo que há uma necessidade urgente de novas tecnologias para a eliminação destes resíduos. Nos últimos anos, foi reportada a biodegradação de plásticos por microrganismos, o que poderá potenciar o desenvolvimento destas novas tecnologias. Os microrganismos conseguem converter os polímeros de plástico em compostos assimiláveis, fornecendo assim um ponto de partida para a economia global circular destes materiais.

Neste trabalho, quatro microrganismos distintos, leveduras e bactérias, foram avaliados pelo seu potencial de biodegradação do polietileno (PE). Este polímero foi selecionado, dada a sua reduzida biodegradabilidade e enorme impacto poluente. O processo de rastreio envolveu a utilização de vários meios seletivos/diferenciais, para a determinação da capacidade de utilização de fontes de carbono específicas. O crescimento nestes meios será indicativo da produção de enzimas extracelulares envolvidas na biodegradação do PE e.g. oxidase, protease, lacase, cutinase, lipase e esterase. Apenas a *Yarrowia lipolytica* e *Pseudomonas aeruginosa* apresentaram crescimento nestes meios, não tendo sido detetado em *Debaryomyces hansenii* e *Alcanivorax borkumensis*. A capacidade de biodegradação de PE pela *Y. lipolytica* e *P. aeruginosa* foi avaliada durante 90 dias em meio líquido suplementado com o filme, tendo sido considerados vários parâmetros: crescimento microbiano, formação de biofilme, perfil proteico extracelular; enquanto os filmes de PE foram caracterizados por ATR-FTIR, SEM e perda de peso. Os resultados indicam a capacidade dos microrganismos para se fixarem à superfície do plástico, formando biofilmes e produzindo enzimas com atividade catalítica do PE. Além disso, as análises por ATR-FTIR e SEM revelaram alterações significativas nos filmes após o período de incubação. Este trabalho evidenciou o potencial da *P. aeruginosa* e *Y. lipolytica* na biodegradação de plásticos recalcitrantes como o PE.

Palavras-chave: Biodegradação microbiana; Biorremediação; Plásticos; Polietileno.

Abstract Plastic degrading microorganisms: towards microbial biorecycling factories

Plastic polymers are very resistant and have low maintenance and cost, which makes them preferable to other materials such as glass, wood and metals. Nowadays, plastics are essential and ubiquitous in modern society, having varied applications, ranging from packaging, construction, automobiles, electronics, household, sports, agriculture, among others. The increased use and production of plastics in recent years, combined with their high stability and low biodegradability, causes millions of tons of plastic to accumulate annually, threatening ecosystems and public health, while posing a real and growing environmental problem.

The plastic waste produced is mainly eliminated by 3 different methods: landfill, incineration, and recycling. These plastic waste treatment methods have enormous costs, are unsustainable and have several negative consequences. Thus, there is an urgent need for new technologies for the disposal of plastic waste. In recent years, there have been several reports on the biodegradation of plastics by microorganisms which could promote the development of these new technologies. Microorganisms can convert plastic polymers into assimilable compounds, providing a starting point for a global circular economy of plastics.

In this work, four different microorganisms, yeasts and bacteria, were evaluated for their potential to biodegrade polyethylene (PE), which was selected for its very low biodegradability, and huge impact as pollutant. To do this, a screening process involving the use of several different selective/differential media was performed to assess the potential of the microorganisms to use specific carbon sources, indicative of the ability to produce extracellular enzymes, e.g. oxidase, proteases, laccase, cutinases, lipases and esterases, involved in polyethylene biodegradation. While *Debaryomyces hansenii* and *Alcanivorax borkumensis* were unable to utilize the substrates, *Yarrowia lipolytica* and *Pseudomonas aeruginosa* used them as carbon and energy source. The potential plastic biodegradation capacity of *Y. lipolytica* and *P. aeruginosa* was then evaluated for 90 days in liquid media supplemented with PE film. During this period, several parameters were investigated namely, microbial growth, biofilm formation and extracellular protein profile, whereas the PE films were characterized by ATR-FTIR, SEM and weight loss. Results indicate the ability of the microorganisms to attach and adhere to the surface of the plastic materials, forming biofilms and producing enzymes with PE catalytic activity. Moreover, ATR-FTIR and SEM analyses revealed significant changes in the PE films after the incubation period. Thus, this work demonstrates the potential of using *P. aeruginosa* and *Y. lipolytica* for the biodegradation of recalcitrant plastics such as PE.

Keywords: Bioremediation; Microbial biodegradation; Plastics; Polyethylene.

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List of abbreviations

ATR-FTIR	Attenuated total reflectance - Fourier transform infrared
BHET	Bis(2-hydroxyethyl) terephthalate
bt	Billion tons
CMC	Critical micellar concentration
Davis	Davis Minimal Broth without Dextrose
Davis + blue	Davis Minimal Broth without Dextrose + methylene blue
HDPE	High density polyethylene
LDPE	Low density polyethylene
MB	Marine Broth
MBP	Marine Broth + 1 % Sodium Pyruvate
Mt	Million tons
Mw	Molecular weight
OD	Optical density
PBS	Phosphate buffer saline
PCL	Polycaprolactone
PE	Polyethylene
PEG	Poly(ethylene glycol)
PET	Polyethylene terephthalate
PP	Polypropylene
PS	Polystyrene
PSF	Polysulfone
PVA	Poly(vinyl alcohol)
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Scanning electron microscopy
UV	Ultraviolet

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1. Introduction

1.1. A brief history of plastics

Plastic is a term commonly used for synthetic polymers, which consist of large molecules comprising several identical or similar subunits bonded together [1, 2]. Due to the rapid development of urbanization, especially over the last century, there was the need to develop cheaper and more efficient materials [3]. In this sense, in 1869 John Hyatt, inspired by a company that was looking for a substitute for ivory, synthesized celluloid, a polymer developed from nitrocellulose and camphor [4, 5]. This material could be molded into a variety of shapes and demonstrated to possess good physical/mechanical properties, representing a cheap alternative to natural materials. Thus, the pressure on the supply of natural ivory obtained through the slaughter of wild elephants has been reduced [6]. This discovery was revolutionary as, for the first time, human fabrication was not constrained by the limits of nature, and Human society could now create new materials [7].

In 1907, Leo Baekeland looking for a synthetic replacement for shellac, a natural electrical insulator, invented Bakelite, the first fully synthetic plastic [1]. The successes of celluloid and bakelite led chemical companies to invest in research and development of new polymers, and new plastics emerged.

World War II, along with the need to preserve scarce natural resources, led to a massive expansion of the plastics industry since the production of synthetic alternatives was a priority and plastics were these substitutes [6]. Polyethylene (PE) was created in England in 1933 and was used to insulate radar cables, making them light enough to be placed on planes and giving Britain's planes a significant advantage against the Germans [8]. Nylon, invented in 1935, was used during the war for parachutes, ropes, vests, helmet liners, and more. The glass for aircraft windows was replaced by Plexiglas [6]. Since then, different synthetic polymers have been invented either using natural substances such as cellulose or using plentiful carbon atoms provided by petroleum and other fossil fuels [9].

Thus, plastic came to be seen as a cheap, safe, highly stable and resistant material that could be molded by man according to his needs, taking the place of traditional materials [7, 10].

1.2. Uses of plastics

The production of plastics has continued to increase to this day, becoming a fundamental material for modern life. Plastics made possible the development of computers, cell phones, and most of the life-saving advances in modern medicine [11, 12]. In addition, it has raised the standards of living, as the substitution of natural materials for plastic has made many products cheaper, lighter, safer and more

resistant [6, 10]. Currently, plastics have applications in several areas, such as building, automobile, electronic, household, sports, and agriculture. The packaging industry is the largest of them, representing more than 40% of the world production of plastics in 2020 (Figure 1) [13].

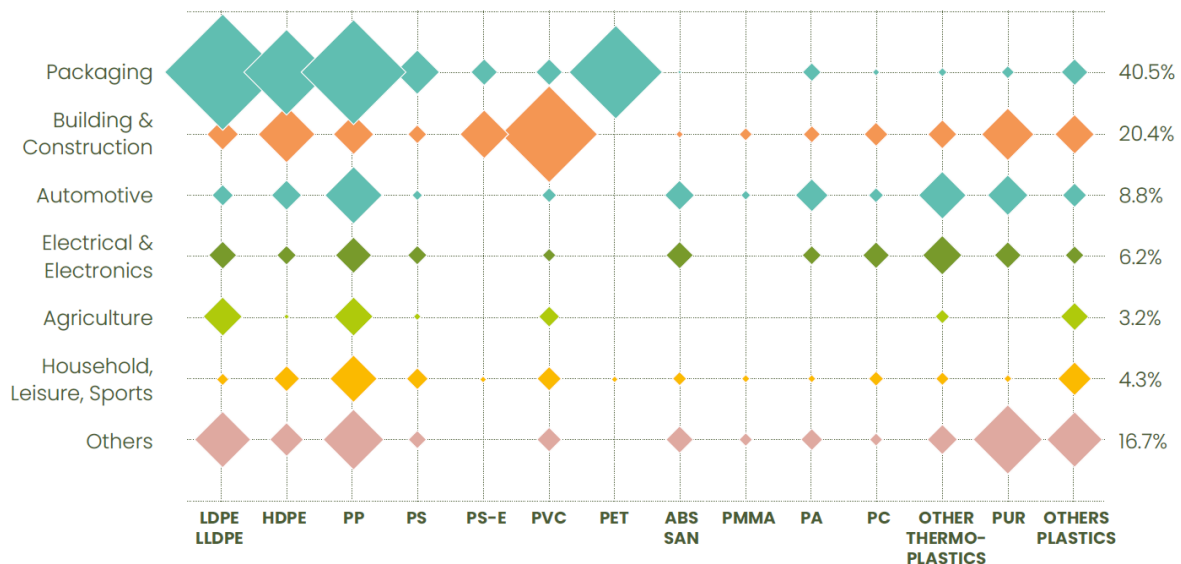


Figure 1. Demand for each type of plastic in the various application areas. Adapted from [13].

1.2.1. Packaging

The use of plastic packaging is increasing due to population growth and market expansion [14-17]. Product packaging, especially in the food and beverage industry, is very important as it protects the product from external factors, e.g. light, moisture loss/incorporation, heat, physical aggression, among others, and prolongs its shelf life by preserving its organoleptic properties throughout the logistics chain from the manufacturer to the end user [18, 19]. In addition, it plays an important role in product branding and marketing and provides information and safety, since the packaging must contain important information about the product and the care to be taken with it [16].

However, packaging has a very short shelf life - usually less than 6 months - in contrast to the significantly longer shelf life elsewhere: civil construction (35 years), industrial machinery (20 years), transport (13 years), electronics (8 years), textiles (5 years) and consumer and institutional products (3 years) [20]. The packaging industry, besides being the largest application of plastics, also stands out as the biggest generator of plastic waste, and is therefore a major contributor to the problems associated with plastic consumption [20, 21].

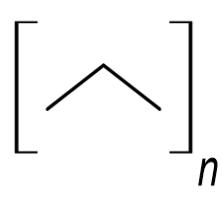
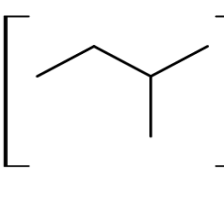
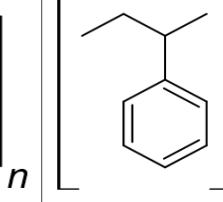
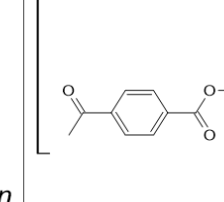
1.2.2. Main plastics used in packaging

Each plastic is designed with specific characteristics that make it ideal for its intended application [22]. The most used plastics in the packaging industry include PE (low density – LDPE and high density – HDPE), polypropylene (PP), polyethylene terephthalate (PET) and polystyrene (PS) [13].

Plastics can be further classified into two categories: thermosets and thermoplastics, according to their behaviour at high temperatures. Thermosets are plastics that harden during their manufacturing and hot molding processes. They solidify forming a solid and stable body, but once molded, they cannot be transformed again by softening and molding, nor recycled by thermal action, which prevents their later reuse [10]. Their structure is highly cross-linked and their main chains are made of hetero-atoms, e.g. ester or amide bonds, making them potentially more susceptible to biodegradation [23]. Thermosets are not widely used in the packaging industry, and their use declined sharply with the commercial availability of thermoplastic resins that offered easier processing at a lower price [24]. The thermoset forming process allows for very high precision along with great definition and is thereby used for closures with a highly detailed, embossed brand logo, as found on some alcoholic beverages. Other packaging applications for thermosets are cross-linked acrylic, polyurethane and epoxy lacquers and adhesives [24].

Thermoplastics are very high molecular weight polymers, which structure can be modified by heating or cooling repeatedly. Thus, they can be molded many times, returning to a solid state after cooling [10]. This characteristic is due to the fact that the monomers are linked end-to-end in a series of long carbon chains independent of each other allowing them to be recycled and reused [9]. Also, this structure with a high-carbon backbone makes thermoplastics resistant to degradation or hydrolytic cleavage of the chemical bonds [23]. However, recycled thermoplastic materials cannot be used in food packaging to avoid contamination by paints and/or toxic products. This category includes the main families of plastics, namely, PE, polypropylene (PP), polystyrene (PS) and polyethylene terephthalate (PET) [25] (Table 1).

Table 1. Structural representation of the monomers of main plastics used in packaging. PE – polyethylene; PP – polypropylene; PS – polystyrene; PET – polyethylene terephthalate.

<i>PE</i>	<i>PP</i>	<i>PS</i>	<i>PET</i>
			

Polyethylene

PE accounts for 30.3 % of plastic production, being the most produced plastic in the world with over 100 million tons per year as of 2017 [13, 26, 27]. It consists of long chains of ethylene monomers and is chemically synthesized through the polymerization of ethane [28]. PE is easily processed, has good mechanical properties, high tensile strength, and high production at low cost, which makes it widely used for various applications [29]. Among the main areas of use for this polymer is the packaging industry, and in particular for the packaging of products with a short shelf life, such as plastic bags, bottles, storage cups and containers, food packaging film, etc. [30, 31].

Many types of PE are known varying in their density and branching. The two main ones are high density polyethylene (HDPE) and low density polyethylene (LDPE) (Figure 2) [28, 32]. In LDPE, the chains have more side branches, which prevents them from aligning closely and thus leads to a lower density. HDPE, on the other hand, has greater rigidity and durability, as the polymer chains are regularly aligned to form a more crystalline structure [29].

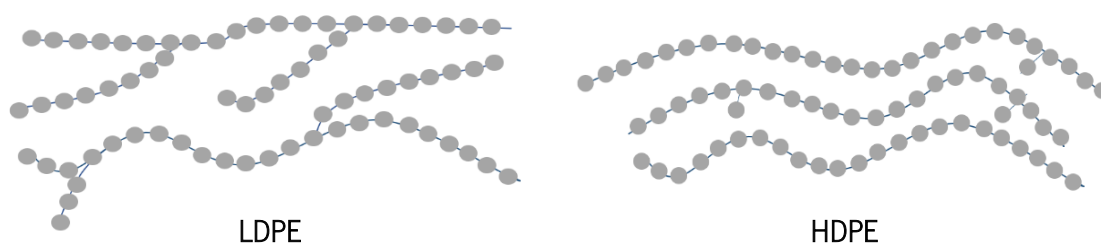


Figure 2. Schematic representation of the structure of HDPE and LDPE.

Polypropylene

PP is the second most produced polymer and has the chemical formula $(C_3H_6)_n$ being composed of propane-1,2-diyl repeating units [33]. Due to its low cost, versatile physical properties, and good heat resistance, it is widely used in various areas. Among the packaging, which represents its largest market demand in 2020, it is used in the packaging of surgical equipment, medicines, cosmetics, electronic products, and others [13, 30, 31]. In addition, PP has a moisture barrier property, making it an effective packaging solution for the food and beverage industry, that represents an important application for this polymer [34].

Polystyrene

PS is a synthetic hydrophobic polymer that consists of styrene monomers [28]. It is one of the main plastics used, with a demand in Europe of 6.1 % by 2020 [13]. Among its main attractive features are its clarity, dimensional stability, and adaptability to radiation sterilization [35].

Additionally, it is also produced in the expanded PS form, which is widely used not only in the construction industry for building insulation, but also in the packaging industry [13]. Packaging applications include packaging foam, such as meat trays and egg cartons, and film for food packaging. Furthermore, it is commonly used as molded foam for packaging electronic equipment such as TV's, audio equipment, and computers [36].

Polyethylene terephthalate

PET is formed from the condensation reaction of dimethyl terephthalate and ethylene glycol [37]. It has the molecular formula $(C_{10}H_8O_4)_n$ and is a polar copolymer of ethylene glycol and terephthalic acid. PET has high resistance to chemical and physical agents and is therefore widely used in the packaging industry especially for single-use packaging (water bottles, soft drinks, processed meat packaging, food containers, etc.) [13, 28, 38]. The annual production of PET exceeded 30 million tons in 2019, making this synthetic polymer in the top of plastics that generates the largest amount of waste [39].

1.3. The plastic crisis

1.3.1. Plastics production

Nowadays, plastics represent one of the most used materials in the world with a global production estimated at 9 billion tons (bt) from 1950 to 2020, with an increase every year [7, 13, 20, 40, 41]. To

note that the slight decrease assisted in 2020, is due to the impact of the Covid-19 pandemic which led to lower production and use of plastics (Figure 3) [42]. Nevertheless, it is expected that in the coming years more than 400 Mt of plastic will be produced annually and it should reach 800 Mt by 2035 and 1600 Mt by 2050 [41].

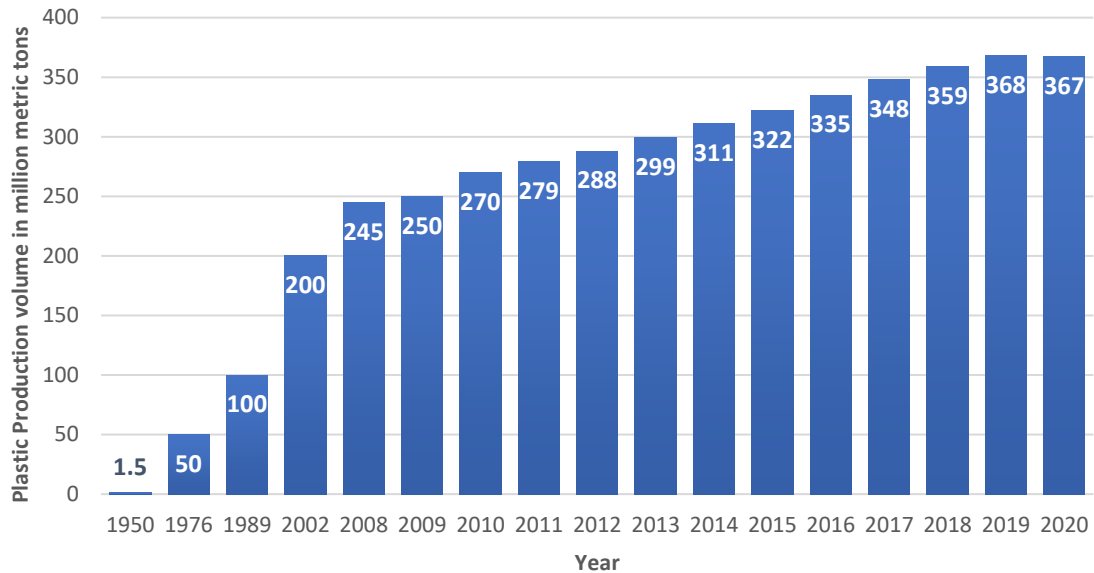


Figure 3. Global plastics production from 1950 to 2020 (based on [13]).

1.3.2. Bioplastics

The increasing production of plastics has been followed by a growing environmental awareness of its consequences, leading to an attempt to make plastics more sustainable, both in production, by reducing fossil fuel consumption, and with respect to waste [43]. Thus, bioplastics emerged, which can be defined as a plastic material that is either biologically based or biodegradable, or has both properties [11]. As for the source, bioplastics are divided into bio-based when derived from biomass or renewable resources, or fossil-based when derived from petrochemicals [44, 45]. In addition, some bioplastics can be biodegradable when there are microorganisms in the environment that, under suitable conditions, can convert them into natural substances such as water and carbon dioxide; otherwise, they are considered non-biodegradable if this is not possible [11, 46]. Considering the aforementioned, there are three main types of bioplastics (Figure 4): bio-based and non-biodegradable, fossil-based and biodegradable, or bio-based and biodegradable [46]. Plastics that are both non-biodegradable and fossil-based are classified as conventional plastics.

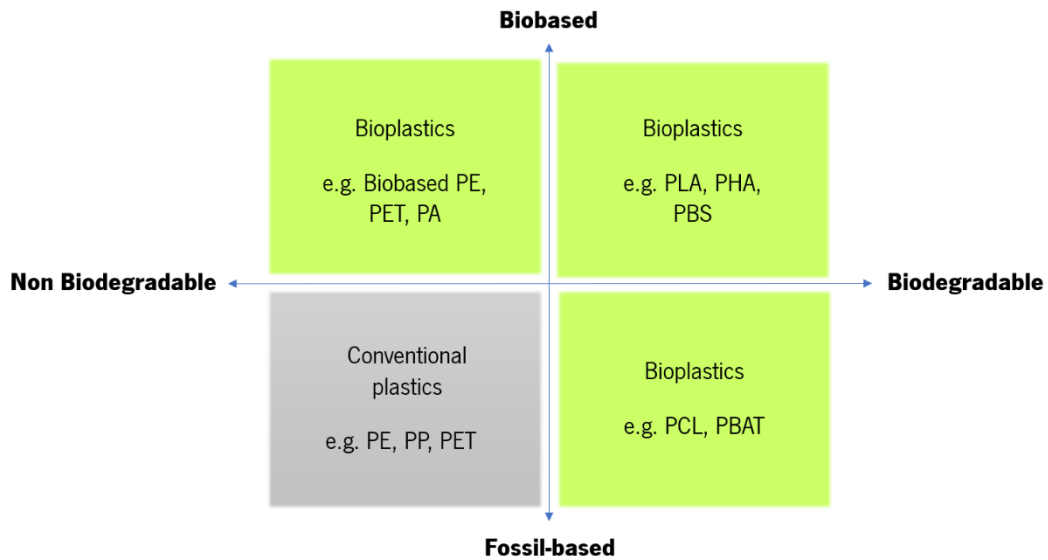


Figure 4. Classification of bioplastics by source and biodegradability (based on [46]).

Although bioplastics are apparently more environmentally friendly materials, they also have some limitations, such as higher production costs and worse mechanical properties [47]. For these reasons, today, conventional plastics are still the most widely used as bioplastics represent less than one percent of the 367 Mt of plastic produced in 2020 [13, 48].

1.3.3 Plastic waste disposal

With the unsuccessful attempt to make plastics more sustainable through bioplastics, and the increasing use and production of conventional plastics over the years, the problem of post-consumer disposal of these materials has become a central issue of the 21st century [49]. The properties that make plastics such an interesting product, with so many different applications, also threatens the sustainability of our planet. Plastics are resistant and non-reactive, which makes them remain in the environment for a long time [7]. Of the global plastic production from 1950 to 2018, 6.3 bt ended up as waste, with over 4 bt being used just once before disposal [50]. Nowadays, plastic waste is produced at a rate near 400 Mt per year [41, 51]. Current methods of disposing plastic waste are landfills or its release into the environment (72 %), incineration (14 %), and recycling (14 %) [52, 53], each of these presenting drawbacks (Figure 5).

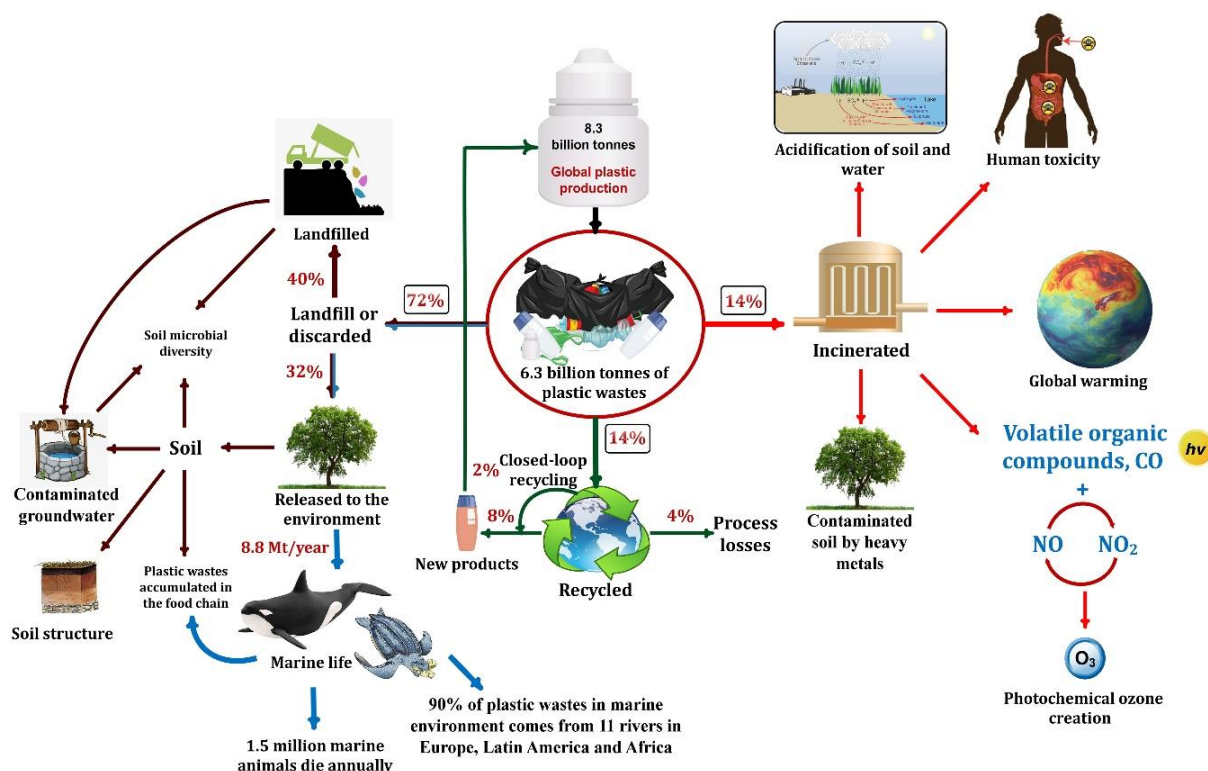


Figure 5. Schematic representation of the methods used for plastic waste disposal and the associated consequences. Reproduced from [53].

1.3.3.1. Landfills and accumulation in the environment

Landfills, widely used due to their operability and low cost, require a large area and have shown harmful effects of plastics on the activity, reproduction, and diversity of soil microbiota, leading to soil infertility, since the abiotic degradation of plastic can release highly toxic compounds, deteriorating soil quality and contaminating groundwater [54-58]. In addition to the damage of terrestrial ecosystems, when plastics enter the marine environment they can cause a series of problems, such as endocrine disruption and lower fecundity in animals, and reduced light transmission on the sea surface. As an example, plastics can decrease the photosynthetic efficiency of algae but also affect plankton growth, which contributes to global warming [7, 59]. Besides that, their ingestion, and the entanglement of organisms with plastics can lead to health problems, intestinal obstruction, parasites and injury to the stomach or intestine wall, and death. Moreover, invasive species can use ocean plastics to travel long distances [60, 61].

Beyond the environmental impact, plastic waste also presents socioeconomic impacts on tourism and fishing, maritime navigation, and affects human food security [62, 63]. In terms of tourism, less dirty beaches are preferred by people, so there will be a loss of tourism in places with more waste. Furthermore, cleaning up plastic waste from beaches has a considerable economic cost [64]. Marine

litter is also a hazard to all maritime traffic, from shipping to recreational users. Entanglement in fishing gear, ropes and lines is a major concern, but plastic bags can also block water intakes in cooling systems and can damage anchors and equipment, putting both the vessel and its crew at risk [64].

Another high impact consequence of plastic waste in the environment is the accumulation of nano and microplastics. The exposure of plastic waste to ultraviolet (UV) light and mechanical erosion caused by waves and winds or grinding on marine rocks and sediments, eventually causes breakages of larger plastics into smaller pieces of micro- (size between 0.1 μm and 5 mm) and nanoplastics (size $<0.1 \mu\text{m}$) [28, 65]. These small pieces of fragmented plastic can be translocated more deeply into the soil by plants and the activity of soil organisms, contaminating groundwater and deteriorating soil properties [66, 67]. This fragmentation of plastic waste increases and amplifies its dangerousness, as they can easily spread, enter trophic chains and gain the potential to affect various ecosystem functions [7, 57]. In animals, ingestion of microplastics can decrease energy storage, induce an inflammatory response, reduce reproduction, cause liver inflammation and oxidative stress, and decrease nutrition [68-71]. Microplastics also attract a wide variety of microbes to their surface, affecting the composition of aquatic microbial communities on a global scale [64].

Once exposed to mechanical UV weathering and wave action, microplastics can break down into nanoplastics. Today, the largest accumulation is believed to be in the nanoplastic range, but current analytical methods are unable to identify and isolate nanoparticles from environmental matrices [64]. Thus, these smaller pieces of plastic easily enter food chains, both terrestrial and aquatic, and eventually reach the human body, harming health by causing inflammation, genotoxicity, apoptosis, oxidative stress, and necrosis [59, 72-74]. Continued exposure leads to more serious problems such as tissue damage, fibrosis and carcinogenesis [53, 75]. Recently, Ragusa *et al.* (2021) reported, for the first time, the presence of microplastics in human placenta and the following year, another study detected their presence in human breast milk confirming that micro- and nanoplastics can enter the human body and potentially lead to serious negative consequences [76, 77].

1.3.3.2. Incineration

Incineration reduces the demand for landfills but, this process releases several pollutants into the atmosphere and is considered one of the main sources of air pollution today [54]. Among the main pollutants released by incineration are compounds considered toxic and potentially carcinogenic, harming human health. They include particulate matters, metals, nitrogen oxides, methane, carbon monoxide, furan, polyaromatic hydrocarbons, volatile organic compounds, and massive emissions of carbon dioxide

that can contribute to global warming and climate change [78, 79]. In addition, a huge amount of ash and slag containing dangerous and toxic compounds are produced, as well as emission of numerous chemicals with unknown toxicity and effects on human health.

The health and environmental impacts of incineration vary widely and depend, among other things, on incineration technology, temperature, operation, and emission control technology [25]. However, most incinerators still operate without air pollution control devices, leading to a large release of harmful gases into the atmosphere [79].

1.3.3.3. Recycling

Nowadays, recycling is the most consensual process to reduce the harmful impacts of plastic waste in the environment. Still, despite the momentum in plastic recycling, this process presents limitations. For instance, the adoption of plastic recycling processes for all the types of plastic produced can be cost-ineffective, and plastic waste is often contaminated with other materials requiring separation by polymer type [25, 54, 80]. Additionally, recycled plastic needs to compete with lower-cost primary plastics with superior technical specifications, which can represent an obstacle for its global adoption, despite the current recycling trend [54].

The recycling of plastic waste can be done mechanically or chemically. In mechanical recycling, plastic waste is mechanically shredded, cleaned and decontaminated and reassembled into granulate [81]. In chemical recycling, the pre-treated plastic waste is depolymerized, that is, separated into its monomers, which can then be repolymerized into the original plastic. Chemical recycling generates recycled material of higher value but is more expensive and has higher environmental impacts than mechanical recycling [25]. Until now, almost all recycling is done mechanically. In addition, it is important to note that recycling does not prevent final disposal, as the properties of most recycled materials are significantly compromised after a series of processing cycles. Moreover, some uses of plastic are inherently dissipative, meaning it cannot be collected after use and therefore ends up in the environment. Some examples are plastic sheets and films used in agriculture, gardening or landscaping, which begin to disintegrate during use and partially lost to the environment, and microbeads in cosmetics and personal care products [25].

1.4. Degradation by microorganisms

Considering the limitations associated with current plastic disposal methods and the global impacts of plastic on the environment, wildlife, and human health, there is an urgent need for new technologies targeting the remediation of plastic waste [3]. In recent years, the utilization of microorganisms or enzymes for the biodegradation of plastics have been the focus of several research projects, offering the possibility to develop new technologies for the biological treatment of plastic waste [54]. Some microbes are able to excrete enzymes that can depolymerize and convert plastic polymers into assimilable compounds that can be used as a source of carbon and energy for them [82-85]. However, despite all the potential of microorganisms for the degradation of macro- but also micro- and nanoplastics, it is important to consider that maintaining the ideal conditions for its performance are necessary for a faster and more efficient biodegradation. Once this is achieved, these microbial biodegradation systems/platforms will offer a starting point for overcoming plastic pollution, enabling a global circular economy for these recalcitrant pollutants [86-89].

1.4.1. Biodegradation process

The process of plastic degradation by microorganisms comprises the following main steps: i) colonization, ii) biodeterioration, iii) biofragmentation, iv) assimilation and v) mineralization (Figure 6) [53].

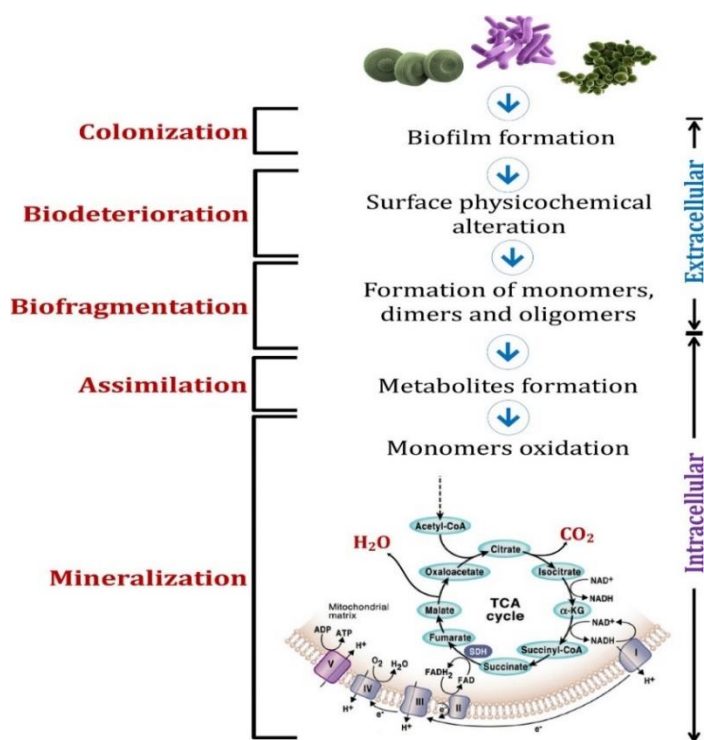


Figure 6. Process of degradation of plastic polymers by microorganisms. Reproduced from [53].

First, there is the colonization of the plastic surface by microbes through the production of various proteins and polysaccharides that facilitate adhesion [58]. In plastics with greater surface hydrophobicity, such as polyethylene, the formation of biofilms is necessary to increase the interaction of the polymeric surface with the microorganism, which improves degradation compared with planktonic cells [90, 91]. Once microbes attach to the plastic's surface, they continue to proliferate using the polymers as the sole carbon source [7]. These events lead to biodeterioration stage in which, microbial adhesion and activity on the plastic surface result in major damages to the polymer surface [92]. This phase includes not only the physical biodeterioration carried out by microorganisms (bacteria and fungi) that can invade the polymer, but also chemical biodeterioration through the secretion of extracellular enzymes, resulting in pores and cracks [93-96]. At this stage, prolonged exposure to external conditions (light, humidity, temperature and chemicals in the environment) increases biodegradation, as it reduces the high resistance characteristic of plastics [7]. Then, biofragmentation occurs, a depolymerization step characterized by the conversion of polymers into oligomers and monomers by the action of extracellular enzymes, and free radicals generated by microbes [9]. This process involves the reduction of the molecular weight of the polymer and oxidation of the lower weight molecules [83]. These reactions are necessary to facilitate the subsequent action of microbial enzyme systems that are more capable of attacking lower molecular weight compounds. The produced oligomers or monomers are then transported to the cytoplasm of the microorganism in a process called assimilation [7]. Once they are successfully transported into the cells, a series of enzymatic reactions occur, leading to their complete degradation into oxidized metabolites in a process called mineralization [97]. This phase can occur aerobically or anaerobically. Under anoxic conditions (such as landfills and composting), polymer deterioration yields methane, microbial biomass, water and carbon dioxide. On the other hand, under aerobic conditions, microorganisms assist in the degradation of complex materials into end products such as carbon dioxide, water and microbial biomass [98, 99].

1.4.2. Factors that influence biodegradation

Microbial biodegradation of plastic polymers, either in nature or under controlled conditions, is affected by a wide variety of factors that can be grouped into environmental factors and physicochemical properties of the polymer. These factors play an important role in facilitating subsequent microbial action, increasing surface area, hydrophilicity, as well as reducing molecular weight (Figure 7) [100].

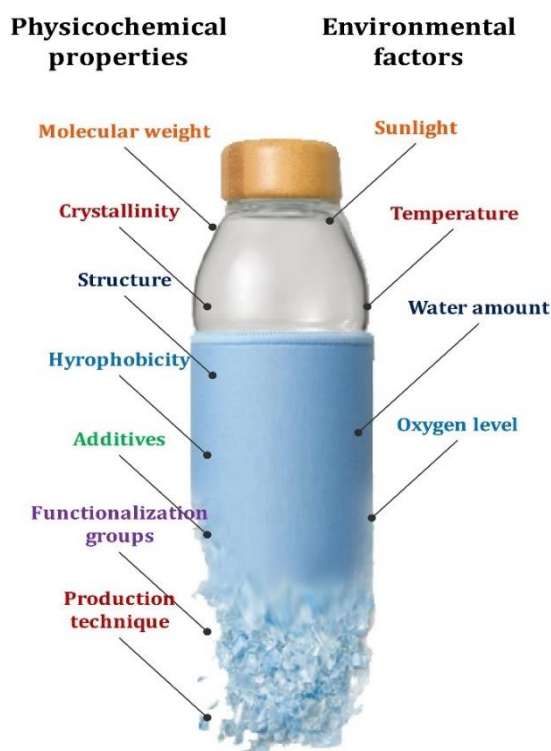


Figure 7. Factors that affect the ability of microorganisms to biodegrade plastics. Reproduced from [53].

Regarding the physicochemical properties of the polymer, a long carbon chain like PE and PP turns these polymers more resistant, while the incorporation of heteroatoms, as in PET and polyurethane (oxygen-containing polymers), makes them more susceptible to biodegradation [101-103]. Additionally, the higher the hydrophobicity or degree of crystallinity of the polymers, the lower the rate of biodegradation [104, 105].

However, the surrounding environment also plays a key role in the kinetics of biodegradation. Factors including UV light, heat, moisture and pH have been reported to enhance bond scission [106]. For example, environmental weathering factors such as exposure to UV radiation can lead to the formation of polar functional groups in plastics, increasing hydrophilicity and greatly facilitating the ability of microorganisms to biodeteriorate them [107, 108]. Thus, the significantly different characteristics of each environment alter the rate of microbial action. Indeed, Chamas *et al.* (2020) reported that with all other factors held constant, degradation in the marine environment is greater than on land [51].

1.4.3. Microorganisms and enzymes involved in plastic biodegradation

Due to the alarming increase and the environmental impacts of plastic waste accumulation, the potential use of microorganisms as platforms for the bioreremediation/biorecycling of plastics have been the subject of great research. Over the past years, several microorganisms, namely bacteria and fungi,

have been identified as potential biodegraders of widely adopted plastics such as PE, PP, PS and PET (Table 2). Similarly, research efforts and advances allowed the identification of the main enzymes involved in the biodegradation process (Table 3), which are grouped into three main classes [7, 28, 30, 53, 54, 109-119]:

- Hydrolases that catalyze bond cleavages and comprise lipases, esterases, cutinases, proteases, PETases, MHETase.
- Oxidoreductases which are a broad group of enzymes that catalyze the transfer of electrons from one molecule to another. These include oxidases, reductases, alkane hydroxylases, hydroquinone peroxidase, monooxygenase, dehydrogenase.
- Isomerases that catalyze reactions involving a structural rearrangement of a molecule, of which styrene oxide isomerase is included.

Table 2. Microorganisms with potential for the biodegradation of plastics [7, 28, 30, 53, 54, 114-119].

Plastic type	Microorganism	Kingdom
PE	<i>Aspergillus clavatus</i>	Fungi
	<i>Aspergillus flavus</i>	Fungi
	<i>Aspergillus fumigatus</i>	Fungi
	<i>Aspergillus japonicus</i>	Fungi
	<i>Aspergillus niger</i>	Fungi
	<i>Aspergillus terreus</i>	Fungi
	<i>Penicillium simplicissimum</i>	Fungi
	<i>Bacillus cereus</i>	Bacteria
	<i>Bacillus pumilus</i>	Bacteria
	<i>Bacillus subtilis</i>	Bacteria
	<i>Brevibacillus</i>	Bacteria
	<i>Comamonas acidovorans</i>	Bacteria
	<i>Pseudomonas</i> spp.	Bacteria
	<i>Pseudomonas chlororaphis</i>	Bacteria
	<i>Pseudomonas putida</i>	Bacteria
	<i>Streptomyces</i> spp.	Bacteria
	<i>Rhodococcus ruber</i>	Bacteria
	<i>Rhodococcus rhodochrous</i>	Bacteria
PP	<i>Aspergillus niger</i>	Fungi
	<i>Phanerochaete chrysosporium</i>	Fungi
	<i>Rhizopus oryzae</i>	Fungi
	<i>Bacillus</i>	Bacteria
	<i>Brevibacillus</i>	Bacteria
	<i>Rhodococcus</i>	Bacteria

PS	<i>Azotobacter beijerinckii</i>	Bacteria
	<i>Bacillus</i> sp.	Bacteria
	<i>Bacillus subtilis</i>	Bacteria
	<i>Pseudomonas</i> sp.	Bacteria
	<i>Pseudomonas aeruginosa</i>	Bacteria
	<i>Pseudomonas putida</i>	Bacteria
	<i>Rhodococcus ruber</i>	Bacteria
	<i>Rhodococcus zopfii</i>	Bacteria
PET	<i>Aspergillus</i> sp.	Fungi
	<i>Fusarium solani</i>	Fungi
	<i>Penicillium citrinum</i>	Fungi
	<i>Thermomyces insolens</i>	Fungi
	<i>Saccharomonospora viridis</i>	Fungi
	<i>Ideonella sakaiensis</i>	Bacteria
	<i>Pseudomonas mendocina</i>	Bacteria
	<i>Thermobifida fusca</i>	Bacteria
	<i>Streptomyces scabies</i>	Bacteria

Table 3. Enzymes required for biodegradation [7, 28, 30, 53, 54, 114-119].

Plastic	Enzymes
PE	Alkane hydroxylase, cutinase, lipase, esterase, protease, oxidase, laccase
PS	Hydroquinone peroxidase, esterase, monooxygenase, dehydrogenase, styrene oxide isomerase
PP	Lipase
PET	PETase, MHETase, lipase, cutinase, esterase

1.4.4. Polyethylene biodegradation mechanism

Among the various plastics, PE is of special interest since it is the most widely used, with more than 100 Mt per year, also generating a higher amount of plastic waste compared to other polymers. In addition, the identification of microbial species capable of effectively biodegrading PE is still relatively unexplored [27, 120]. However, some studies have identified enzymes capable of biodegrading PE, namely laccase from *Rhodococcus ruber* strain C208 which was reported to degrade UV-treated PE films,

and a recombinant alkane hydroxylase from *Pseudomonas* sp. E4 that was able to convert 20 % of LDPE to CO₂ in 80 days [121, 122]. Additionally, proteases have been shown to be responsible for polyethylene degradation in *Brevibacillus* spp. and *Bacillus* spp. [114]. Tribedi and co-workers reported higher activity of esterases and lipases in *Pseudomonas* sp. AKS2 in biofilm on LDPE surface when compared to planktonic cells, and another study revealed that cutinase derived from *Thermobifida fusca* WSH03-11 can reduce the average molecular weight of LDPE by 47.4 % in 7 days [91, 123].

Nevertheless, it is suggested that abiotic and biotic factors play a fundamental role in the biodegradation of PE in the environment. This is justified by the fact that PE is a plastic with low susceptibility to microbial attack, which poses a challenge to biodegradation. PE consists of a long carbon chain and has a high surface hydrophobicity, making the biodegradation process relatively slow [120]. However, this can be accelerated by exposure of the plastic to abiotic factors such as UV radiation, temperature, etc. [124]. Although the biodegradation mechanism of PE is not yet fully elucidated, figure 8 depicts the expected PE biodegradation mechanism [120, 124].

Initially, PE biodegradation is triggered by photo-oxidation. UV radiation leads to the formation of radicals which, followed by oxygen uptake, give rise to molecules with carbonyl groups. Subsequent oxidation reduces the number of carbonyl groups and generates carboxylic acids. Then, microbial extracellular enzymes further induce polymer fragmentation. After the initial cleavage of the polymer into oligomers of 10-50 carbon atoms, these are transported into the cell for further metabolism [120].

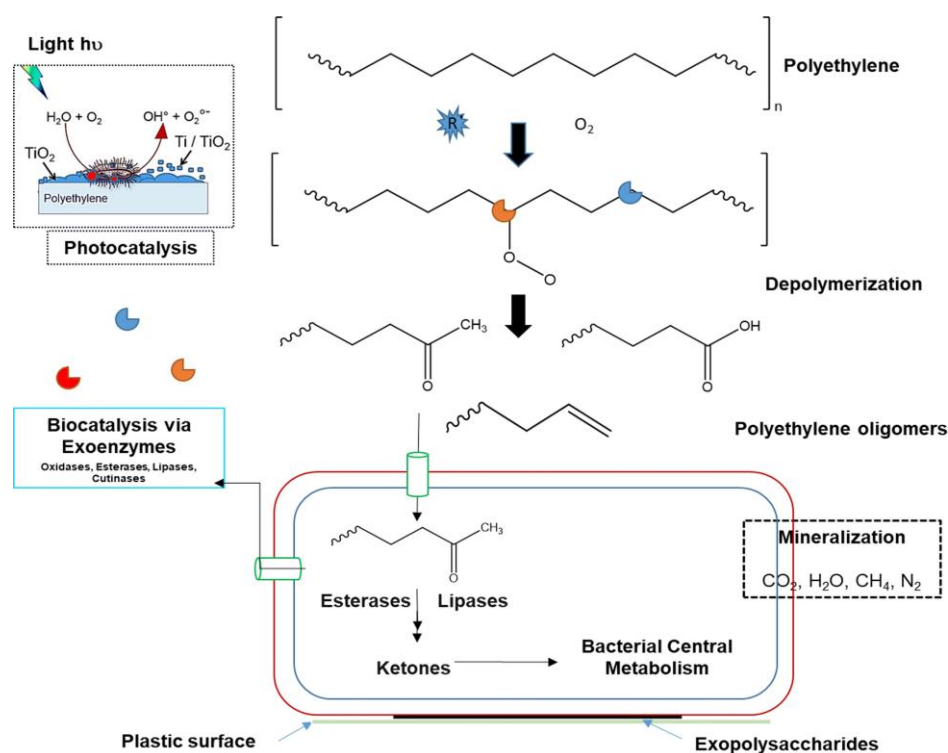


Figure 8. Mechanism of abiotic and biotic degradation of polyethylene. Adapted from [120].

2. Objectives

The present work was carried out at the Centre of Molecular and Environmental Biology (CBMA, University of Minho) and the Institute of Science and Innovation for Bio-Sustainability (IB-S, University of Minho), and developed in the scope of the River2Ocean project and ongoing collaborations with an industrial partner.

The overall main objective of this work was to establish and implement methodologies for the screening of plastic-degrading microorganisms (yeasts and bacteria) and evaluate their biodegradation performance. Due to its very low biodegradability, high representativeness in the packaging industry and impact as a pollutant, PE was selected as the base plastic material to carry out the experimental work. The CBMA holds a large collection of microorganisms from environmental samples that can be screened for their capability to biodegrade plastics. This work thus represents the basis to explore the CBMA collection to set up a platform of microbial biorecycling factories. To achieve the proposed goal, this work proposes the following main objectives:

- Definition and establishment of adequate screening methodologies;
- Evaluation of the biodegradation performance of PE films over short-term microbial treatment.

3. Materials and Methods

3.1. Microbial strains

Four different microorganisms were studied regarding their potential for plastic biodegradation: two species of bacteria (*Alcanivorax borkumensis* SK2 and *Pseudomonas aeruginosa* PAO1), and two yeasts (*Debaryomyces hansenii* PYCC 2968 and *Yarrowia lipolytica* PYCC 4811).

3.2. Culture media

Screening and growth assays were made in Marine Broth 2216 medium (MB; Millipore, Germany), MB supplemented with 1 % (w/v) of sodium pyruvate (MBP), Davis Minimal Broth without Dextrose (Davis; Sigma-Aldrich, Germany) and Davis supplemented with 0.0025 % (w/v) methylene blue (Davis + blue). Sodium pyruvate (≥ 99 %) was purchased from ReagentPlus® (Sigma-Aldrich, Germany) and methylene blue from Merck (Germany). For the preparation of solid media, 2 % agar (w/v) (LabChem, Portugal) was added to each medium.

3.3. Pre-inoculum and microbial maintenance

Prior to the assays described in the following sections, a pre-inoculum was prepared, in which both bacteria and yeast were inoculated in 50 mL of MBP in 250 mL Erlenmeyer flask (volumetric ratio of 1:5) and incubated at 30 °C, 200 rpm for 24 h (*P. aeruginosa* and *Y. lipolytica*) or 48 h (*A. borkumensis* and *D. hansenii*). Similarly, maintenance of the solid medium cultures was performed in MBP medium supplemented with 2 % agar. After this, they were stored at 4 °C and used within a maximum period of 10 days.

3.4. Assessment of microbial cell growth

The microorganisms referred to in section 3.1 were evaluated for their ability to grow in 3 different liquid media: MB, MBP and Davis. For this, the concentration of the microorganism in the pre-inoculum was normalized to a starting OD of 0.05, followed by incubation at 30 °C and 200 rpm for 48 h. Cell growth was followed by assessing the optical density of the culture at 600 nm for bacteria and 640 nm for yeast, by means of a spectrophotometer (Thermo Spectronic 4001/4). The respective sterile culture media was used as the blank.

3.5. Screening assays and selection of the most promising microorganisms for PE biodegradation

To assess the potential of the microorganisms to act as plastic biodegraders, microbial growth was evaluated using media supplemented with different substrates. For this, the culture media (MBP; Davis; Davis + blue) was supplemented with different substrates at a concentration of 3 g/L (Table 4).

Table 4. Media used for the screening assays.

Base Media	Substrate
Marine Broth supplemented with 1 % sodium pyruvate (MBP)	PE powder (3 g/L)
	Poly(vinyl alcohol) (3 g/L)
	Poly(ethylene glycol) (3 g/L)
	Coconut oil (3 g/L)
	Polycaprolactone (3 g/L)
	Polysulfone (3 g/L)
Davis Minimal Broth without dextrose (Davis)	PE powder (3 g/L)
	Poly(vinyl alcohol) (3 g/L)
	Poly(ethylene glycol) (3 g/L)
	Coconut oil (3 g/L)
	Polycaprolactone (3 g/L)
	Polysulfone (3 g/L)
Davis Minimal Broth without dextrose supplemented with 0.0025 % methylene blue (Davis + blue)	PE powder (3 g/L)
	Poly(vinyl alcohol) (3 g/L)
	Poly(ethylene glycol) (3 g/L)
	Coconut oil (3 g/L)
	Polycaprolactone (3 g/L)
	Polysulfone (3 g/L)

3.5.1. Formulation of media supplemented with polyethylene

Initial experiments aiming at creating PE emulsions were carried out to evaluate the solubility of the plastic in different solvents namely, benzene, acetone and toluene (data not shown). As PE demonstrated to be stable in all the tested solvents, screening assays were performed with a suspension

of PE powder with 500 micron (low density polyethylene, $\rho = 0.92 \text{ g/cm}^3$, Alfa Aesar, Germany). The powder was washed with 70 % ethanol for 30 min to remove impurities, and further filtered overnight. The next day, the dried powder was sterilized with UV radiation in the flow chamber for 20 min. After, 3 g/L of PE were added to autoclaved base media (Table 4), stirred for 10 minutes at 70 °C and transferred to a preheated ultrasonic bath (LBX instruments ULTR-6L5-001) for 5 min at 70 °C. Finally, the media was stirred and poured into sterile Petri dishes.

3.5.2. Formulation of media supplemented with poly(vinyl alcohol) and poly(ethylene glycol)

Since poly(vinyl alcohol) (PVA) and poly(ethylene glycol) (PEG) are water soluble, media were prepared by adding the substrate directly to the culture broths. PVA (98 % hydrolyzed, Sigma-Aldrich, Germany) with an average molecular weight (MW) of 13,000 - 23,000, and PEG (Sigma-Aldrich, Germany) with an average MW of 3,350 were added to the base media at a concentration of 3 g/L (Table 4) and sterilized by autoclaving.

3.5.3. Formulation of media supplemented with coconut oil, polycaprolactone and polysulfone

The preparation of media supplemented with coconut oil was based on the procedure from Molitor *et al.* [125]. Coconut oil was heated by incubation at 37 °C for 30 minutes, and used to prepare a 50 % (v/v) coconut oil (Acros Organics, Belgium) emulsion in preheated water at 60 °C containing 50 g/L of gum arabic (M&B, UK) and 0.35 g/L rhodamine B (Merck, Germany). The solution was then homogenized at 8,000 rpm using a vortex. The emulsion was added to the base culture media to a substrate concentration of 3 g/L (Table 4), and shaken vigorously (i.e. 13,000 rpm) for 5-10 minutes at room temperature, using a stir plate. Due to the presence of rhodamine B, it is possible to track esterase/lipase activity.

The media supplemented with polycaprolactone (PCL, MW $\approx$ 80,000; Thermo Scientific, UK) and Polysulfone (PSF; Acros Organics, Belgium) were prepared according to the protocol described by Park *et al.* [126]. Briefly, 3 g of the polymer were resuspended in 40 mL of dichloromethane (Merck, Germany) for 1 hour at 700 rpm. Surfactant and water (100 mL) were added to the solution and sonicated (BioBlock Scientific Vibra-Cell™ 75043) for 20 minutes with 9 seconds pulse on, 9 seconds pulse off and amplitude 40 %. The solution was then heated at 80 °C for 2 hours and finally added to the base medium (Table

4). For these, an optimization was firstly carried out regarding the best concentration (0.35 % or 0.01 %) and surfactant to use, sarkosyl (Thermo Scientific, UK) or sodium dodecyl sulfate (SDS; PanReac AppliChem, Spain). To evaluate the interference of the surfactant in microbial cell growth, solid media without substrate (PCL or PSF) was used as control. Table 5 describes the different conditions tested for the PCL and PSF emulsions.

Table 5. Conditions tested for the optimization of PCL and PSF emulsions in solid media.

	MBP + 2 % agar	Davis + 2 % agar	Davis + blue + 2 % agar
Substrates	PCL + 0.35 % SDS	PCL + 0.35 % SDS	PCL + 0.35 % SDS
	PCL + 0.35 % Sarkosyl	PCL + 0.35 % Sarkosyl	PCL + 0.35 % Sarkosyl
	PCL + 0.01 % SDS	PCL + 0.01 % SDS	PCL + 0.01 % SDS
	PSF + 0.35 % Sarkosyl	PSF + 0.35 % Sarkosyl	PSF + 0.35 % Sarkosyl
	0.35 % SDS	0.35 % SDS	0.35 % SDS
	0.01 % SDS	0.01 % SDS	0.01 % SDS
	0.35 % Sarkosyl	0.35 % Sarkosyl	0.35 % Sarkosyl
	0.01 % Sarkosyl	0.01 % Sarkosyl	0.01 % Sarkosyl

3.5.4. Visualization of clear halos in solid media

Due to the lack of opacity, the media containing PVA and PEG as substrates did not allow the visualization of clear halos around the colonies. To solve this, two different ways for the visualization of clear halos were tested: through 0.20 % Coomassie blue staining solution (Fluka, USA) and by adding methylene blue into the medium at a concentration of 0.0025 % (medium Davis + blue, as mentioned above in section 3.2 and in Table 4). Coomassie blue staining was performed in solid media plates after 7 days of cell growth, and consisted of carefully adding the Coomassie blue solution to the Petri dish to cover the entire plate and leaving it under agitation for 20 min. After removing the solution, a destaining solution (7.5 % acetic acid and 20 % ethanol) was added, followed by incubation for 1 hour. Finally, the destaining step is repeated until it sufficiently bleached.

3.5.5. Inoculation of microorganisms on solid media and visualization of degradation halos

Three different inoculation methods were used to evaluate the best method for visualizing the formation of a clear halo: non-perforation, perforation and by layering a drop of microbial cell culture (Figure 9). For the non-perforation, a sterile toothpick was used to remove the biomass of a single microbial colony and transferred to the surface of the solid medium to be inoculated (Figure 9A). For the perforation method, a toothpick was used to remove the biomass of a single microbial colony and used to perforate the solid culture medium up to approximately 2-thirds (Figure 9B). For the drop method (Figure 9C), a pre-inoculum was made and its OD was adjusted to 0.6. Then, using a micropipette, a 3 μ L drop was placed onto the surface of the plate.

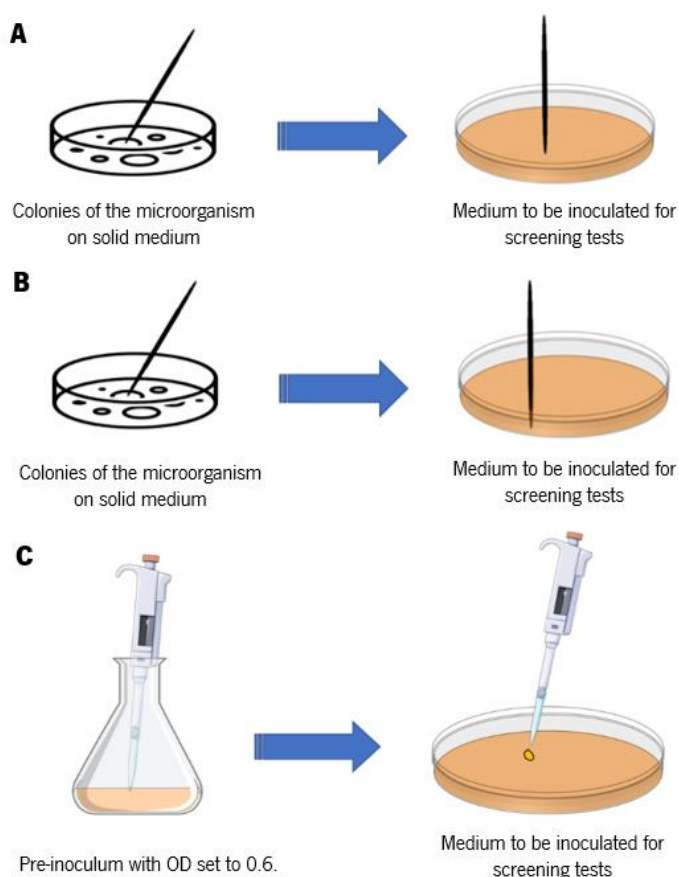


Figure 9. Schematic representation of the three methods used to inoculate the microorganisms. A – non-perforation, B – perforation and, C – drop.

After inoculation, the Petri dishes were incubated at 30 °C and digitally recorded after 1, 3 and 7 days. For the plates containing the coconut oil, the activity of the lipase enzymes was evaluated based on the fluorescence of rhodamine B under UV light (254 nm and 365 nm) on a 2020E UV/White

transilluminator (Stratagene). The ImageJ software ([127]) was used to obtain a semi-quantitative assessment of the clear halos (degradation halos) and microbial growth. The digital images of each plate were analyzed and measurements of the clear halos and size of microbial colonies were enumerated as shown in figure 10. Each value is the average of four measurements. In order to make the results more intuitive, the values obtained for the controls (the corresponding media without the supplements) were subtracted. Additionally, a color scale was applied where green represents the values at which the highest microbial growth/clear halos were obtained, and red represents the lowest microbial growth/clear halos. The values obtained were analysed using Microsoft Excel (version 2209 to Microsoft 365 MSO).

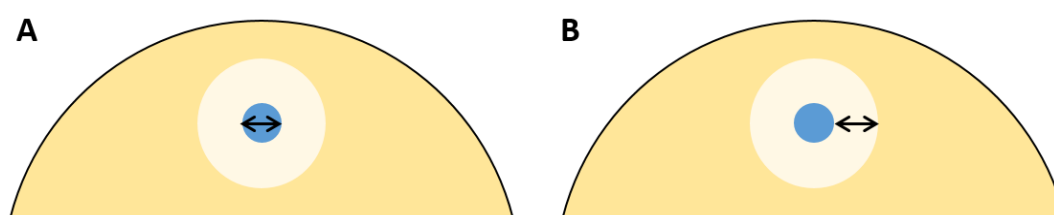


Figure 10. Schematic representation of the measurement on petri dishes for A) microbial colony size and B) clear halos.

3.6. Biodegradation of PE films

Following the screening assays, the most promising microorganisms (*P. aeruginosa* and *Y. lipolytica*) were used to evaluate their ability to degrade or induce fracture effects on PE film samples. The biodegradation assays were performed in triplicate, in liquid culture media (MBP and Davis) of *P. aeruginosa* and *Y. lipolytica* incubated with a PE film sample over a period of 90 days. Samples were collected after 15, 30, 60 and 90 days of incubation, and characterized for microbial growth, biofilm formation/quantification, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis of the culture supernatant, attenuated total reflection-Fourier transform infrared spectroscopy (ATR-FTIR) of the PE film, film weight loss and film inspection by scanning electron microscopy (SEM).

3.6.1. PE samples and incubation with microbial cell cultures

PE films were obtained from polyethylene Pasteur transfer pipettes (Labbox, Spain), which contain a low amount of additives. Pieces of 2.5 cm x 2 cm (thickness $\approx$ 200 μ m) were cut from the upper part of the Pasteur pipette. For sterilization, the PE samples were immersed in 70 % ethanol for 30 minutes and allowed to dry in the flow chamber. After, they were weighed on a four-digit sensitive

digital balance (Quintix224-1S), and sterilized under UV light for 20 minutes on each side. The sterile PE films samples were then transferred to 150 mL Erlenmeyer flasks containing 30 mL of culture medium (MBP and Davis) and inoculated with *P. aeruginosa* or *Y. lipolytica* for an initial OD (600 nm for *P. aeruginosa* and 640 nm for *Y. lipolytica*) of 0.05. Cells were incubated at 30 °C with an agitation of 120 rpm for 15, 30, 60 and 90 days. PE films immersed in sterile medium were used as control for abiotic degradation whereas, media inoculated with microorganisms but without the PE films were used as controls for cell growth. The controls were subjected to the same treatments as the other samples. All experiments were performed in triplicate.

3.6.2. Analysis of cell growth and biofilm formation

Growth of cells in the planktonic stage were monitored by OD measurements, 600 nm for *P. aeruginosa* and 640 nm for *Y. lipolytica*, (Thermo Spectronic 4001/4). The results obtained were analyzed using the Graphpad Prism software (version 9.4.1).

For analysis of biofilm formation, the PE films were collected in 6-well plates (VWR, Portugal) and washed with 1X phosphate buffer saline (PBS) (NaCl, 8 g/L; Na₂HPO₄, 1.2 g/L; KH₂PO₄, 0.24 g/L; KCl, 0.2 g/L) for 5 min. Then, the biofilms were fixed with 100 % methanol (Fisher Scientific, UK) for 5 min, washed with PBS for 5 min and incubated with 0.2 % crystal violet in PBS solution for 5 min. The excess of crystal violet was removed, followed by two additional washing steps (5 min each) with PBS. Finally, the crystal violet bound to the biofilm was dissolved in 7.5 mL of 33 % acetic acid (Panreac, Spain). Biofilm formation was then recorded by measuring the absorbance at 570 nm using a spectrophotometer (Thermo Spectronic 4001/4), with the 33 % acetic acid solution as a blank control. The results were analyzed using the Graphpad Prism software (version 9.4.1).

3.6.3. Determination of film weight loss

For determination of weight loss, the PE films were recovered in 6-well plates (VWR, Portugal) from the liquid media and washed overnight at 37 °C with a 2 % (v/v) SDS solution to remove adhered microbial cells. The next day, films were extensively washed with deionized water, followed by 70 % ethanol and, finally, deionized water again for the maximum possible removal of cells and debris. Each of the washing steps was performed for about 2 min for each film. The washed films were allowed to dry for 24 h at room temperature and protected from sunlight. The films were then weighted by a four-digit

sensitive digital balance (Quintix224-1S). The results obtained were analyzed using the Graphpad Prism software (version 9.4.1).

3.6.4. SDS-PAGE analysis

During the process of biodegradation, microorganisms produce an assortment of extracellular enzymes (see sections 1.4.1 and 1.4.3) to turn the plastic matrix amenable to assimilation. Accordingly, the supernatant of the microbial cell cultures at the end of the experiment (90 d) was evaluated by SDS-PAGE using a 10 % polyacrylamide gel (Appendix 1) and compared with cultures without PE film. Prior to SDS-PAGE, the cell cultures were centrifuged at 4 °C for 10 min at 7,585 xg. The supernatant was transferred to new tubes and the pelleted cells were discarded. With exception for *P. aeruginosa* in MBP, the supernatants were then subjected to ammonium sulfate precipitation at 90 % saturation to concentrate proteins, while maintained on ice under constant stirring for 30 min. The precipitated solution was centrifuged at 20,000 xg for 30 min at 4 °C, and the supernatant discarded. The pellet was resuspended in ice-cold water (100 µL for samples grown in Davis; 200 µL for samples grown in MBP) and used for SDS-PAGE analysis. For electrophoresis, 24 µL of the sample was added to 6 µL of loading buffer (Appendix 1). From this solution, 25 µL were loaded into the gel. The gels were run at 15 mA per gel, stained with BlueSafe (NZYTech, Portugal) and digitally recorded. After Coomassie staining, the gels were destained with destaining solution and further stained with silver nitrate according to the method described in Appendix 2.

3.6.5. Attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectroscopy analysis

To inspect chemical changes in the films incubated with the microorganisms, the PE samples were analysed by ATR-FTIR. Prior to FTIR analysis, the films were subjected to a washing step with 2 % (v/v) SDS solution by following the same procedure described in section 3.6.3. The washed PE films were analyzed by ATR-FTIR using a Perkin Elmer Spectrum Two™ IR spectrometer coupled with an UATR accessory (Perkin Elmer) with diamond crystal (single reflection), in the range of 4000-400 cm⁻¹, with a resolution of 4 cm⁻¹, accumulating 64 scans. Graphical representation of the FTIR spectra was performed with OriginPro 2022b (Originlab).

3.6.6. Morphological evaluation using Scanning Electron Microscopy (SEM)

The PE films subjected to 90 d of incubation with the microorganisms, were submitted to SEM analysis. Before visualization by SEM, the PE films were washed with 2 % SDS (v/v) solution by following the procedure indicated in section 3.6.3. This washing step was necessary for a better visualization of surface erosion. The samples were then coated with a thin gold layer using a sputter coater (Fision Instruments, Polaron Sputter Coater SC502) and visualized by SEM (Leica Cambridge S360) with an accelerating voltage of 10 kV.

4. Results and Discussion

4.1. Selection of microorganisms and culture conditions

As mentioned in the Introduction, there are several studies reporting the capability of bacteria and yeasts to biodegrade plastics. In this work, the bacteria *A. borkumensis* and *P. aeruginosa*, and the yeasts *D. hansenii* and *Y. lipolytica*, were selected as potential biodegraders, on the basis of literature review and the interest of the research group to expand their biotechnological potential. *A. borkumensis* occurs naturally in marine environments, being able to feed on hydrocarbons and alkanes, and therefore thrives in open and coastal waters polluted with oil, reaching 80-90 % of these microbial communities. For this reason, it has been pointed out as a potential PE degrader [128, 129]. *P. aeruginosa* is a Gram-negative bacterium capable of forming biofilms and has been highlighted by the scientific community as a potential degrader of various types of plastics, including PE, PP, and PS [130, 131]. The yeast *D. hansenii* is well-known for its applications in the food industry, but its biotechnological potential remains to be fully explored. Although poorly studied for its ability to degrade synthetic plastics polymers, it has already been validated as a degrader for PHB, a biodegradable polymer and one of the most promising alternatives to petroleum-based plastics [132-134]. Also, there is an interest of the research group to further explore the potential of *D. hansenii*. Finally, *Y. lipolytica* is notable for its ability to degrade lipids and proteins and growth on *n*-paraffins as the sole carbon source, and has been associated as a potential PP plastic degrader [135].

After selecting the microorganisms to be studied, it is essential to select adequate growth media for bacteria and yeast, that will allow to set up the experimental conditions for the subsequent screening and biodegradation assays. For this purpose, we tested a rich medium that allows the microorganisms to thrive and a minimal medium, in which the growth of the microorganism will depend largely on its ability to utilize the added substrate. The growth of the selected microorganisms was evaluated in minimal medium (Davis) (Figure 11).

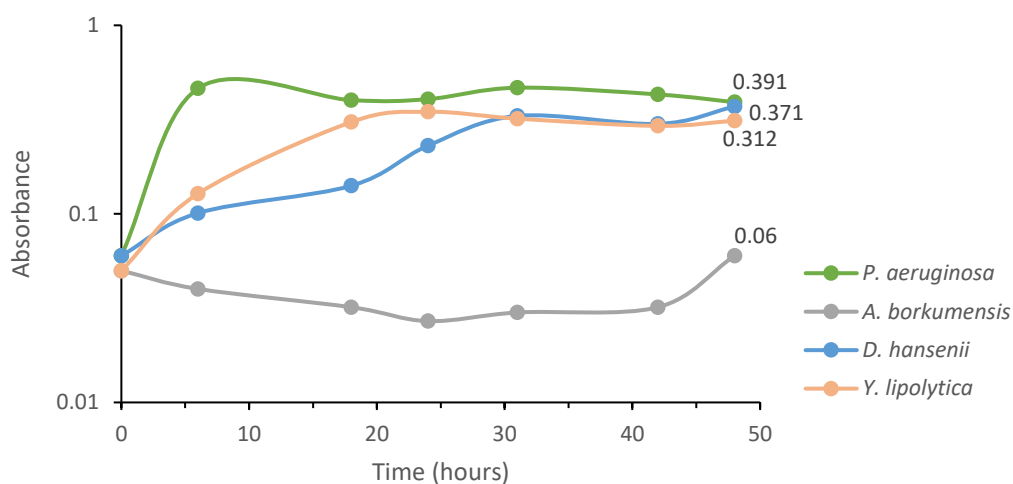
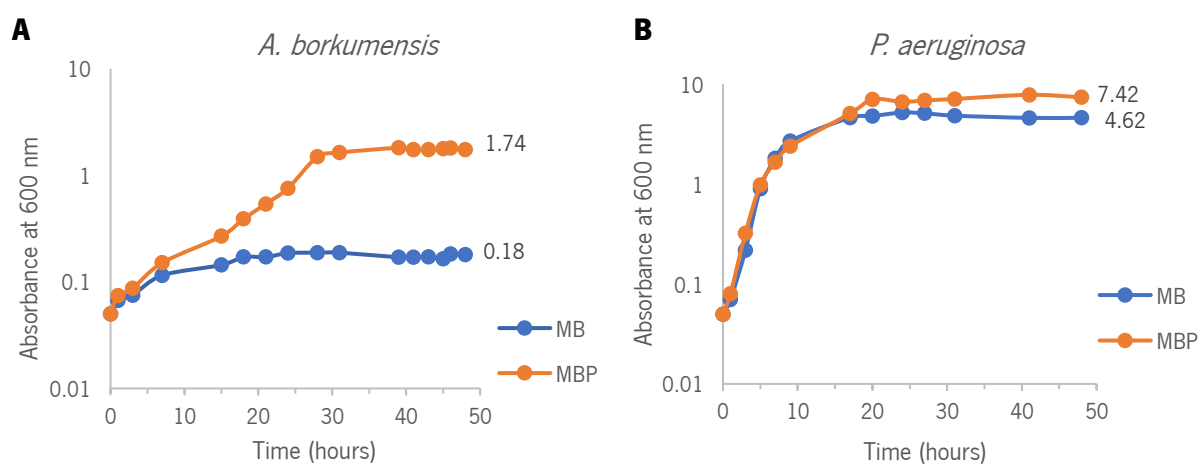


Figure 11. Growth of *P. aeruginosa* (green), *A. borkumensis* (gray), *D. hansenii* (blue) and *Y. lipolytica* (orange) in Davis media over 48 hours. Absorbance values are expressed as $\log_{10}$ and were obtained by measuring the OD at 600 nm and 640 nm for bacteria and yeasts, respectively.

As expected, all strains grew poorly in this medium reaching a final OD between 0.3 and 0.4, and *A. borkumensis* was unable to grow, reaching a final OD of 0.06. Therefore, this medium was selected as the minimum medium to be used in this study. Regarding the rich medium, the MB medium was selected because previous studies conducted by the research group revealed the difficulty of *A. borkumensis* to grow in Lysogeny Broth and Nutrient Broth (data not shown). It is described in the literature, that either hydrocarbons or pyruvate, are necessary in the culture medium for the growth and reproduction of this bacterium [136]. So 1 % pyruvate was added to the MB medium and growth was monitored for 48 hours (figure 12).



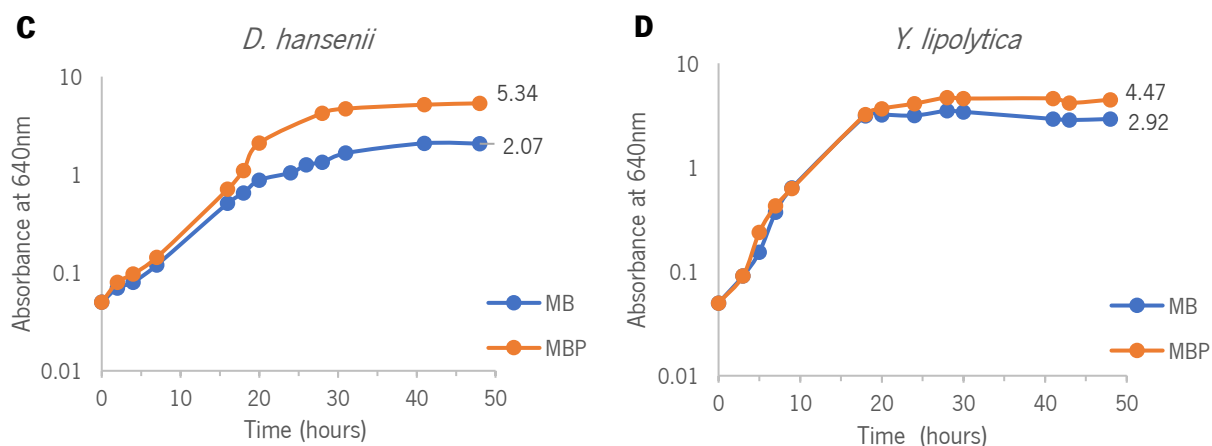


Figure 12. Growth of *A. borkumensis* (A), *P. aeruginosa* (B), *D. hansenii* (C), and *Y. lipolytica* (D) in MB (blue) and MBP (orange) media over 48 hours. Absorbance values are expressed as Log_{10} and were obtained by measuring the OD at 600 nm and 640 nm for bacteria and yeasts, respectively.

All microorganisms showed an increased growth in MBP medium, when compared to MB. This variation is of special interest for *A. borkumensis*, where a large difference in growth was reached after 48 h (from 0.183 in MB to 1.738 in MBP). For this reason, MBP was chosen as rich medium since all the selected microorganisms revealed the highest growth.

Incubations of the microorganisms were carried out at 30 °C, as this temperature demonstrated to be suitable for the growth of the studied microorganisms. *D. hansenii* has an optimal growth temperature of 25 °C, while for *Alcanivorax* spp. this is between 25 and 30 °C [137, 138]. Furthermore, Sekova *et al.* tested the growth of *Y. lipolytica* at various temperatures and showed that the maximum growth of this yeast occurred at 29 °C [139]. On the other hand, *P. aeruginosa* has the highest growth at 37 °C, although it can grow well at 30 °C [140].

4.2. Screening assays and assessment of enzymatic-related activity

As mentioned in the early sections, synthetic plastics can be degraded through the action of microorganisms. For this process to occur, enzymes play a paramount role in polymer depolymerization leading to the formation of low molecular weight molecules (i.e. oligomers and monomers) that can be further assimilated by the cell. To evaluate the microorganism's ability to secrete extracellular enzymes associated with the biodegradation process, a screening using media supplemented with selected substrates was performed to assess microbial growth. In this assay, the measurement of the colony size

and the presence of clear halos around the colonies indicates that the microorganism is able to utilize that substrate for growth, i.e. has the enzymes required for its biodegradation. This method is widely used and was chosen because it provides a quick and easy way to assess the potential of a microorganism [141-143]. However, it is very important that the supplement is equally distributed in the medium so it is available for the inoculated microbe.

Initial screening assays were considered using media supplemented with PE emulsions due to its insolubility in water. But despite the use of different solvents (benzene, acetone and toluene), the synthetic polymer demonstrated to be insoluble, thus hindering the possibility of using it for the microbial growth screening assays and presence of clear halos. Similarly, the supplementation of culture media with PE powder revealed to be unreliable due to the low density of PE ($\rho = 0.92 \text{ g/cm}^3$) which resulted in an uneven distribution of the plastic particles in the media (Appendix 3, Figure S1). To overcome the constraints associated with the use of PE, the screening assays were extended to other compounds, indicative of the ability of the microorganisms to produce extracellular enzymes. Several substrates were tested, according to the type of chemical bonds present in the plastic polymers. PVA is a vinyl polymer joined only by carbon-carbon bonds, the same as typical plastics such as PE, PP and PS [144]. It is a biodegradable polymer and has been indicated as a replacement for PE in screening tests [141]. Enzymes such oxidases [145], laccases [146] and esterases [147] are involved in its biodegradation. PEG is an ethylene oxide polymer and has been reported as a potential substitute for PE in biodegradation screenings [148-150]. Coconut oil allows the evaluation of the presence of lipases and esterases when the microorganisms are able to degrade this substrate [151]. PCL is a biodegradable polyester and the enzymes involved in its biodegradation process are cutinases, lipases, esterases, and proteases [109, 152-156]. Despite the limited body of work associated with the use of PSF for biodegradation screening assays, this synthetic polymer was also included in the screening assays. PSF is a highly stable, chemical-resistant and non-biodegradable thermoplastic, widely used for technical applications and membrane production, with notable impact at the environmental level [157-159].

Table 6 presents the enzymes that are required for the biodegradation of the aforementioned substrates to occur, as well as those identified so far for the degradation of PE. To note that, to the best of our knowledge, no enzymes have been reported as involved in the biodegradation PSF.

Table 6. List of enzymes involved in the biodegradation process of PVA (poly(vinyl alcohol)), PEG (poly(ethylene glycol)), PCL (polycaprolactone), PSF (polysulfone) and PE (polyethylene).

Substract	Enzymes						
	alkane hydroxylase	cutinase	lipase	esterase	protease	oxidase	laccase
PVA	—	—	—	X	—	X	X
PEG	—	—	—	—	—	—	—
Coconut oil	—	—	X	X	—	—	—
PCL	—	X	X	X	X	—	—
PSF	—	—	—	—	—	—	—
PE	X	X	X	X	X	X	X

4.2.1. Evaluation of microbial growth in solid media

The screening assays were conducted over a period of 7 days and evaluated for microbial growth by assessing the size of colonies in the different media at day 1 (Appendix 4), day 3 (Appendix 5) and day 7 (Table 7-11). Determination of growth values was performed by subtracting the size of the corresponding control colonies (microorganisms grown in media without substrate) to the size of the colonies grown in the media supplemented with the substrates. This assessment provides a reasonable indication on the ability of the microbes to grow using the selected substrates. Accordingly, a positive value indicates that the microorganism showed increased growth than the control and is able to thrive on the substrates at some extent, while negative values represent the opposite.

To facilitate data interpretation of this semi-quantitative analysis, a color scale was applied for a more intuitive visualization and analysis of the results (Tables 7-11). In this scale, the intensity of the green color represents values in which a higher microbial growth was observed whereas, the red color represents the opposite.

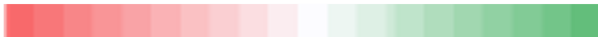
4.2.1.1. Media supplemented with PEG, PVA and coconut oil

The microbial growth on solid media supplemented with PEG, PVA, and coconut oil substrates is presented in Table 7. The three inoculation methods (non-perforation, perforation and by layering a drop of microbial cell culture) are also detailed. For the non-perforation, a sterile toothpick was used to remove the biomass of a single microbial colony and transferred to the surface of the solid medium to be

inoculated. On the other hand, for the perforation method, the biomass of a single microbial colony was removed with a toothpick and used to perforate the solid culture medium up to approximately 2-thirds. For the drop method, a 3 μ L drop with the microbial culture to be inoculated, was placed onto the surface of the plate.

Table 7. Mean size of the colonies after 7 days of growth in MBP, Davis and Davis + blue, supplemented with PEG, PVA or coconut oil (C.O.) using three different inoculation methods: non-perforation (non-perf.), perforation (perf.) and drop. Results are expressed in millimetres.

Medium		MBP			Davis			Davis + blue		
Microorganism		PEG	PVA	C.O.	PEG	PVA	C.O.	PEG	PVA	C.O.
<i>Y. lipolytica</i>	Non-perf.	0.67	1.41	2.53	-0.86	2.93	8.43	1.39	-0.03	1.14
	Perf.	0.78	3.41	2.42	-0.32	2.69	10.60	0.58	-2.15	-1.10
	Drop	0.56	4.15	2.25	0.54	1.94	15.63	0.94	1.30	0.88
<i>D. hansenii</i>	Non-perf.	0.10	0.88	2.46	-0.32	-0.41	0.22	0.05	-1.01	-0.08
	Perf.	0.26	1.67	2.06	1.13	1.84	0.05	0.24	0.86	0.36
	Drop	0.34	1.39	1.18	4.23	3.57	2.54	n.a.	n.a.	n.a.
<i>P. aeruginosa</i>	Non-perf.	1.95	-1.67	4.05	0.12	0.44	1.47	4.12	6.24	1.92
	Perf.	1.78	-1.08	3.86	-0.78	3.93	0.94	4.07	6.05	-0.30
	Drop	0.48	-1.37	1.87	-1.14	1.20	-1.92	1.62	2.92	2.76
<i>A. borkumensis</i>	Non-perf.	-0.07	0.24	1.48	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Perf.	-0.31	0.36	0.09	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Drop	2.22	2.23	1.30	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

- Growth  + Growth

n.a. indicates that no microbial growth was detected. For the non-perforation (non-perf.), a sterile toothpick was used to remove the biomass of a single microbial colony and transferred to the surface of the solid medium to be inoculated. For the perforation (perf.), a toothpick was used to remove the biomass of a single microbial colony and used to perforate the solid culture medium up to approximately 2-thirds. For the drop method, a pre-inoculum was made and its OD was adjusted to 0.6. Then, using a micropipette, a 3 μ L drop was placed onto the surface of the plate.

Regarding *Y. lipolytica*, the higher growth of colonies in MBP and Davis media supplemented with PVA and coconut oil is remarkable, especially for the latter, as the growth in Davis with the drop inoculation method was 15.63 mm higher than for the control. This indicates that colonies were 4 times larger comparing with the medium without the addition of supplements. For PEG, no significant differences in growth were observed. *D. hansenii*, on the other hand, was not able to utilize any of these supplements as a carbon and energy source to growth. In relation to *P. aeruginosa* and comparing with the control, colonies were 6 mm larger in Davis + blue + PVA, and 4 mm larger in MBP + coconut oil and Davis + blue + PEG, at the end of 7 days of incubation. For *A. borkumensis*, no growth was observed on Davis and Davis + blue medium. This result can be explained by the difficulty of this microorganism to grow in the Davis minimal medium since it requires complex media with higher salt concentration. For instance,

A. borkumensis demonstrated to grow on ONR7 medium, which is a much more complex medium and with a set of compounds that is not present in Davis medium [160]. Moreover, ONR7 has a higher salt concentration in its composition making it more suitable for this marine bacterium.

4.2.1.2. Media supplemented with PCL and PSF

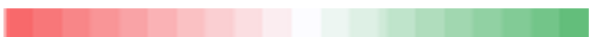
For the screening with media supplemented with PCL and PSF emulsions, an initial test was carried with two surfactants – Sarkosyl and SDS. Surfactants are amphiphilic molecules that decrease the interfacial tension between two immiscible phases [161]. Due to the presence of both hydrophilic and hydrophobic moieties, surfactants create self-assembled molecular clusters, called micelles, in a solution containing two immiscible phases (e.g. water/oil). Thus, depending on the susceptibility of the microorganism and the type of surfactant, they can exert an antimicrobial effect by interfering with cell membrane components [162].

Here, in addition to the surfactants (Sarkosyl or SDS), two different concentrations (0.01 % or 0.35 %) were also tested, and assessed by visual inspection for the ability to produce adequate emulsions for the screening assays. Moreover, the effect of the surfactants on the growth capacity of the microorganisms was also studied. It should be noted that, to the best of our knowledge, there are no reports on the use of SDS as emulsifying agent for biodegradation screening assays. Among the different groups of surfactants, SDS is classified as an anionic surfactant since the hydrophilic group dissociates into anions in aqueous solutions [163, 164]. Similarly, Sarkosyl belongs to the family of anionic surfactants [165]. The main difference between the two is that Sarkosyl is a milder detergent than SDS and may therefore have a less inhibitory effect on the growth of some microorganisms [166]. The concentration of 0.35 % was selected as a value above the critical micellar concentration (CMC) and the concentration of 0.01 % was based on previous works reporting the formation of PCL emulsions with Sarkosyl [167].

Results obtained by assessment of microbial growth in terms of colony size in solid media are presented in Table 8. Overall, SDS promoted higher growth inhibition than Sarkosyl. Accordingly, the lowest concentration tested of 0.01 % was the one that least impeded microbial growth.

Table 8. Mean size of the colonies after 7 days of growth in MBP, Davis and Davis + blue in the presence of the surfactants SDS and Sarkosyl at a concentration of 0.35 % or 0.01 %, using three different inoculation methods: non-perforation (non-perf.), perforation (perf.) and drop. Results are expressed in millimetres.

Medium Microorganism		MBP				Davis				Davis + blue			
		0.35 %		0.01 %		0.35 %		0.01 %		0.35 %		0.01 %	
		SDS	Sarkosyl	SDS	Sarkosyl	SDS	Sarkosyl	SDS	Sarkosyl	SDS	Sarkosyl	SDS	Sarkosyl
<i>Y. lipolytica</i>	Non-perf.	-7.90	-3.07	0.95	0.72	-0.86	1.65	3.92	2.44	-1.04	0.78	0.71	1.25
	Perf.	-8.29	-1.37	1.90	1.64	-0.93	1.93	3.54	3.46	-4.12	-1.01	0.20	1.32
	Drop	-9.25	1.14	1.83	1.26	0.17	3.31	4.22	4.19	-4.23	1.57	2.21	1.55
<i>D. hansenii</i>	Non-perf.	-2.99	-1.25	0.29	0.66	-2.02	-2.02	-0.25	-0.46	-2.00	-2.00	-2.00	-2.00
	Perf.	-4.80	-0.96	0.42	0.65	-1.96	-3.79	2.07	0.66	-2.28	-2.28	-2.28	-2.28
	Drop	-5.73	1.28	0.64	0.89	-0.78	-2.02	3.94	3.66	n.a.	n.a.	n.a.	n.a.
<i>P. aeruginosa</i>	Non-perf.	1.64	1.88	2.32	1.73	3.55	0.92	1.51	2.53	4.43	0.85	1.26	1.75
	Perf.	1.13	1.06	2.61	1.79	1.51	0.71	1.66	1.98	2.43	0.66	1.34	1.86
	Drop	1.58	1.44	0.60	2.14	1.60	0.37	0.21	0.85	4.42	0.79	0.92	0.30
<i>A. borkumensis</i>	Non-perf.	-4.97	-3.46	-0.42	-0.21	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Perf.	-6.54	-4.72	-0.58	0.51	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Drop	-6.04	-0.03	1.50	1.74	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

- Growth  + Growth

n.a. indicates that no microbial growth was detected. For the non-perforation (non-perf.), a sterile toothpick was used to remove the biomass of a single microbial colony and transferred to the surface of the solid medium to be inoculated. For the perforation (perf.), a toothpick was used to remove the biomass of a single microbial colony and used to perforate the solid culture medium up to approximately 2-thirds. For the drop method, a pre-inoculum was made and its OD was adjusted to 0.6. Then, using a micropipette, a 3 μ L drop was placed onto the surface of the plate.

From these results it is possible to highlight the large decrease in growth for the cultures in MBP media with the use of 0.35 % SDS. However, *P. aeruginosa* did not experience growth inhibition by any of the conditions used. In fact, in some situations it was possible to observe higher growth than the control. This can be explained by the ability of some microorganisms to degrade surfactants. Shahbazi and co-workers demonstrated that *P. aeruginosa* was able to degrade 84 % of SDS at the end of 4 days of incubation [168]. As in the previous assays, *A. borkumensis* was unable to grow in Davis and Davis + blue. The results for *D. hansenii* suggest lower growth in relation to the control, mainly at concentrations of surfactant of 0.35 %, and absence of growth in Davis + blue supplemented with either surfactants.

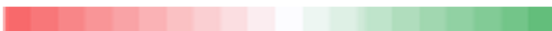
Then, PCL was used as a reference to test the efficacy of these surfactant and concentration combinations for emulsion preparation. However, at the lowest surfactant concentration (0.01 %) the emulsion demonstrated to be unstable, changing from a homogeneous dispersion solution to a two-phase solution, making it inadequate for the screening assays (Appendix 6, Figure S2).

Considering the results obtained and the stability of the emulsions, the use of Sarkosyl at a concentration of 0.35 % was selected as the best condition to perform the emulsions of PCL and PSF. To note that this condition showed no relevant effects on the microbial growth; thus, the remaining assays

were done using this combination. The microbial growth on solid media supplemented with PCL and PSF emulsions are presented in Table 9.

Table 9. Mean size of the colonies after 7 days of growth in MBP, Davis and Davis + blue with PCL emulsions by SDS and Sarkosyl surfactants at the concentration of 0.35 % and PSF (Sarkosyl 0.35 %), using three different inoculation methods: non-perforation (non-perf.), perforation (perf.) and drop. Results are expressed in millimetres.

Medium Microorganism		MBP			Davis			Davis + blue		
		PCL (SDS)	PCL (Sarkosyl)	PSF	PCL (SDS)	PCL (Sarkosyl)	PSF	PCL (SDS)	PCL (Sarkosyl)	PSF
<i>Y. lipolytica</i>	Non-perf.	n.a.	-0.30	0.67	n.a.	n.a.	-0.97	5.07	0.05	0.20
	Perf.	n.a.	-0.69	-0.22	-1.84	n.a.	-1.61	5.71	1.20	0.95
	Drop	n.a.	-0.33	-0.48	-0.75	n.a.	-3.44	7.31	0.69	1.39
<i>D. hansenii</i>	Non-perf.	n.a.	0.35	1.23	n.a.	n.a.	n.a.	2.15	n.a.	n.a.
	Perf.	n.a.	0.13	0.42	n.a.	n.a.	n.a.	4.58	n.a.	n.a.
	Drop	n.a.	0.39	-1.02	n.a.	n.a.	n.a.	1.73	n.a.	n.a.
<i>P. aeruginosa</i>	Non-perf.	-0.82	0.50	1.52	-2.07	1.00	-1.24	-3.38	-0.94	1.14
	Perf.	-0.29	-0.09	0.93	0.31	5.02	-1.96	-1.55	-1.36	1.04
	Drop	-1.11	0.97	0.49	-0.84	4.84	0.77	-2.33	0.59	2.74
<i>A. borkumensis</i>	Non-perf.	n.a.	1.05	2.25	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Perf.	n.a.	0.44	0.95	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Drop	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

- Growth  + Growth

n.a. indicates that no microbial growth was detected. For the non-perforation (non-perf.), a sterile toothpick was used to remove the biomass of a single microbial colony and transferred to the surface of the solid medium to be inoculated. For the perforation (perf.), a toothpick was used to remove the biomass of a single microbial colony and used to perforate the solid culture medium up to approximately 2-thirds. For the drop method, a pre-inoculum was made and its OD was adjusted to 0.6. Then, using a micropipette, a 3 µL drop was placed onto the surface of the plate.

In Davis + blue supplemented with PCL (0.35 % SDS) it was observed much larger growth for *Y. lipolytica* and *D. hansenii*, comparing with the control. For *Y. lipolytica* using the drop method, this was 7.31 mm higher while for *D. hansenii*, the perforation method had 4.58 mm larger colonies. Nevertheless, *D. hansenii* showed difficulty in growing on these supplements, something previously reported for media with surfactants (Table 8). Similarly, it was not possible to conclude on the ability of *A. borkumensis* to use these supplements, because of the lack of growth in most conditions.

With respect to PSF, *P. aeruginosa* indicated better results than the control, showing larger colonies in MBP and Davis + blue. However, the differences observed are not enough to ensure the ability of this microorganism to use PSF as a carbon and energy source for growth.

4.2.2. Assessment of enzymatic-related activity

As mentioned above, the clear/degradation halo approach represents a convenient approach to evaluate the capability of microorganisms to utilize certain substrates, thus providing an indication of the extracellular enzymes that are secreted. The formation of clear halos around the colonies of the four microorganisms tested in this work was evaluated in the same solid media cultures used for evaluation of microbial growth, after 7 days of incubation. Representation of halo size was performed by subtracting the values of the respective control to the size of the samples. As in section 4.2.1, a red-to-green color scale was adopted to facilitate result analysis and comparison in which, red represents smaller clear halos, and green larger clear halos.

4.2.1.1. Media supplemented with PEG, PVA and coconut oil

Results of the measurements of the clear halos for media containing the substrates PEG, PVA and coconut oil are presented in Table 10. The formation of degradation halos by *Y. lipolytica* was observed in Davis + blue medium with the coconut oil substrate. Similarly, *P. aeruginosa* obtained large degradation halos with coconut oil for both Davis and Davis + blue, although for MBP medium with this substrate, the size of the halo around the colony was similar to that seen for the control. Regarding PVA, it is apparent that when exposed to *P. aeruginosa* it leads to the formation of clear halos of larger size than the control for all media and inoculation method used. *D. hansenii* and *A. borkumensis* did not demonstrate the ability to utilize these substrates.

Table 10. Mean size of the clear halos around the colonies of *Y. lipolytica*, *D. hansenii*, *P. aeruginosa* and *A. borkumensis* after 7 days of growth in MBP, Davis and Davis + blue, with the substrates PEG, PVA or coconut oil (C.O.). Results are expressed in millimetres.

Medium		MBP			Davis			Davis + blue		
Microorganism		PEG	PVA	C.O.	PEG	PVA	C.O.	PEG	PVA	C.O.
<i>Y. lipolytica</i>	Non-perf.	-0.10	0.10	0.07	n.a.	n.a.	n.a.	n.a.	-0.06	1.60
	Perf.	-0.53	-0.10	-0.40	n.a.	1.23	1.55	n.a.	0.02	2.93
	Drop	-0.15	0.95	0.35	n.a.	n.a.	n.a.	n.a.	0.10	1.25
<i>D. hansenii</i>	Non-perf.	0.12	-0.07	0.00	0.17	0.06	0.02	n.a.	n.a.	n.a.
	Perf.	-0.25	-0.27	-0.35	0.28	0.66	0.29	n.a.	n.a.	n.a.
	Drop	-0.17	0.14	-0.31	1.04	1.00	n.a.	-1.54	n.a.	n.a.
<i>P. aeruginosa</i>	Non-perf.	-0.42	1.35	-0.31	0.03	0.36	4.87	0.31	0.87	5.97
	Perf.	-0.16	2.90	-0.06	0.26	1.60	2.92	0.38	1.06	5.74
	Drop	-0.49	1.93	-0.03	0.10	1.06	4.19	-0.02	0.37	7.93
<i>A. borkumensis</i>	Non-perf.	-0.10	-0.26	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Perf.	-0.49	-0.02	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Drop	-0.19	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Smaller clear halo  Larger clear halo

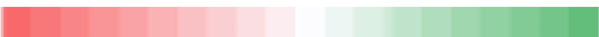
n.a. indicates that no clear halos were detected. For the non-perforation (non-perf.), a sterile toothpick was used to remove the biomass of a single microbial colony and transferred to the surface of the solid medium to be inoculated. For the perforation (perf.), a toothpick was used to remove the biomass of a single microbial colony and used to perforate the solid culture medium up to approximately 2-thirds. For the drop method, a pre-inoculum was made and its OD was adjusted to 0.6. Then, using a micropipette, a 3 µL drop was placed onto the surface of the plate.

4.2.1.2. Media supplemented with PCL and PSF

Results on the measurements of clear halos in solid media supplemented with PCL and PSF are presented in Table 11. As mentioned above, the surfactant Sarkosyl at 0.35 % concentration was selected as the best condition for emulsions. Therefore, microorganisms were evaluated for their ability to utilize the substrates PCL and PSF in emulsions with 0.35 % Sarkosyl. From these results, it is noteworthy that the clear halo obtained on Davis + blue medium with the PCL substrate is 5.35 mm larger than that observed for the control. In addition, *Y. lipolytica*, was shown to form clear halos larger than the control on MBP medium with the substrate PSF. However, the same was not seen on Davis and Davis + blue media, possibly due to the lower growth of the colonies on these media.

Table 11. Mean size of the clear halos around the colonies of *Y. lipolytica*, *D. hansenii*, *P. aeruginosa* and *A. borkumensis* after 7 days of growth in MBP, Davis and Davis + blue, with the substrates PCL and PSF both in emulsion with 0.35 % Sarkosyl. Results are expressed in millimetres.

Microorganism	Medium	MBP		Davis		Davis + blue	
		PCL	PSF	PCL	PSF	PCL	PSF
<i>Y. lipolytica</i>	Non-perf.	0.35	1.04	n.a.	n.a.	n.a.	n.a.
	Perf.	0.14	1.41	n.a.	n.a.	n.a.	n.a.
	Drop	0.57	2.55	n.a.	n.a.	n.a.	n.a.
<i>D. hansenii</i>	Non-perf.	0.02	0.03	n.a.	n.a.	n.a.	n.a.
	Perf.	0.10	0.24	n.a.	n.a.	n.a.	n.a.
	Drop	0.08	-0.03	n.a.	n.a.	n.a.	n.a.
<i>P. aeruginosa</i>	Non-perf.	0.40	0.50	n.a.	n.a.	3.33	0.59
	Perf.	0.33	0.81	n.a.	n.a.	5.35	0.82
	Drop	0.41	0.69	n.a.	n.a.	3.85	0.75
<i>A. borkumensis</i>	Non-perf.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Perf.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Drop	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Smaller clear halo  Larger clear halo

n.a. indicates that no clear halos were detected. For the non-perforation (non-perf.), a sterile toothpick was used to remove the biomass of a single microbial colony and transferred to the surface of the solid medium to be inoculated. For the perforation (perf.), a toothpick was used to remove the biomass of a single microbial colony and used to perforate the solid culture medium up to approximately 2-thirds. For the drop method, a pre-inoculum was made and its OD was adjusted to 0.6. Then, using a micropipette, a 3 μ L drop was placed onto the surface of the plate.

4.2.1.3. Assessment of lipase activity

In addition to the clear halo measurements, a fluorescence assay was also carried out to detect lipases in the media supplemented with coconut oil, containing rhodamine B. This highly specific and sensitive test for the detection of lipases resorts to the formation of a fluorescent complex that can be detected under UV light. If the microorganisms secrete lipases to the medium, this will lead to lipolysis of the coconut oil, resulting in the generation of free fatty acids. The free fatty acids will form a fluorescent complex with the rhodamine B present in the medium and, as a result, colonies capable of producing lipases will give rise to a yellow fluorescent halo that is visible under UV light, e.g. 254 nm [125, 170]. Considering the results obtained with the clear halo assays, *Y. lipolytica* and *P. aeruginosa* were evaluated for the presence of lipases as these were the only to exhibit an halo in the solid media supplemented with coconut oil (Davis and Davis + blue). Figure 13 and Figure 14 show the results obtained for *Y. lipolytica* and *P. aeruginosa*, respectively, after exposure of the solid media plates to UV light at 254 nm radiation.

As observed in Figure 13, the media inoculated with *Y. lipolytica* revealed the presence of a yellow fluorescent halo, as a result of the lipolysis of coconut oil by this microorganism, which is absent in the control plates without coconut oil and rhodamine B.

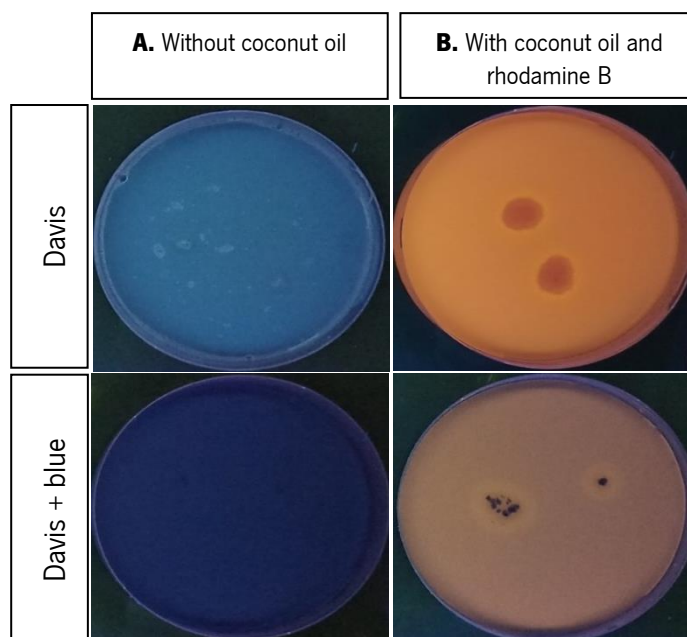


Figure 13. Digital images of *Y. lipolytica* plated on solid media under UV light at 254 nm, for demonstration of lipase activity. No fluorescence is formed in the plates without coconut oil (A). Due to the presence of rhodamine B in the media (B), a yellow fluorescent halo is formed around the colonies demonstrating lipolytic activity.

Regarding *P. aeruginosa* (Figure 14), no yellow fluorescent halo was detected under UV light; instead, a halo of blue fluorescence is perceived for both the controls and the media with coconut oil, although for the latter the blue fluorescence is much stronger. To clarify the reason for a blue fluorescence and not yellow, the petri dishes were further evaluated under the emission of radiation at a wavelength of 365 nm, following the procedure previously described by Vague and co-workers (Figure 15) [171]. As a result, blue fluorescence was visible in all media, independently of the presence of coconut oil. This phenomenon is attributed to the autofluorescence of some *Pseudomonas* species when exposed to UV radiation, which overrides a possible fluorescence caused by coconut oil lipolysis, making an evaluation by this test more difficult [125, 171]. Nevertheless, these results for *P. aeruginosa* are in agreement with those previously described by Vague *et al.* in which, the authors report the formation of a similar blue fluorescence for *Pseudomonas* species exposed to UV light at 365 nm [171]. Moreover, in agreement with our observations, the authors also demonstrated increased fluorescence intensity in the medium incorporating rhodamine B and vegetable oil, with larger and more intense fluorescent halos being consistent with isolates showing greater lipase activity [171]. In this work, the halo observed for *P. aeruginosa* in media supplemented with coconut oil and rhodamine B was much larger and intense than that of the conditions without supplementation. This suggests that changes in fluorescence intensity are not attributed solely to autofluorescence and point out to a lipase positive confirmation.

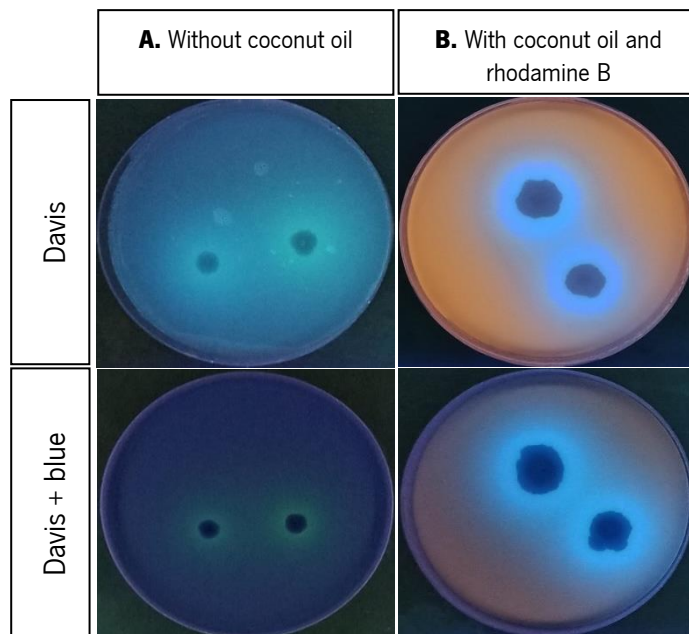


Figure 14. Digital images of *P. aeruginosa* plated on solid media under UV light at 254 nm, for demonstration of lipase activity. The plates without coconut oil (A) show the natural fluorescence of *P. aeruginosa*. The coconut oil and rhodamine B plates (B) show intense blue fluorescent halos.

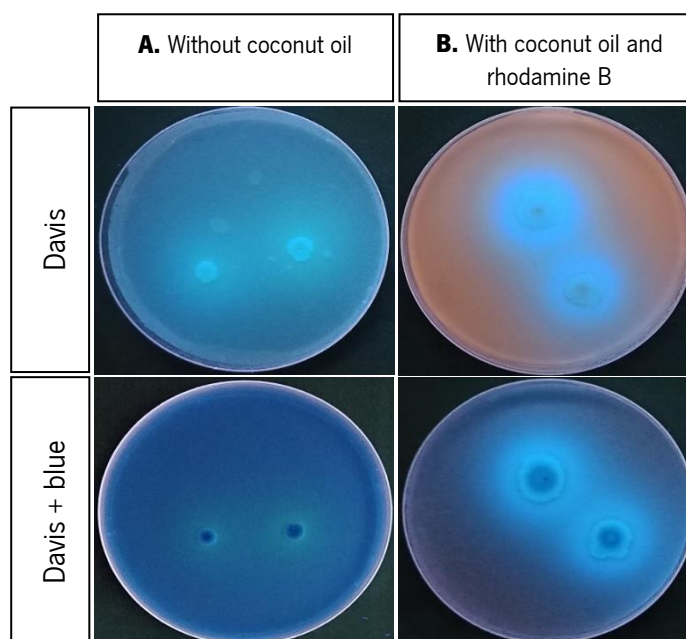


Figure 15. Digital images of *P. aeruginosa* plated on solid media under UV light at 365 nm, for demonstration of lipase activity. The plates without coconut oil (A) show the natural fluorescence of *P. aeruginosa*. The coconut oil and rhodamine B plates (B) show intense blue fluorescent halos, indicative of lipolytic activity.

4.2.3. Considerations on the screening assays

The clear halos formed around the colonies were evaluated in three different ways (No staining, Coomassie Blue Staining and Methylene Blue). No staining (Appendix 7, Figure S3) may be used when the solid media is opaque, and upon enzyme activity the media becomes translucent and a halo is visible; however, when using substrates such as PVA and PEG that are water soluble, translucent media are obtained, in which visualization of the clear halos around the colonies becomes very difficult. For these cases, it is necessary to add a dye to the media, to facilitate the visualization process. One of the options available was the Coomassie Blue Staining (Appendix 7, Figure S4), but this can only be done at the end of the microbial growth, so it is not possible to follow the halo formation over time. Methylene blue (Appendix 7, Figure S5), on the other hand, is easy to use, which is important in a process such as in a screening that, by its nature, encompasses a large number of samples, and is intended to select as quickly as possible the species that are relevant from those that are not.

In addition, these screening assays tested 3 different inoculation methodologies, all of which have their advantages and disadvantages. The non-perforation method, as well as the perfuration method, are convenient, however, the amount of biomass inoculated can vary as it is not possible to quantify the exact amount of biomass removed by the toothpick. The perforation method allows a three-dimensional analysis, but it can lead to water release and possible spreading of the microorganism on the plate. The drop method, on the other hand, is more likely to have contaminations as it requires an extra step of making a pre-inoculum in liquid medium. Additionally, it requires more attention to the growth phase as it is important that inoculation is performed during the exponential growth phase. The main advantage of this method is that the amount of inoculated biomass is always the same in all situations, since it employs defined conditions namely, a specific volume of cell culture (3 μ L in this work) at a defined OD (typically, 0.6). Thus, the best method to use varies depending on the objective of the screening. For a faster analysis with a large number of samples it may be better to opt for non-perforation or even perfuration, while for a test that aims for a precise comparison or quantification, the drop method may be more appropriate.

Considering the results of the screening tests, the most promising microbial species were *Y. lipolytica* and *P. aeruginosa*. For *D. hansenii* no significant growth or clear halo on any substrate was observed while for *A. borkumensis* no growth was observed on Davis and Davis + blue medium as described above. Regarding *Y. lipolytica*, it is possible to highlight its potential for plastic biodegradation as results obtained with PVA and coconut oil demonstrate the ability of this microorganism to assimilate both substrates. To the best of our knowledge, *Y. lipolytica* revealed for the first time the capability to

biodegrade PVA. This microorganism was already reported as being able to grow in media with vegetable oils [172, 173]. Both the results obtained by evaluating the microbial growth in the different media and the formation of clear halos around the colonies, as well as through the fluorescence assay, are in agreement with these indications and report the potential of this microorganism for PE biodegradation. The ability of this microorganism to biodegrade PE is not described in previous studies; there is only reference to the potential of *Y. lipolytica* to degrade alkanes and thermolysis residues from PE [173, 174].

From the bacteria tested, *P. aeruginosa* is the most versatile and was able to utilize coconut oil, PVA, PEG and PCL. Regarding PVA, this ability has already been identified for the species *Pseudomonas alcaligenes*, that belongs to same genus and shares high similarity with *P. aeruginosa* [175]. Similarly, *P. aeruginosa* has been described to assimilate both PEG and coconut oil [148, 176-178]. Finally, its ability to degrade PCL has already been reported, which is supported in this work by differences in its growth and the appearance of clear halos [179, 180].

Overall, the screening assays revealed that *P. aeruginosa* and *Y. lipolytica* are able to utilize substrates associated with enzymatic activities that are promising for the biodegradation of PE; therefore, were selected for the subsequent biodegradation tests.

4.3. Biodegradation of PE films

Based on the screening results, both *P. aeruginosa* and *Y. lipolytica* demonstrated to be equipped with enzymes that, in principle, are able to attack PE, which is promising for biodegradation. The ability of these microorganisms to colonize the surface of PE films and exert a microbial attack was studied for 90 days and included evaluation of microorganism-related parameters namely, microbial growth, biofilm formation and extracellular protein/enzyme profile; and characterization of the PE film through evaluation of film weight loss, assessment of chemical modifications by FTIR, and analysis of film surface morphology by SEM.

4.3.1. Microbial growth in planktonic stage

An indirect way to assess the ability of microorganisms to utilize PE is through the evaluation microbial growth in the planktonic phase. Once the incubation is done for long periods of time, the carbon sources present in the media are soon depleted. In order to grow, the microorganisms have to be able to

utilize the PE film added to the medium as a source of carbon and energy. Therefore, the growth in the planktonic phase was monitored over 90 days in Davis (Figure 16) and in MBP medium (Figure 17).

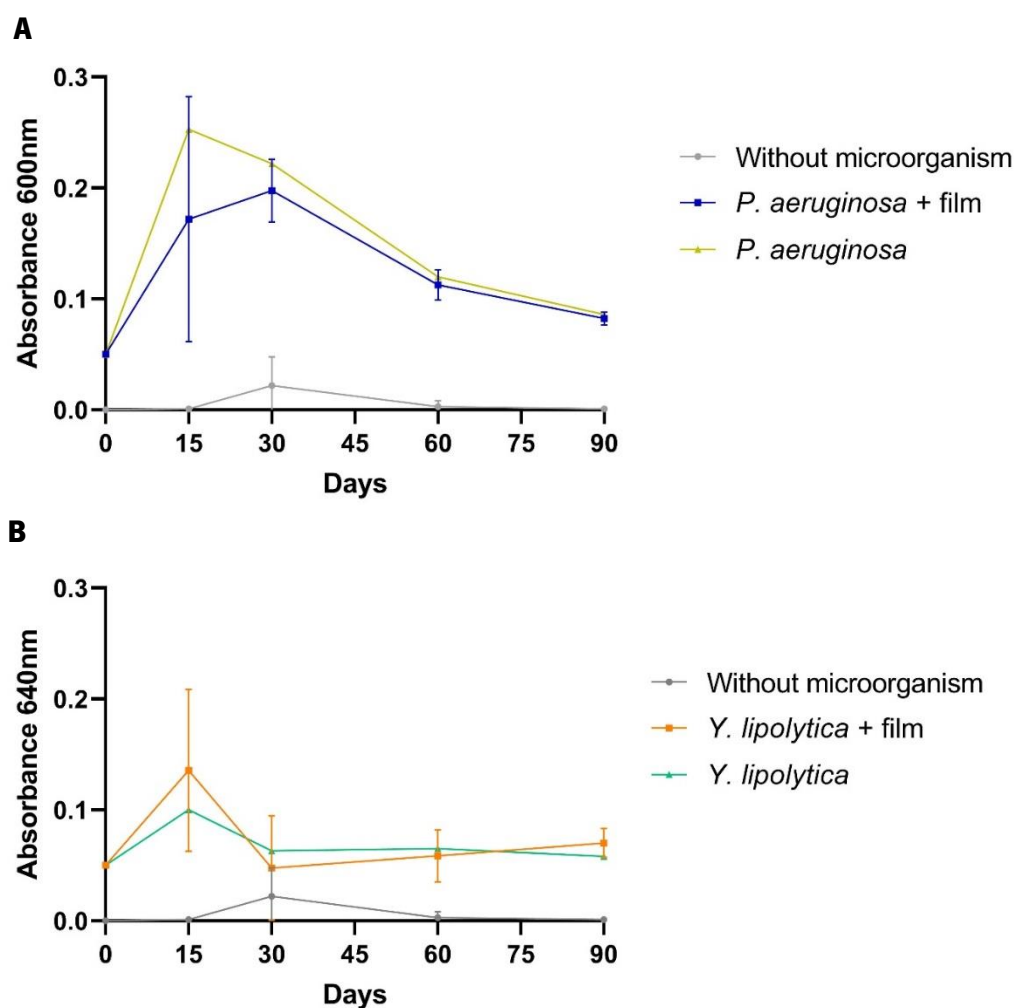


Figure 16. Microbial growth of *P. aeruginosa* (A) and *Y. lipolytica* (B) in Davis medium with and without PE film, during 90 days.

It is noticeable that microbial growth in the planktonic phase is very similar for the media with and without film for both microorganisms, except for the initial phase in which the film seems to slightly inhibit growth. After the 90 days of incubation, *Y. lipolytica* showed a slightly higher absorbance for the medium with film, reaching a value of 0.070, in opposition to the value of 0.058 obtained in the condition without film. Nevertheless, this result does not represent a significant difference. As for *P. aeruginosa*, the values of 0.082 and 0.086 were obtained with and without the addition of PE film to the medium, respectively.

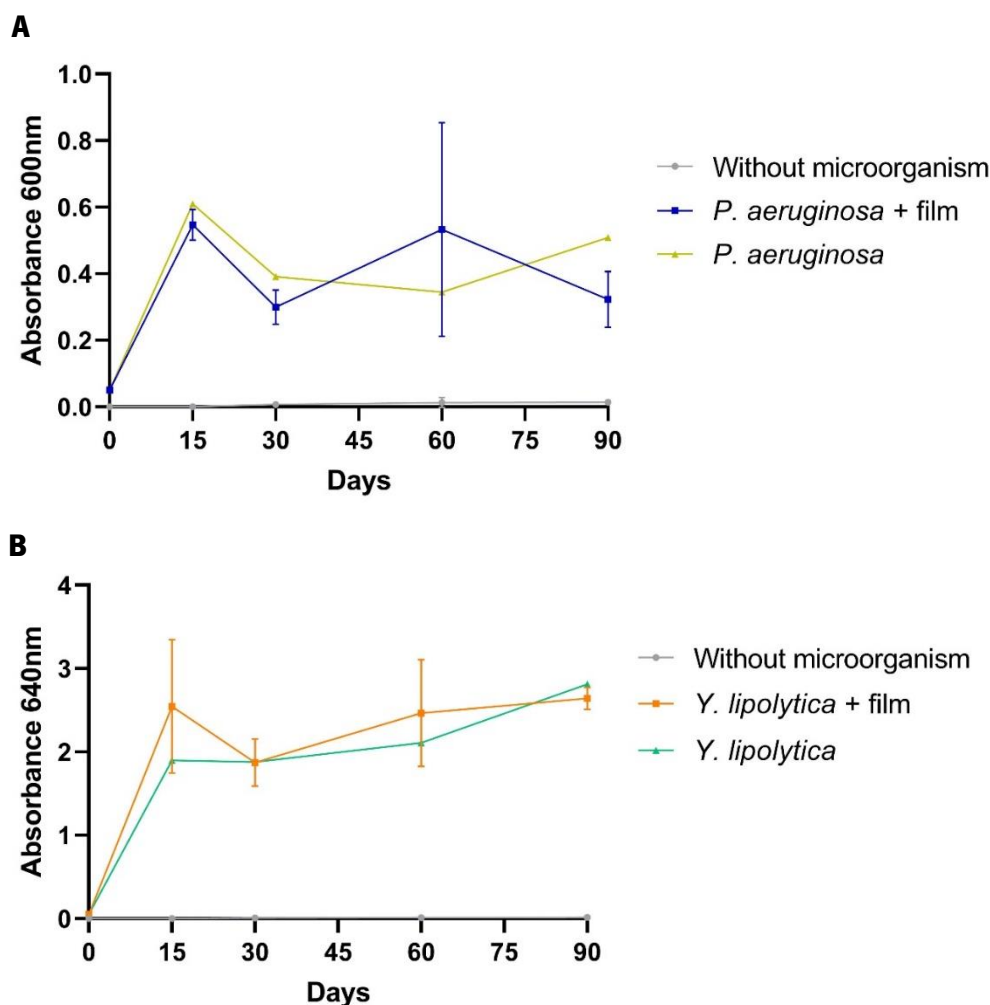


Figure 17. Microbial growth of *P. aeruginosa* (A) and *Y. lipolytica* (B) in MBP medium with and without PE film, during 90 days

Regarding the growth in MBP medium, no major differences were observed. For the yeast *Y. lipolytica*, at the end of 90 days of incubation, an OD of 2.81 and 2.64 was reached for the media without film and with film respectively. For *P. aeruginosa*, a lower growth was observed, reaching values of 0.509 and 0.323 without film and with film, respectively. Also, *P. aeruginosa* formed cell aggregates over time, which, along with biofilm formation, could explain the low OD obtained by this microorganism in this medium.

Considering these results, no significant difference was noted in the growth of the microbes. Nevertheless, the absence of significant results at this point does not prove that the microorganisms under analysis do not have the ability to biodegrade PE, since previous studies report that microbial growth can be observed through the formation of biofilms in the film and not so much in an increased growth in the planktonic state [181, 182]. Moreover, one should consider that the period of the experiment

(90 days) may be insufficient for the microorganisms to effectively depolymerize the polymer and utilize the oligomers/monomers as energy source.

4.3.2. Biofilm formation

As mentioned earlier, the first step in the biodegradation process is the colonization of the plastic surface by microorganisms. To do this, the microorganisms excrete extracellular polymeric substances (typically polysaccharides, DNA, proteins, and lipids) upon adherence to the surface, on which they grow [183-185]. The resulting film is called a biofilm. In plastics with a large hydrophobic surface area such as PE, biofilm formation is especially important to increase the interaction of the polymer surface with the microorganism and allows for greater degradation of the plastic than in the planktonic phase [91].

One way to assess biofilm formation is by using the crystal violet assay. Crystal violet is a basic dye that binds non-specifically to negatively charged surface molecules such as polysaccharides and DNA found in the extracellular matrix [186]. Therefore, crystal violet marks on both viable and dead cells, so it is not possible to quantify the actual number of viable cells, being this assay widely used to assess biofilm biomass as a whole [187]. Using this methodology, it was possible to observe the formation of biofilms on the surface of the PE films (Figure 18). Overall, *Y. lipolytica* was distributed throughout the plastic film, while *P. aeruginosa* was mainly located in the most irregular areas, i.e. the shear zone.

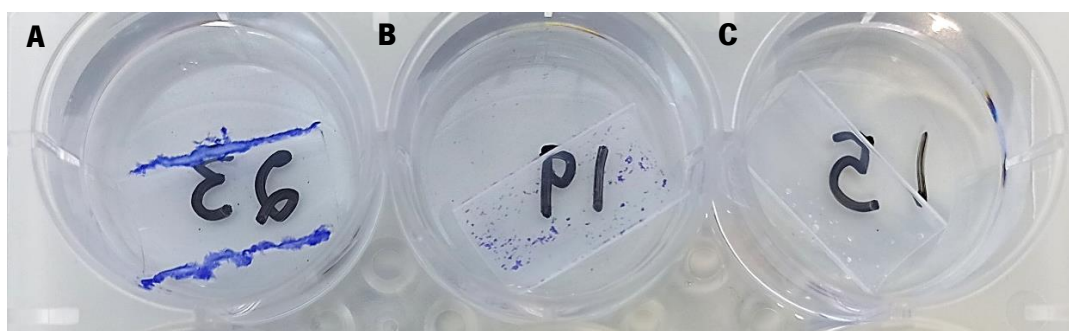


Figure 18. Biofilm formation assessed using the crystal violet assay on PE film when incubated in MBP for 60 days and exposed to: A) *P. aeruginosa*; B) *Y. lipolytica*; C) no microorganisms.

In addition to the visual inspection of the crystal violet stained biofilms, the amount of biofilm formed was quantified by measuring the absorbance at 570 nm, and compared with films without incubation with the microorganisms (Figure 19).

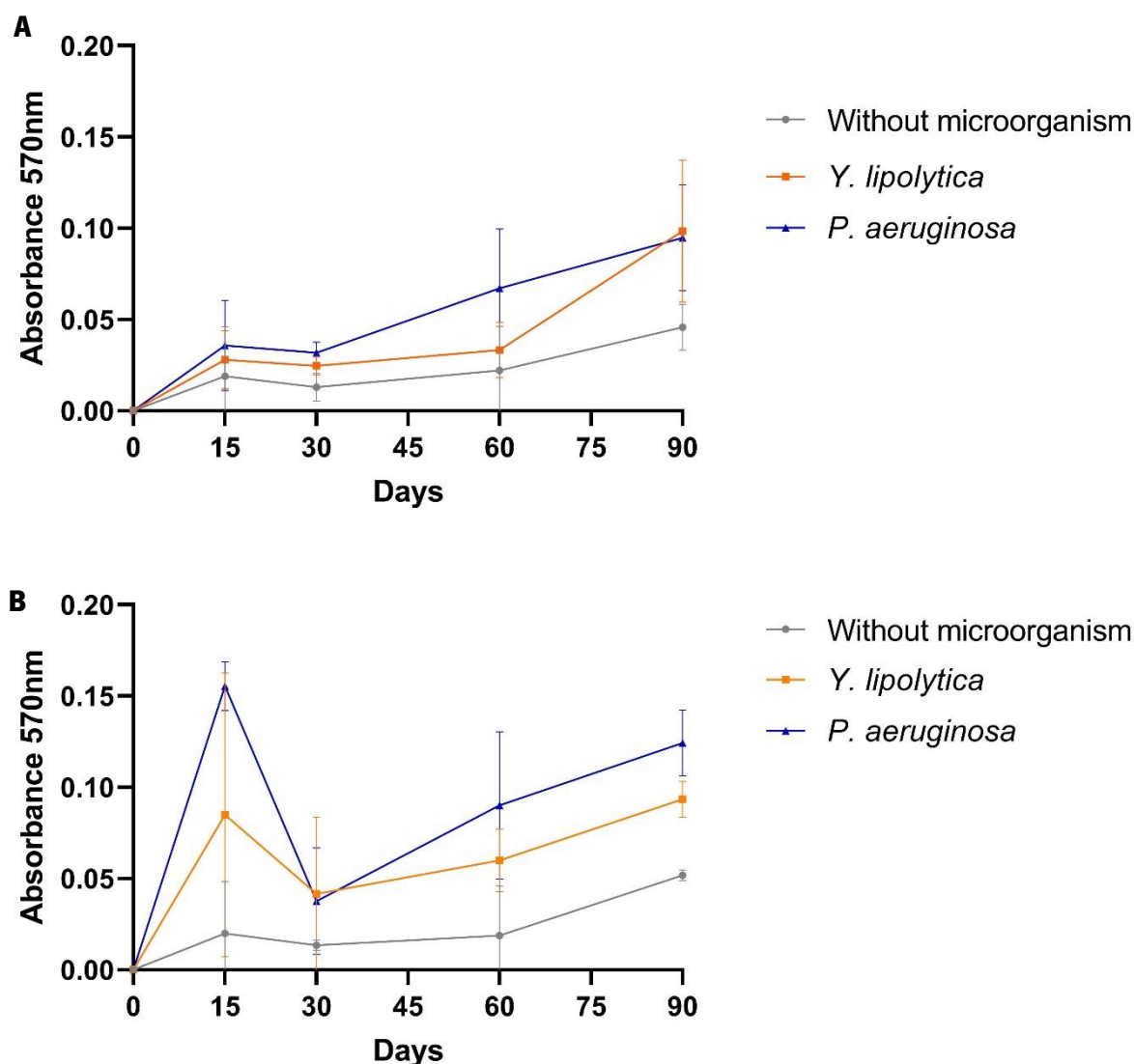


Figure 19. Biofilm formation determined by measuring the absorbance at 570 nm after crystal violet staining. Growth in Davis (A) and MBP (B) media of *Y. lipolytica* (orange) and *P. aeruginosa* (blue). The control (gray) was done without the inoculation of microorganisms.

Considering the values obtained for the quantification of biofilm and performing a comparative analysis, the amount of biofilm present in the samples incubated in the Davis media was similar between the two microbes at the end of the 90 days. The highest difference was observed after 60 days, with *P. aeruginosa* and *Y. lipolytica* presenting mean absorbance values of 0.067 and 0.033, respectively. As for the MBP media, although the growth in the planktonic phase of *P. aeruginosa* was much lower than *Y. lipolytica*, it was observed that *P. aeruginosa* presented a greater amount of biofilm, when compared with *Y. lipolytica*. At the end of the 90 days, the absorbance at 570 nm was 0.124 and 0.093 for *P. aeruginosa* and *Y. lipolytica* respectively. Additionally, an increase in biofilm formation is perceived over time, apart from 15 days in MBP. This suggests that the microorganisms could still grow and form a

biofilm on the PE film at the end of the 90 days, which may indicate their potential for biodegradation of these samples.

In summary, both microorganisms were able to form biofilms on the PE film when growing in Davis and MBP media. These results reveal the potential of these microbes to biodegrade PE since they perform the first step of the biodegradation process which is colonization. Nonetheless, results also suggest that increasing the incubation period beyond 90 days could result in enhanced biofilm formation.

4.3.3. Analysis of the protein expression profile

Incubation of the microorganism with the PE film as carbon source may lead to the secretion of certain proteins (i.e. enzymes) that promote the extracellular degradation of these polymers. The protein profile present in the supernatants of culture media was evaluated by SDS-PAGE, when *Y. lipolytica* and *P. aeruginosa* cells were grown in the presence and absence of PE film (control).

To visualize the protein profile in the supernatants of culture media, an initial test was performed with BlueSafe staining of the SDS-PAGE gels (data not shown). Also, a silver nitrate staining which has higher sensitivity was tested (data not shown). Using these stainings methods we were unable to visualize an adequate protein profile. Thus, the proteins present in the supernatants were concentrated by ammonium sulfate precipitation, except for *P. aeruginosa* grown in MBP media, in which a visualization of the protein profile was already possible with both BlueSafe and silver nitrate staining.

Figure 20 presents the SDS-PAGE gels for the protein-concentrated supernatants of *Y. lipolytica* cultures, stained with BlueSafe. It is possible to visualize two bands around 48 kDa for the samples incubated with the film in MBP medium, especially after 30 days of incubation, that are not observed in the control. Since what varies between the control and the other samples is the addition of PE film, this could be the result of the expression of extracellular enzymes that are required for the biodegradation of the PE film. Regarding analysis of the supernatants in Davis medium, despite the use of ammonium sulphate to concentrate proteins, no bands were detected on the gel.

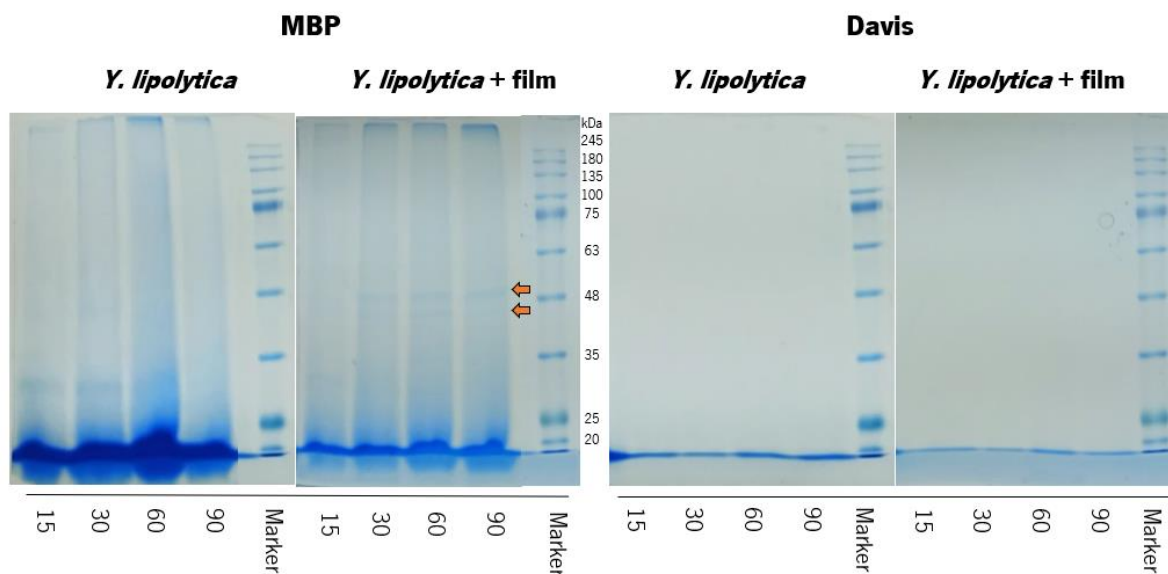


Figure 20. SDS-PAGE from the supernatants of *Y. lipolytica* cultures after incubation for 15, 30, 60 and 90 days in MBP and Davis media, with and without addition of the PE film (control). The arrows indicates the presence of bands around 48 kDa which are absent in the control. Gels were stained with BlueSafe.

Based on the results, the gels were further stained with silver nitrate to confirm the presence of other proteins that could be present at lower levels (Figure 21). Unexpectedly, the bands around 48 kDa demonstrated to be also present in the control samples, although at lower intensity. Of remarked interest is the presence of a relatively intense band near 63 kDa that appears in the supernatants of *Y. lipolytica* cultures after 15 days of incubation in MBP, and is absent in all the control samples. This could represent the production of an extracellular enzyme potentially involved in the attack to the plastic material. As for the cultures grown in Davis, it is possible to observe a band of low intensity slightly above 48 kDa, which appears to be in the same size range as that observed in MBP.

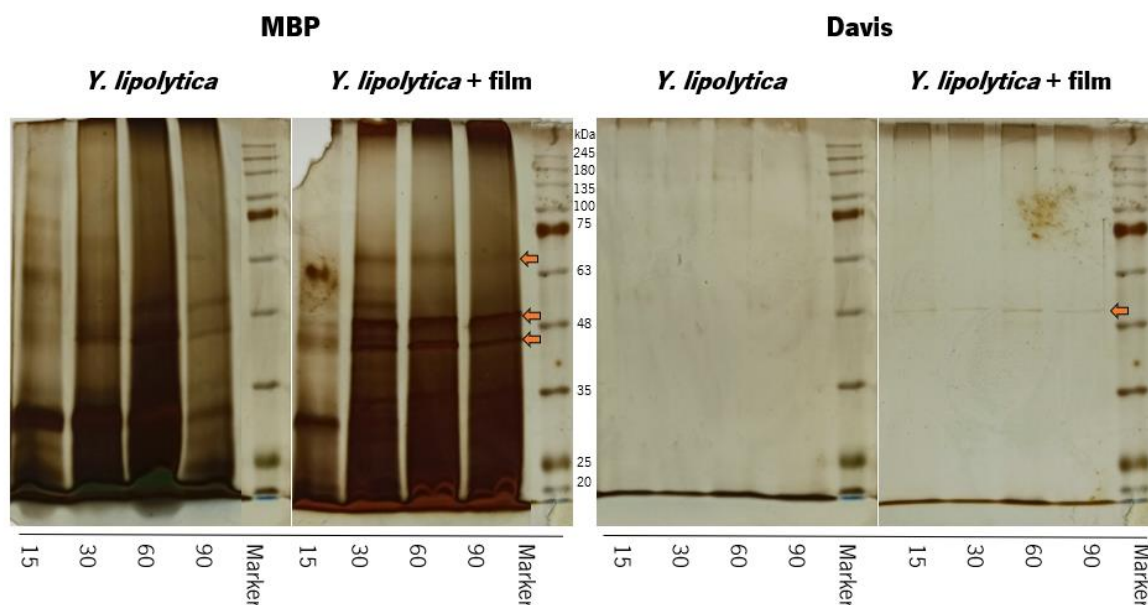


Figure 21. SDS-PAGE from the supernatants of *Y. lipolytica* cultures after incubation for 15, 30, 60 and 90 days in MBP and Davis media, with and without the addition of the PE film (control). The arrows point to bands of interest: bands near 48 kDa in MBP were not observed in the gels stained with SafeBlue; band between 63 and 75 kDa in MBP and band at near 48 kDa in Davis are absent in the control. Gels were stained with silver nitrate.

Regarding *P. aeruginosa*, the gels stained with BlueSafe demonstrate the presence of a band between 63 kDa and 75 kDa for the cultures in Davis medium with film, which is present throughout the 90 days of the experiment, and is not present in the control (Figure 22). After silver nitrate staining (Figure 23), the presence of this band between 63 and 75 kDa is even more evident and is missing in the control samples. It must be noted that a band of similar molecular weight, although at a much lower intensity, was also observed for the cultures of *Y. lipolytica* in MBP with the PE film (Figure 21). This further supports and provides indications on the production of an extracellular enzyme in the presence of the plastic sample, with putative involvement in the biodegradation of PE.

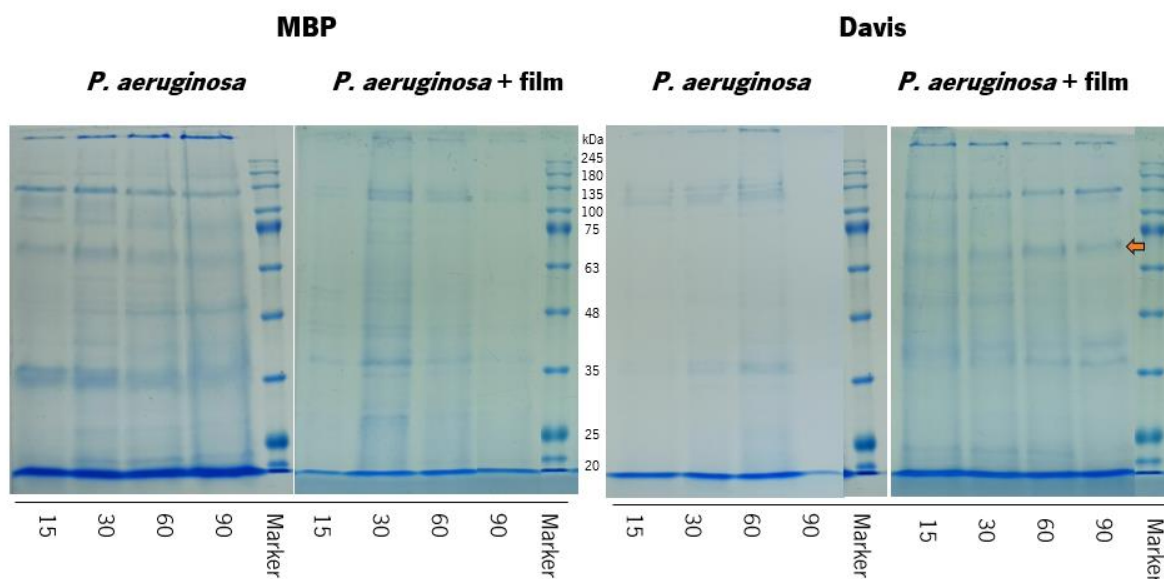


Figure 22. SDS-PAGE from the supernatants of *P. aeruginosa* cultures after 15, 30, 60 and 90 days of incubation in MBP and Davis media, with and without the addition of the PE film (control). The arrow indicates the presence of a protein band between 63 and 75 kDa bands which is not present in the control. Gels were stained with BlueSafe.

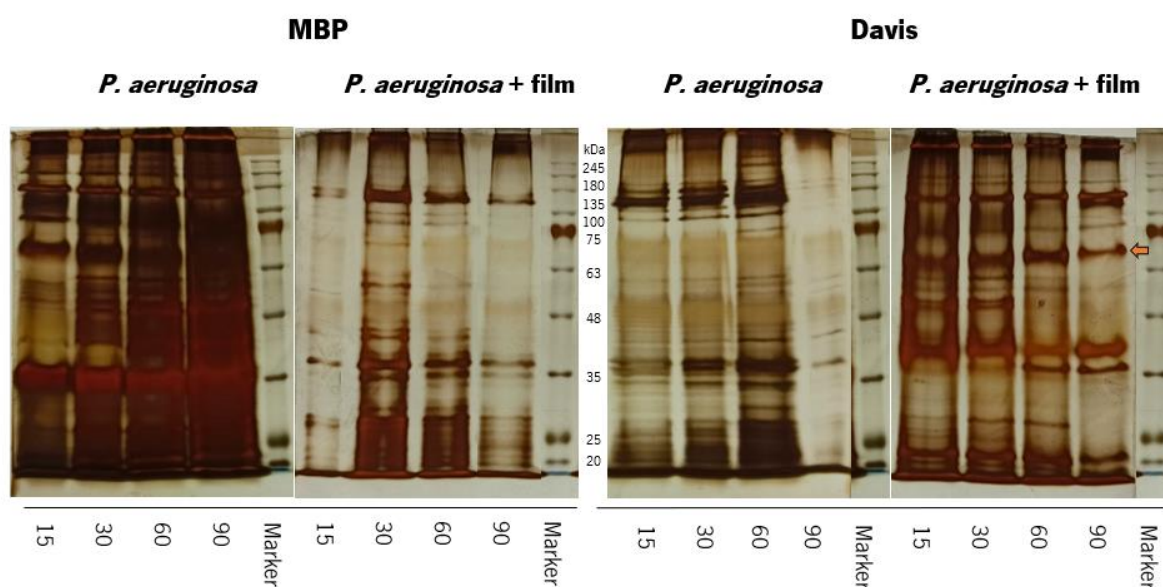


Figure 23. SDS-PAGE from the supernatants of *P. aeruginosa* cultures after 15, 30, 60 and 90 days of incubation in MBP and Davis media, with and without the addition of the PE film (control). The arrow indicates the presence of a protein band between 63 and 75 kDa bands which is not present in the control. Gels were stained with silver nitrate.

In summary, the analysis of the extracellular protein profile of the microbial cultures revealed an expression, or overexpression, of specific proteins when these microorganisms are incubated with PE. This result suggests that these microorganisms are able to secrete enzymes that could biodegrade PE

films. Also, the differences observed in the electrophoretic profile for cultures in MBP or Davis are most likely related with the increased biomass in MBP than in Davis. The more cells present in the culture, the more extracellular enzymes being produced, which allows for a better detection by SDS-PAGE.

4.3.4. Determination of weight loss

A simple and quick way to evaluate the biodegradation of polymers is by determination of the film weight loss after incubation with microorganisms. Once microbes initiate the biodegradation process through depolymerization of the plastic and its use as a carbon and energy source, this leads to a loss of integrity and consequently, weight loss. This difference in weight will be proportional to the surface area of the film since biodegradation is usually initiated at the polymer surface. However, one should take into account that microbes adhered to the plastic surface will lead to an overestimation of the weight. Thus, for this method, washing and removal of microbes are very important steps, so microorganisms and substances present in the media that can adhere to the film do not influence the final weight value.

The variations in the weight of the films are presented in Table 12 and expressed as weight loss percentage. The differences reported were not significant although there is a slight tendency for the films incubated with the microorganisms, especially for the conditions in MBP media and *P. aeruginosa* in Davis. PE is highly recalcitrant and inert, making it very difficult to degrade in the environment even after being buried for several years in landfills [120]. Under laboratory conditions, promising results have been obtained such as Muhonja and coworkers who showed a percent weight loss of 35.2 % in 30 micron film after 120 days of incubation, or Usha *et al.* who reported the degradation of 37.1 % of PE by *Pseudomonas* sp. in 6 month period [188, 189].

Table 12. Weight loss variation of PE films after 90 days of incubation.

Culture media conditions		Initial weight (mg)	Final weight (mg)	Weight loss percentage
Davis	Film (no microorganism)	165.5 ± 5.3	165.4 ± 5.6	0.060 %
	<i>Y. lipolytica</i>	175.6 ± 6.3	175.5 ± 6.3	0.076 %
	<i>P. aeruginosa</i>	163.4 ± 4.5	163.1 ± 4.8	0.143 %
MBP	Film (no microorganism)	174.4 ± 7.8	174.6 ± 7.8	- 0.096 %
	<i>Y. lipolytica</i>	171.8 ± 4.9	171.6 ± 4.8	0.155 %
	<i>P. aeruginosa</i>	163.6 ± 5.1	163.4 ± 5.4	0.102 %

In this work, one must take into consideration that biodegradation was studied over a short-term period of 90 days with ≈ 200 micron PE films, which may imply a prolonged incubation period for the microorganisms to exert an effective action over the plastic material. Another consideration relies on the fact that many researchers have investigated the biodegradation of PE using commercially available polymers, often containing various chemical additives [190]. In this regard, it is unclear if the weight loss is due to the plastic or associated with the biodegradation of the additives, that often make up a large part of it. Thus, the weight loss values obtained in previous works, do not reflect the actual degradation of the PE and therefore may be misleading regarding the ability of this plastic to be biodegraded by microorganisms. Also, some studies reporting on the biodegradation of PE perform pre-treatments, namely by pre-exposing the PE to UV light for several days. The UV radiation can induce significant chemical changes at the levels of carbonyl group production, making PE more susceptible to microbial degradation and thereby achieving higher weight loss [191-193]. In the present study, untreated PE films were used, in which the amount of additives is negligible as demonstrated by FTIR analysis (data not shown); thus, the results obtained are most likely attributed solely to the action of the microorganisms on the PE films.

4.3.5. Analysis of chemical modifications by FTIR

FTIR spectroscopy can be used to study and monitor chemical changes in the structure of a polymeric material, and several studies have reported the importance of FTIR for the chemical characterization of microbial degraded plastics [194-202]. The FTIR spectra provides information on the chemical functional groups of plastic materials, allowing to detect chemical changes deriving from a microbial attack [103, 203].

In this work, PE films exposed to incubation for 90 days with *P. aeruginosa* and *Y. lipolytica* were evaluated in 3 zones to obtain an overall picture of the possible chemical changes in the structure of the plastic sample. These comprise analysis of the cutting zone, inner and outer parts of the films (Figure 24). Evaluation of the inner and outer sides of the plastic samples were carried out, as during incubation cells can adhere to either surfaces in an uncontrolled way. The cutting zone was also analysed, since it presents a more irregular surface which can be preferential for microbial adhesion.

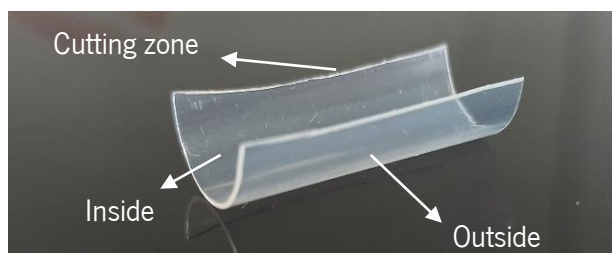


Figure 24. Representation of the three zones of the film used for FTIR analysis (inside, outside and cutting zone).

FTIR analysis of pristine PE samples (before incubation) revealed no differences between the selected zones (Figure 25). The five peaks characteristic of virgin PE plastic stand out, and since PE is made up of only C and H, they correspond to the different vibration modes of the methylene group: the bands at 2914 cm^{-1} and 2847 cm^{-1} correspond to the symmetric and asymmetric stretching of CH_2 , respectively; 1472 cm^{-1} corresponds to the bending vibration of CH_2 ; 730 cm^{-1} and 718 cm^{-1} are attributed to the rocking vibration of CH_2 [204, 205].

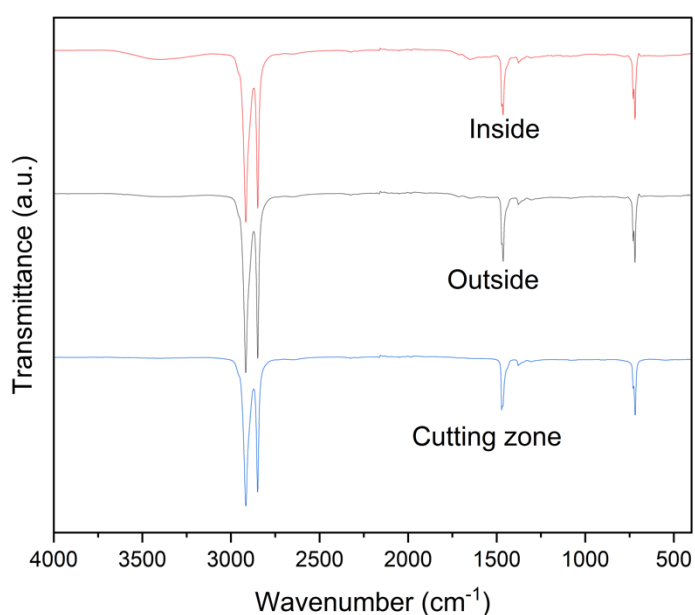


Figure 25. FTIR spectra for the three zones of pristine PE film: inside (red), outside (green) and cutting zone (blue).

The samples incubated with the microorganisms were analyzed over time (Appendix 8). However, for the present discussion, only the samples of 90 days of incubation are presented, as it is expected that these will present more noticeable changes at the level of the chemical structure. For *P. aeruginosa*, the outer side of the film was selected while for *Y. lipolytica* it was the inner side since it was on this side that the biodegradation was most pronounced (Appendix 8).

Figures 26 and 27 present the FTIR spectra of samples after 90 days of incubation with *P. aeruginosa* in Davis and MBP media, respectively. In Figure 26, it is possible to notice some differences compared to the control, namely the appearance of a broad band centered at 3360 cm^{-1} (gray square) that is attributed to stretching vibrations of the hydroxyl group (OH) present in the alcohol groups. During the biodegradation process, a PE molecule containing carbonyl group is initially converted into alcohol (containing -OH group) [206]. Later in this mechanism, the alcohol is oxidized to form aldehydes and ketones, as seen earlier (Figure 8) [198]. In addition, the appearance of a peak at approximately 1060 cm^{-1} (blue square) indicates the formation of new ether bonds (C-O-C) in the PE film, which may arise due to the incorporation of an oxygen atom between weakened C-C bonds, as a consequence of the high electronegativity of oxygen [205]. The bands in the region $963\text{-}914\text{ cm}^{-1}$ show the presence of C=C stretching of alkene groups suggesting that a breakdown of the polymer into oligomers/monomers has occurred, which is characteristic of the biodegradation process [207]. Regarding the sample in MBP (Figure 27), the differences between the control and the sample exposed to *P. aeruginosa* are in the same range of frequencies as those observed for the Davis medium. Of note, is the change in the zone between $3550\text{ to }3160\text{ cm}^{-1}$, attributed to stretching vibrations of the hydroxyl group (OH), revealing the presence of alcohol groups. In addition, the band at 1060 cm^{-1} indicates the formation of a ether bond (-C-O-C-) in the film. However, the differences observed in Davis for the region between $963\text{ - }914\text{ cm}^{-1}$ related with the polymer breakdown into oligomers/monomers are not as noticeable in MBP, which suggests that incubation of the microorganism in minimal medium (Davis) leads to greater biodegradation of the film than in MBP.

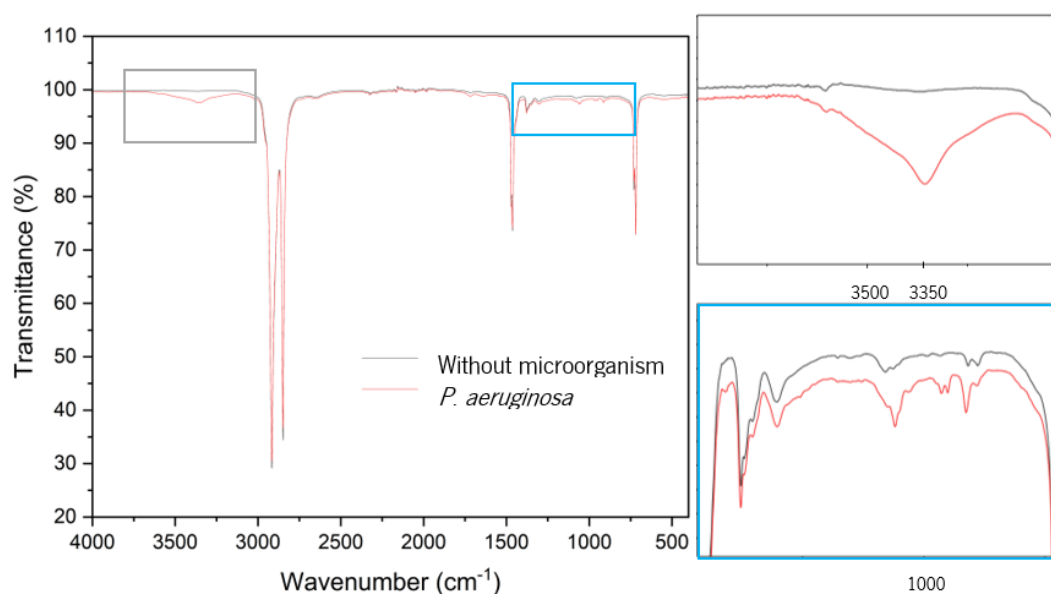


Figure 26. FTIR spectra of PE films incubated for 90 days in Davis with and without *P. aeruginosa*. The insets (gray and blue squares) represent regions showing differences in relation to the control.

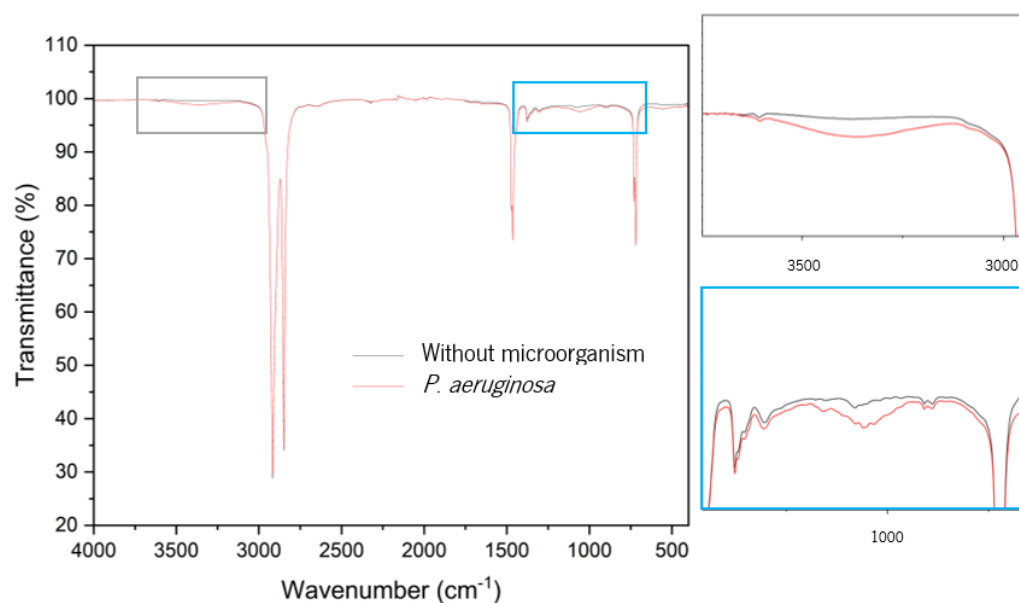


Figure 27. FTIR spectra of PE films incubated for 90 days in MBP with and without *P. aeruginosa*. The insets (gray and blue squares) represent regions showing differences in relation to the control.

Regarding the samples incubated with *Y. lipolytica*, the FTIR spectra for the films incubated for 90 days in Davis and MBP media are presented in Figures 28 and 29, respectively. Similarly to the results obtained with *P. aeruginosa*, the FTIR spectra of the samples incubated with *Y. lipolytica* in Davis medium

also present more dissimilarities in respect to the control, than the samples in MBP (Figure 28). As in the films incubated with *P. aeruginosa*, it is possible to observe the presence of a broad band in the 3300 cm^{-1} region (gray square) attributed to stretching vibrations of the OH group. The band centred at 1655 cm^{-1} (orange square) indicates the formation of new bonds and is associated to stretching vibrations of the carbonyl group ($\text{C}=\text{O}$), that may be the result of the formation of ketone or aldehyde groups [198]. During the biodegradation process, changes in the PE molecule involve chemical modifications to include carbonyl groups and the subsequent formation of aldehydes and ketones as previously described (Figure 8) [198]. Finally, major changes also include the presence of a band at approximately 1080 cm^{-1} (blue square) attributed to the formation of ether groups ($\text{C}-\text{O}-\text{C}$). As for the samples in MBP, changes in the spectrum are much less evident compared with Davis and comprise mainly the presence of bands attributed to OH stretching in the 3300 cm^{-1} (gray square) and stretching vibrations of the carbonyl group at approximately 1655 cm^{-1} (orange square).

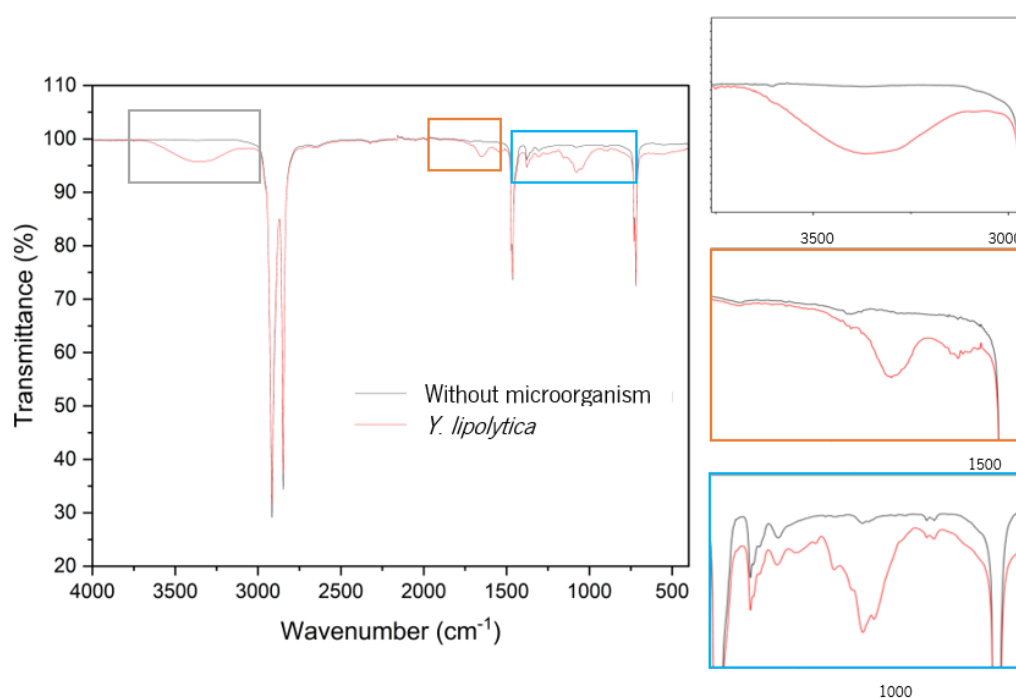


Figure 28. FTIR spectrum of PE films incubated for 90 days in Davis with and without *P. aeruginosa*. The insets (gray, blue and orange squares) represent regions showing differences in relation to the control.

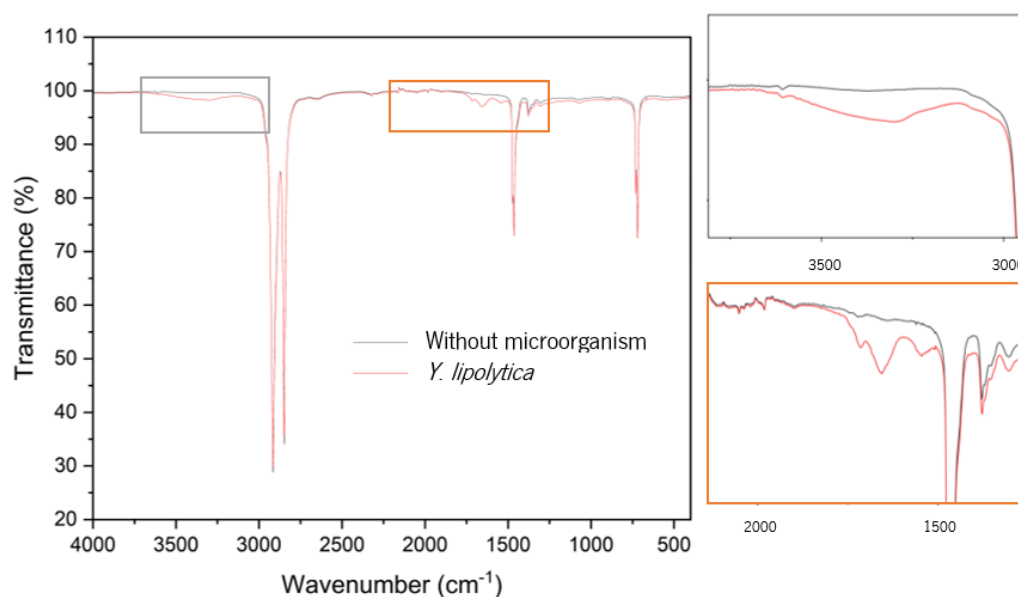


Figure 29. FTIR spectra of PE films incubated for 90 days in MBP with and without *Y. lipolytica*. The insets (gray and orange squares) represent regions showing differences in relation to the control.

Summing up, FTIR analysis revealed that chemical changes occur in the samples incubated with *P. aeruginosa* and *Y. lipolytica* which are potentially related with the process of PE biodegradation. Changes in the spectra showed chemical alterations of the plastic material and the formation of new bonds, demonstrating that both microorganisms are able to exert a transformation in the chemical structure of the synthetic polymer. The detection of new carbonyl and ether groups suggests the occurrence of oxidation reactions, providing evidence of PE biodegradation [208, 209]. Also, the presence of new bands in the 1750–1500 cm^{-1} region, indicates the formation of ketone and/or aldehyde groups that are present in the biodegradation process. Finally, the differences in the spectra were much more evident for the samples in Davis than in MBP, which may be explained by media composition since the only available carbon source in the Davis media is the PE film.

4.3.6. Film surface morphology

During the process of colonization and biodeterioration, microorganisms exert physical and chemical actions that result in plastic erosion, characterized by the formation of pores and cracks in the polymeric material [93-96, 210]. Changes at the surface of the PE films, deriving from a microbial attack by *P. aeruginosa* and *Y. lipolytica*, were assessed by scanning electron microscopy (SEM) in samples after 90 days of incubation.

Figure 30 shows representative micrographs of PE films in the absence of any treatment. As observed in the figure, the PE films present a clear and smooth surface without defects. After 90 days of incubation in media without any microorganisms, the mechanical action resulting from agitation led to the formation of very few, highly disperse holes/pores (Figure 31). This is not surprising as the mechanical stress can induce physical wear in the films, resulting in these defects.

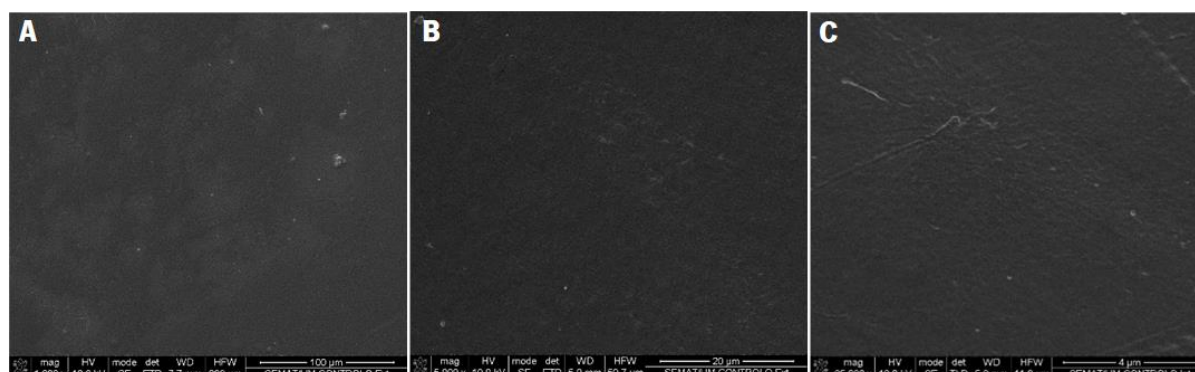


Figure 30. Micrographs of pristine PE films obtained by scanning electron microscopy (SEM) (before incubation). A) 1000x, B) 5000x, and C) 25000x magnification.

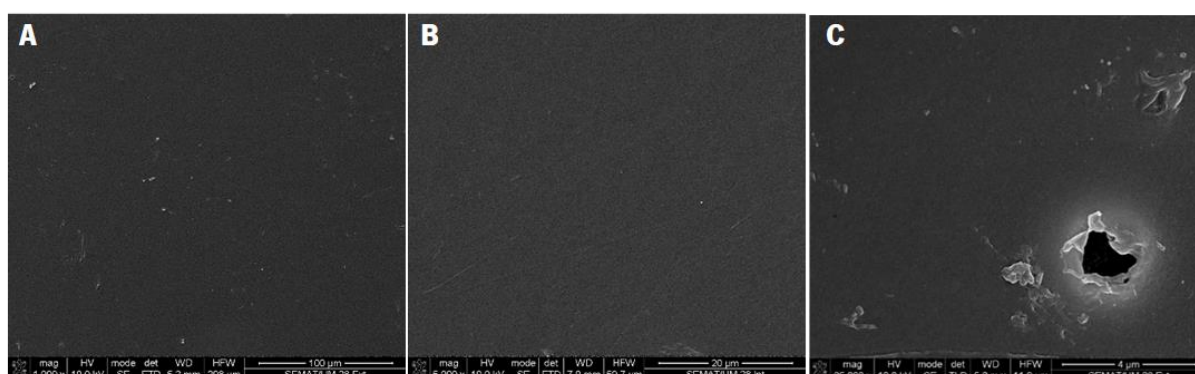


Figure 31. Micrographs of the PE film obtained by scanning electron microscopy (SEM) after 90 days of incubation in Davis media. A) 1000x, B) 5000x, and C) 25000x magnification.

Remarkably, after incubation with *P. aeruginosa*, the signs of wear on the film were highly evident, with the appearance of recurrent signs of erosion, pores and cracks (Figures 32A-D). In addition, it is also possible to observe biofilm formation and cell aggregates (Figures 32E-F) demonstrating that the bacterium strongly adheres to the surface of the film. Sivan *et al.* reported similar biofilm formation on PE by *Rhodococcus ruber*, which is a bacterium referenced for its potential to degrade PE [181]. Nonetheless, the observation of biofilm and cells in the samples was somewhat unexpected, as the films

were extensively washed with 2 % SDS prior to SEM analysis; this can explain the low values obtained for weight loss (section 4.3.4), resulting from an underestimation due to the contribution of cells and biofilm.

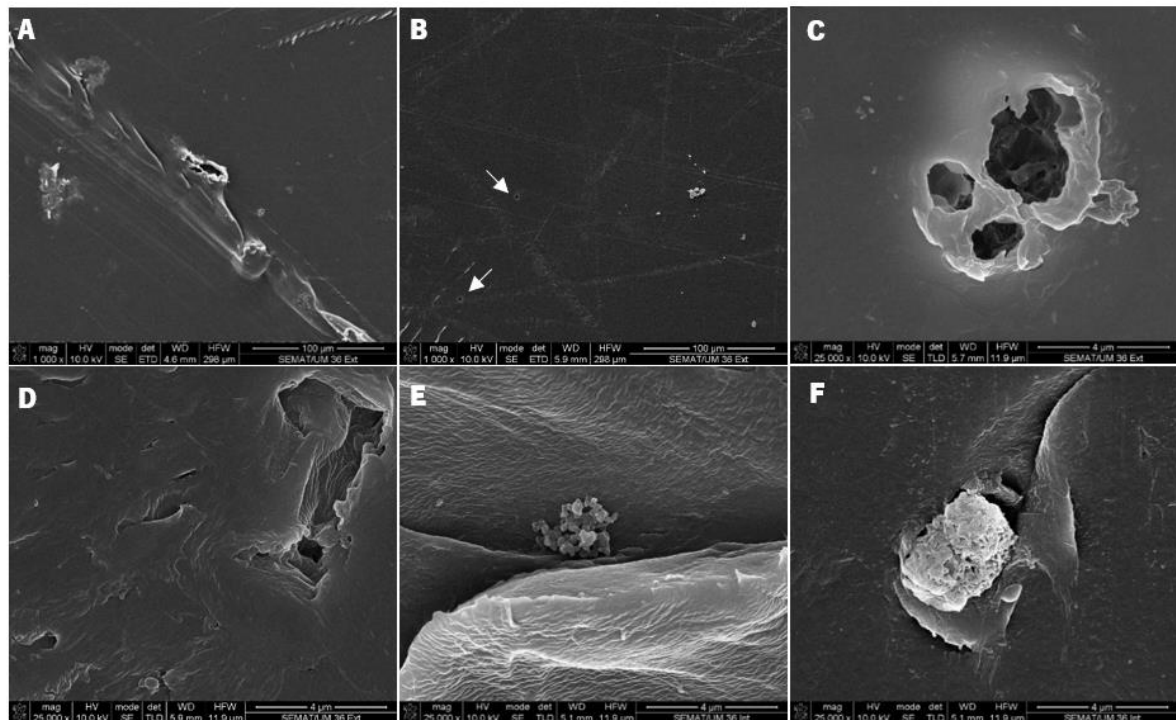


Figure 32. Micrographs of the PE film obtained by scanning electron microscopy (SEM) after 90 days of incubation with *P. aeruginosa* in Davis medium. A) 1000x, B) 1000x, C) 25000x, D) 25000x, E) 25000x, and F) 25000x magnification. Cracks can be observed in A) and D), pores in B) (white arrows) and C) and cells aggregates and biofilm in B, E), and F).

Likewise, the films exposed to *Y. lipolytica* showed a high wear compared to that observed in the control (Figure 30), with frequent signs of deterioration and holes in the surface (Figures 33D and 33F). Moreover, the presence of yeasts that resisted the washing process is clearly visible at the surface of the film, indicating the ability of *Y. lipolytica* to adhere onto the plastic material (Figures 33B-C and 33E). Again, the presence of cells at the surface of the film may have resulted in an underestimation of the weight loss mentioned in section 4.3.4. Finally, it is also possible to observe the formation of hyphae that allows the fungi to adhere more strongly to the plastic surface, which has been evidenced by other authors as an indicator for the microbial degradation of plastics [211-213].

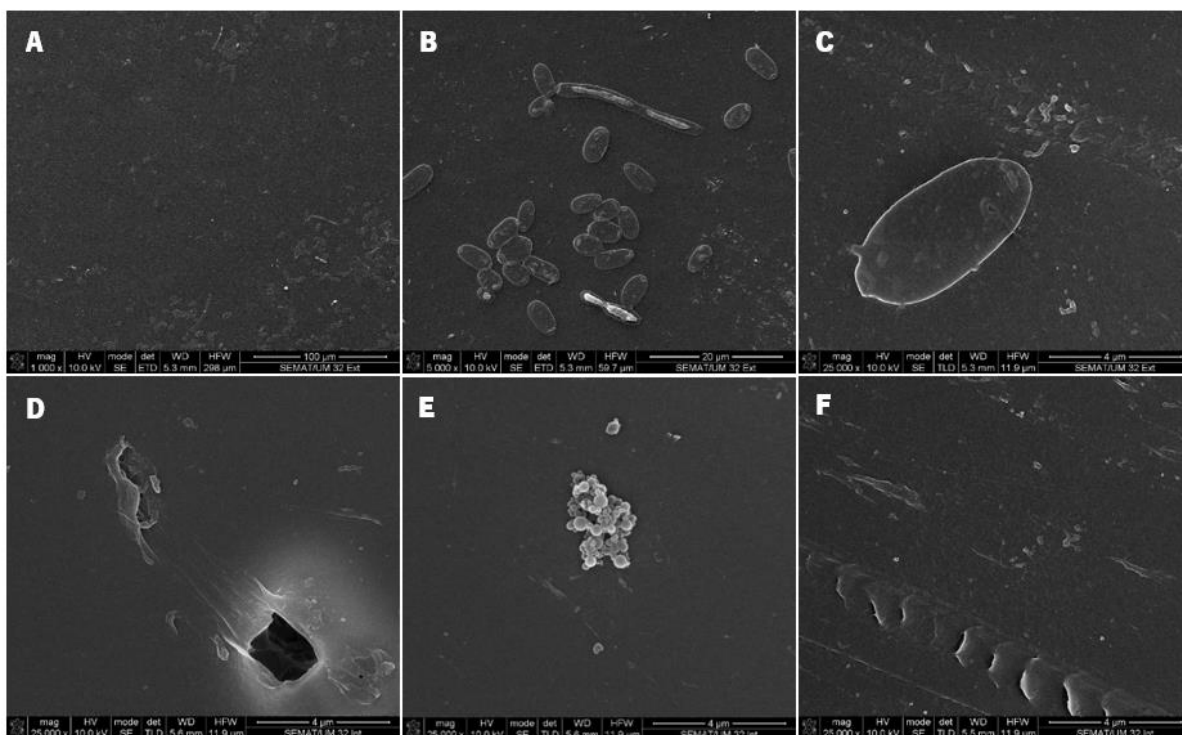


Figure 33. Micrographs of the PE film obtained by scanning electron microscopy (SEM) after 90 days of incubation with *Y. lipolytica* in Davis medium. A) 1000x, B) 5000x, C) 25000x, D) 25000x, E) 25000x, and F) 25000x magnification. Cells can be observed in A), B), and C); pores in D) cells debris in E), and cracks in F).

Regarding the films incubated in MBP, the micrographs of the control reveal some slight signs of wear and erosion (Figure 34). After incubation with *P. aeruginosa* (Figure 35) and *Y. lipolytica* (Figure 36), the signs of deterioration are clear, with the presence of holes and cracks at the surface of the films.

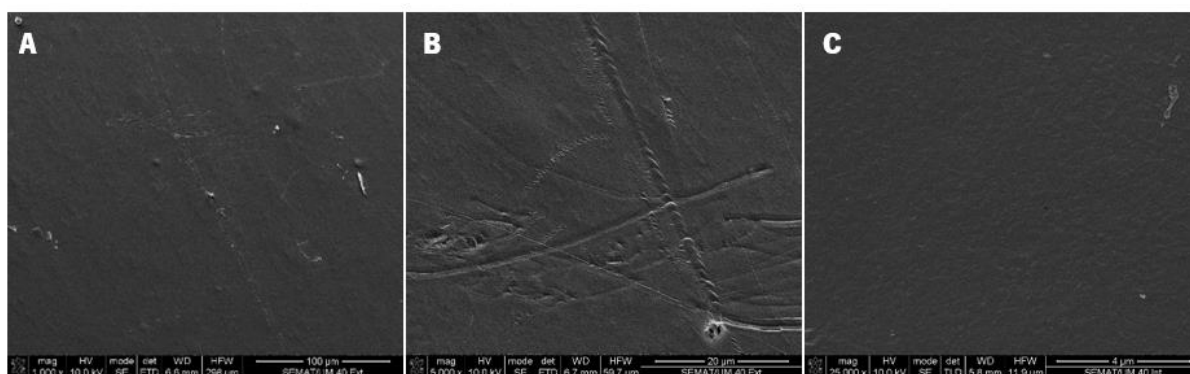


Figure 34. Micrographs of the PE film obtained by scanning electron microscopy (SEM) after 90 days of incubation in MBP media. A) 1000x, B) 5000x, and C) 25000x magnification.

As mentioned earlier, after incubation with *P. aeruginosa*, signs of degradation in the film were highly evident, as can be seen by the erosion, pores and cracks revealed in Figure 35. In addition to the more recurrent presence of these, the film showed overall further degradation.

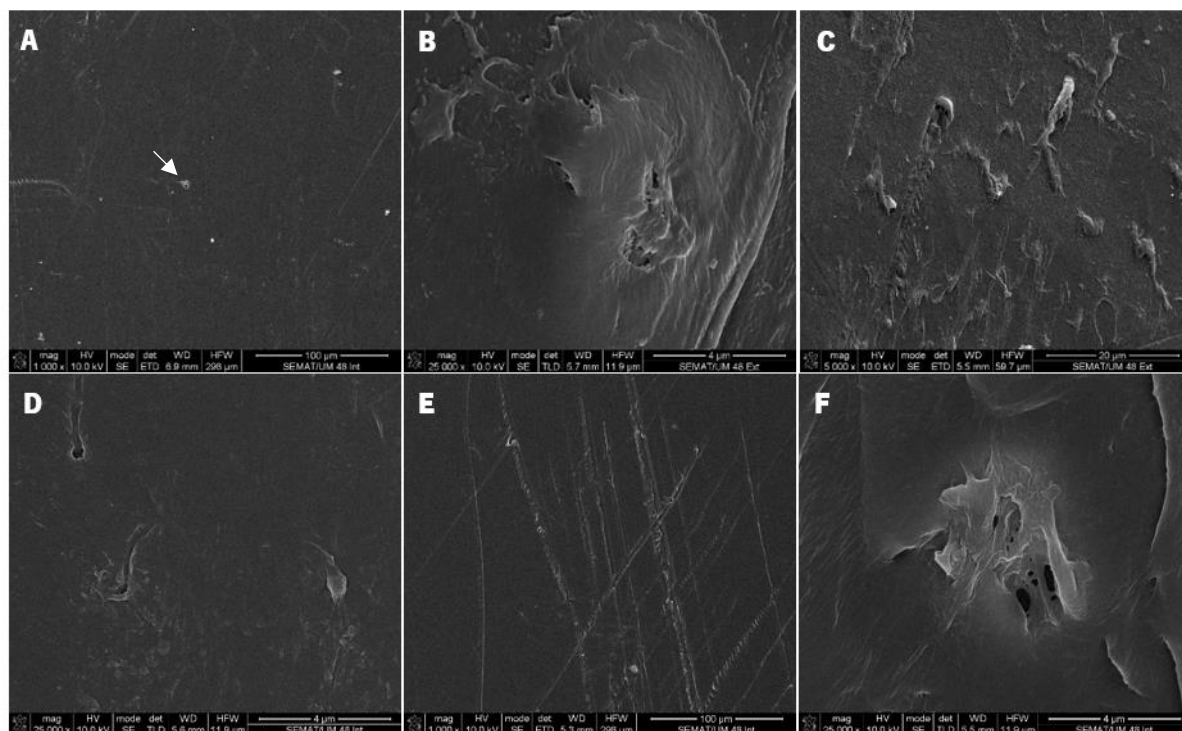


Figure 35. Micrographs of the PE film obtained by scanning electron microscopy (SEM) after 90 days of incubation with *P. aeruginosa* in MBP medium. A) 1000x, B) 25000x, C) 5000x, D) 25000x, E) 1000x, and F) 25000x magnification. Pores can be observed in A) (white arrows), and F); cracks in B)-E).

For the PE films incubated for 90 days with *Y. lipolytica*, it is also observed increased degradation, indicated by the large presence of pores and cracks, compared with the control. Furthermore, it is also noticeable the presence of microbial cells attached around the most eroded areas (Figures 36D-F), which suggests that the degradation was due to microorganisms but also explains the low values obtained for weight loss (section 4.3.4).

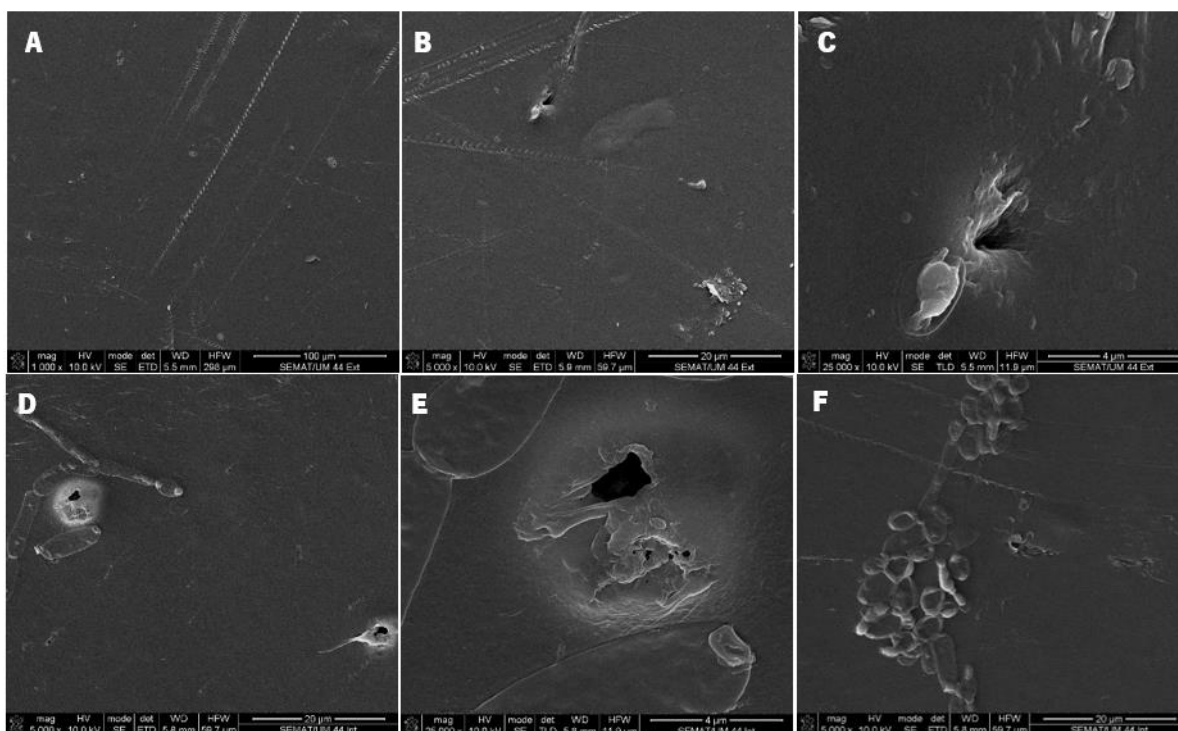


Figure 36. Micrographs of the PE film obtained by scanning electron microscopy (SEM) after 90 days of incubation with *Y. lipolytica* in MBP medium. A) 1000x, B) 5000x, C) 25000x, D) 5000x, E) 25000x, and F) 5000x magnification. Cracks can be observed in A), B), and F); cells in C) - F); pores in C), D) and E); cells debris in E), and F).

Considering the results obtained by SEM, there is strong evidence on the potential of both *P. aeruginosa* and *Y. lipolytica* to degrade PE which is supported by the remaining assays. The samples incubated with the microorganisms revealed not only biofilm formation, but also signs of erosion with frequent cracks and holes at the film surface, indicative of biodegradation. The erosion of plastic materials only occurs if the microorganisms have the ability to degrade it. For instance, Koutny and co-workers reported that even after 100 days of incubation with non-plastic degrading microorganisms, SEM analysis revealed no clear signs of bio-erosion on the surface of the films [214].

In conclusion, the work presented in this thesis clearly points out to the potential of using *P. aeruginosa* and *Y. lipolytica* as biodegraders for recalcitrant plastics, such as polyethylene. Moreover, it also gives the first evidence on the use of *Y. lipolytica* for the biodegradation of polyethylene. This provides the basis to setup a biotechnological platform for the bioremediation of plastic materials.

5. Conclusions and Future Perspectives

In order to identify promising microbial species for PE biodegradation, screening assays were performed with the microorganisms *Y. lipolytica*, *D. hansenii*, *P. aeruginosa* and *A. borkumensis*. For *D. hansenii* no significant growth or clear halos were observed on any substrate tested, while *A. borkumensis* was unable to grow on Davis and Davis + blue medium, which hindered the assessment of its potential for biodegradation of plastics. As for *Y. lipolytica*, it revealed the ability to biodegrade coconut oil and also PVA, being this the first report of PVA biodegradation. *P. aeruginosa* was able to utilize coconut oil, PVA, PEG and PCL as a carbon and energy source. Overall, these results showed that *P. aeruginosa* and *Y. lipolytica* are promising microorganisms for PE biodegradation and were selected for subsequent short-term biodegradation assays with PE films in culture media, during 90 days. In this study, several aspects were analyzed to determine the biodegradation capacity, namely microbial growth, biofilm formation, extracellular protein profile, weight loss of the PE films, assessment of chemical changes in the film, and evaluation of the film surface morphology. From these, several results support the potential of both *P. aeruginosa* and *Y. lipolytica* for the biodegradation of PE including: i) the ability to form biofilms on PE, which indicates that they can perform at least the first step of the biodegradation process; ii) the expression of extracellular proteins in the media incubated with films, not present in the control and most likely corresponding to enzymes; iii) significant changes in the FTIR spectra that show alterations in the chemical structure of the PE film, thus representing a further step in the biodegradation process; iv) visible signs of deterioration (erosion, holes and cracks) at the surface of the PE films. Determination of weight loss variation revealed no significant changes between the control and the films incubated with the microorganisms. However, conclusions on this subject must be considered with care, as SEM analysis demonstrated the presence of cells and biofilm at the surface of the PE films even after extensive washing; this may result in an underestimation of the weight loss, misleading in the interpretation of results. In this regard, although using washing conditions widely reported in the literature for the removal of cells and biofilms, this step should be optimized, especially in the situation of strongly adhered cells and hyphae-forming microorganisms.

Additionally, this work allowed to improve the screening process of microorganisms for plastics biodegradation. Regarding microbial inoculation, three different methods were studied: non-perforation, perforation and drop. The best inoculation method depends on the characteristics of the screening. For a faster analysis with a high number of samples, non-perforation or even perforation are more suitable, while for screenings requiring defined conditions, the drop method is more appropriate. Furthermore, the

addition of methylene blue in solid media, demonstrated to be a convenient approach for the visualization of clear halos, especially in the situation of using translucent media. Screening tests conducted with PE powder revealed to be inappropriate due to the aqueous insolubility of PE and its low density. As such, the screening assays resorted to the addition of substrates related with specific enzyme activities. In order to use some substrates (PCL and PSF) for assessment of the enzymatic-related activity, it was necessary to obtain emulsions through the use of a surfactant. Here, Sarkosyl proved to be less toxic to microorganisms than SDS, and the concentration of 0.35 % was the best condition for obtention of emulsions. Of notice, the use of selective/differential media supplemented with coconut oil and rhodamine B demonstrated to be an appropriate method for the screening of microorganisms capable of secreting extracellular enzymes with lipase activity.

In conclusion, the work presented in this thesis demonstrates the potential of using both *P. aeruginosa* PAO1 and *Y. lipolytica* PYCC 4811 for the biodegradation of a recalcitrant plastic such as PE. To the best of our knowledge, this work represents the first evidence on the potential of *Y. lipolytica* for the biodegradation of PE. Nonetheless, the results obtained in the screening assays also suggest that these microorganisms can be explored for the biodegradation of other plastics such as PET, PP and PS, and also for the biodegradation of emerging polymers such as PVA and PCL.

A limitation of the present study is the time period used for the biodegradation assays. Here, a short-term period of 90 days was employed. Extending this further, could lead to more pronounced differences and should be assessed in the future. Also, future experiments could be focused on the isolation and determination of the catalytic activity of the enzymes involved in the biodegradation process. The extracellular proteins present in the supernatant of microbial cultures conditioned with PE could be concentrated and used for determination of the catalytic activity in specific substrates (e.g. coconut oil) or even PE-based materials. Also, future experiments could consider the identification of these enzymes, their recombinant protein expression and protein engineering for improved activity in the biodegradation process. For this, the protein bands of SDS-PAGE can be excised and sequenced. *In silico* simulations of protein-substrate docking and molecular dynamics can be used to design more efficient enzymes with enhanced plastic degradation performance. These genetically-engineered variants can be expressed using heterologous expression systems and tested for the biodegradation activity on different polymers. Finally, since both *P. aeruginosa* and *Y. lipolytica* reveal the ability to biodegrade PE, a co-culture study with both microorganisms would be an interesting approach to study a potential synergistic effect between them that could accelerate the biodegradation process.

6. References

1. Streit-Bianchi, M., M. Cimadevila, and W. Trettnak, *Mare Plasticum - The Plastic Sea*. 1 ed. 2020: Springer Cham. 252.
2. Reis, F., S. Wu, and K. Potyatynnyk, *Life in Plastic, It's not Fantastic: The Economics of Plastic Pollution*. Science for Sustainability (S4S) Journal, 2017. **1**(1).
3. Muenmee, S., W. Chiemchaisri, and C. Chiemchaisri, *Enhancement of biodegradation of plastic wastes via methane oxidation in semi-aerobic landfill*. International Biodeterioration & Biodegradation, 2016. **113**: p. 244-255.
4. Morris, P.J.T., *Polymer pioneers: a popular history of the science and technology of large molecules*. Vol. 5. 1986: Center for History of Chemistry.
5. Massart, M., C. Dubois, and M. Boileau, *Mechanical properties and morphology of modified celluloid foams*. Journal of Applied Polymer Science, 2021. **138**(21): p. 50490.
6. Institute, S.H. *History and Future of Plastics*. Available from: <https://www.sciencehistory.org/the-history-and-future-of-plastics>.
7. Amobonye, A., et al., *Plastic biodegradation: Frontline microbes and their enzymes*. Science of the Total Environment, 2021. **759**.
8. Association, P.I. *History Of Plastics*. Available from: <https://www.plasticsindustry.org/history-plastics>.
9. Jenkins, S., et al., *Microbial degradation of plastics: New plastic degraders, mixed cultures and engineering strategies*. Soil microenvironment for bioremediation and polymer production, 2019: p. 213-238.
10. Oliveira, J., et al., *Marine environmental plastic pollution: mitigation by microorganism degradation and recycling valorization*. Frontiers in Marine Science, 2020. **7**: p. 567126.
11. Tokiwa, Y., et al., *Biodegradability of plastics*. Int J Mol Sci, 2009. **10**(9): p. 3722-42.
12. Prata, J.C., et al., *Environmental exposure to microplastics: An overview on possible human health effects*. Sci Total Environ, 2020. **702**: p. 134455.
13. PlasticsEurope, *Plastics - the Facts 2021: An analysis of European plastics production, demand and waste data*. 2021.
14. Pira, S., *The Future of Rigid Plastic Packaging to 2022*. 2018, USA.
15. Andrady, A.L. and M.A. Neal, *Applications and societal benefits of plastics*. Philos Trans R Soc Lond B Biol Sci, 2009. **364**(1526): p. 1977-84.
16. Sohail, M., D.W. Sun, and Z. Zhu, *Recent developments in intelligent packaging for enhancing food quality and safety*. Crit Rev Food Sci Nutr, 2018. **58**(15): p. 2650-2662.
17. Thompson, R.C., et al., *Our plastic age*. 2009, The Royal Society Publishing. p. 1973-1976.
18. Muncke, J., *Exposure to endocrine disrupting compounds via the food chain: Is packaging a relevant source?* Sci Total Environ, 2009. **407**(16): p. 4549-59.
19. Manoli, E. and D. Voutsas, *Food containers and packaging materials as possible source of hazardous chemicals to food*. Hazardous Chemicals Associated with Plastics in the Marine Environment, 2016: p. 19-50.
20. Geyer, R., J.R. Jambeck, and K.L. Law, *Production, use, and fate of all plastics ever made*. Sci Adv, 2017. **3**(7): p. e1700782.
21. Phelan, A.A., et al., *Plastic pollution and packaging: Corporate commitments and actions from the food and beverage sector*. Journal of Cleaner Production, 2022. **331**: p. 129827.

22. PlasticsEurope, *Plastics – the Facts 2019: An analysis of European plastics production, demand and waste data*. 2019.
23. Zheng, Y., E.K. Yanful, and A.S. Bassi, *A review of plastic waste biodegradation*. Crit Rev Biotechnol, 2005. **25**(4): p. 243-50.
24. Emblem, A., *13 - Plastics properties for packaging materials*, in *Packaging Technology*, A. Emblem and H. Emblem, Editors. 2012, Woodhead Publishing. p. 287-309.
25. Geyer, R., *A Brief History of Plastics*, in *Mare Plasticum - The Plastic Sea: Combatting Plastic Pollution Through Science and Art*, M. Streit-Bianchi, M. Cimadevila, and W. Trettnak, Editors. 2020, Springer International Publishing: Cham. p. 31-47.
26. PlasticsEurope, *Plastics - the facts 2018: An analysis of European plastics production, demand and waste data*. 2018.
27. Nowlin, T., *Global polyethylene business overview*. Business and technology of the global polyethylene industry. Wiley-VCH, Weinheim, Germany, 2014.
28. Danso, D., J. Chow, and W.R. Streit, *Plastics: Environmental and Biotechnological Perspectives on Microbial Degradation*. Appl Environ Microbiol, 2019. **85**(19).
29. Sam, S.T., et al., *Recent Advances in Polyolefins/Natural Polymer Blends Used for Packaging Application*. Polymer-Plastics Technology and Engineering, 2014. **53**(6): p. 631-644.
30. Shah, A.A., et al., *Biological degradation of plastics: a comprehensive review*. Biotechnol Adv, 2008. **26**(3): p. 246-65.
31. PlasticsEurope, *Plastics – the Facts 2020: An analysis of European plastics production, demand and waste data*. 2020.
32. Graunke, T., K. Schmitt, and J. Wöllenstein, *Organic membranes for selectivity enhancement of metal oxide gas sensors*. Journal of Sensors, 2016. **2016**.
33. Bárány, T. and J. Karger-Kocsis, *Polypropylene Handbook: Morphology, Blends and Composites*. 2019: Springer International Publishing.
34. Allahvaisi, S., *Polypropylene in the industry of food packaging*. 2012: ISSN.
35. Sastri, V.R., *Chapter 6 - Commodity Thermoplastics: Polyvinyl Chloride, Polyolefins, and Polystyrene*, in *Plastics in Medical Devices*, V.R. Sastri, Editor. 2010, William Andrew Publishing: Boston. p. 73-119.
36. Demirors, M., *STYRENE POLYMERS AND COPOLYMERS*, in *Applied Polymer Science: 21st Century*, C.D. Craver and C.E. Carraher, Editors. 2000, Pergamon: Oxford. p. 93-106.
37. Smith, R., H. Inomata, and C. Peters, *Chapter 4 - Historical Background and Applications*, in *Supercritical Fluid Science and Technology*, R. Smith, H. Inomata, and C. Peters, Editors. 2013, Elsevier. p. 175-273.
38. Zanela, T.M.P., E.C. Muniz, and C.A.P. Almeida, *Chemical recycling of poly (Ethylene terephthalate)(PET) by alkaline hydrolysis and catalyzed glycolysis*. Orbital: The Electronic Journal of Chemistry, 2018: p. 226-233.
39. Barnard, E., J.J.R. Arias, and W. Thielemans, *Chemolytic depolymerisation of PET: A review*. Green Chemistry, 2021. **23**(11): p. 3765-3789.
40. Jambeck, J.R., et al., *Plastic waste inputs from land into the ocean*. Science, 2015. **347**(6223): p. 768-771.
41. Barra, R., et al., *Plastics and the Circular Economy: A STAP Document*. Scientific and Technical Advisory Panel (STAP) to the Global Environment Facility (GEF), UN Environment, 2018.
42. Vanapalli, K.R., et al., *Challenges and strategies for effective plastic waste management during and post COVID-19 pandemic*. Sci Total Environ, 2021. **750**: p. 141514.
43. Pathak, S., C. Sneha, and B.B. Mathew, *Bioplastics: its timeline based scenario & challenges*. Journal of Polymer and Biopolymer Physics Chemistry, 2014. **2**(4): p. 84-90.

44. Ahmed, T., et al., *Biodegradation of plastics: current scenario and future prospects for environmental safety*. Environ Sci Pollut Res Int, 2018. **25**(8): p. 7287-7298.
45. Mekonnen, T., et al., *Progress in bio-based plastics and plasticizing modifications*. Journal of Materials Chemistry A, 2013. **1**(43): p. 13379-13398.
46. EuropeanBioplastics, *What are bioplastics? Material types, terminology, and labels – an introduction*. 2018.
47. Emadian, S.M., T.T. Onay, and B. Demirel, *Biodegradation of bioplastics in natural environments*. Waste Manag, 2017. **59**: p. 526-536.
48. EuropeanBioplastics. *Bioplastics market data*. 2021; Available from: <https://www.european-bioplastics.org/market/>.
49. Espinosa, M.J.C., et al., *Toward Biorecycling: Isolation of a Soil Bacterium That Grows on a Polyurethane Oligomer and Monomer*. Front Microbiol, 2020. **11**: p. 404.
50. Geyer, R., J.R. Jambeck, and K.L. Law, *Production, use, and fate of all plastics ever made*. Science Advances, 2017. **3**(7).
51. Chamas, A., et al., *Degradation Rates of Plastics in the Environment*. ACS Sustainable Chemistry & Engineering, 2020. **8**(9): p. 3494-3511.
52. Schulze, G., *Growth Within: A Circular Economy Vision for a Competitive Europe*. Ellen MacArthur Foundation and the McKinsey Center for Business and Environment, 2016: p. 1-22.
53. Ali, S.S., et al., *Degradation of conventional plastic wastes in the environment: A review on current status of knowledge and future perspectives of disposal*. Sci Total Environ, 2021. **771**: p. 144719.
54. Ru, J., Y. Huo, and Y. Yang, *Microbial Degradation and Valorization of Plastic Wastes*. Front Microbiol, 2020. **11**: p. 442.
55. Lahive, E., et al., *Microplastic particles reduce reproduction in the terrestrial worm *Enchytraeus crypticus* in a soil exposure*. Environ Pollut, 2019. **255**(Pt 2): p. 113174.
56. Selonen, S., et al., *Exploring the impacts of plastics in soil - The effects of polyester textile fibers on soil invertebrates*. Sci Total Environ, 2020. **700**: p. 134451.
57. Huerta Lwanga, E., et al., *Field evidence for transfer of plastic debris along a terrestrial food chain*. Sci Rep, 2017. **7**(1): p. 14071.
58. Chen, Q., et al., *Leaching of endocrine disrupting chemicals from marine microplastics and mesoplastics under common life stress conditions*. Environ Int, 2019. **130**: p. 104938.
59. Proshad, R., et al., *Toxic effects of plastic on human health and environment: A consequences of health risk assessment in Bangladesh*. International Journal of Health, 2018. **6**(1): p. 1-5.
60. Thushari, G.G.N. and J.D.M. Senevirathna, *Plastic pollution in the marine environment*. Heliyon, 2020. **6**(8): p. e04709.
61. Gregory, M.R., *Environmental implications of plastic debris in marine settings—entanglement, ingestion, smothering, hangers-on, hitch-hiking and alien invasions*. Philos Trans R Soc Lond B Biol Sci, 2009. **364**(1526): p. 2013-25.
62. Beaumont, N.J., et al., *Global ecological, social and economic impacts of marine plastic*. Mar Pollut Bull, 2019. **142**: p. 189-195.
63. Wyles, K.J., et al., *Factors that can undermine the psychological benefits of coastal environments: exploring the effect of tidal state, presence, and type of litter*. Environment and Behavior, 2016. **48**(9): p. 1095-1126.
64. Steer, M. and R.C. Thompson, *Plastics and microplastics: impacts in the marine environment, in Mare plasticum-the plastic sea*. 2020, Springer. p. 49-72.
65. Horton, A.A., et al., *Microplastics in freshwater and terrestrial environments: Evaluating the current understanding to identify the knowledge gaps and future research priorities*. Sci Total Environ, 2017. **586**: p. 127-141.

66. Zhu, D., et al., *Exposure of soil collembolans to microplastics perturbs their gut microbiota and alters their isotopic composition*. Soil Biology & Biochemistry, 2018. **116**: p. 302-310.
67. Scheurer, M. and M. Bigalke, *Microplastics in Swiss Floodplain Soils*. Environmental Science & Technology, 2018. **52**(6): p. 3591-3598.
68. Wright, S.L., et al., *Microplastic ingestion decreases energy reserves in marine worms*. Curr Biol, 2013. **23**(23): p. R1031-3.
69. von Moos, N., P. Burkhardt-Holm, and A. Kohler, *Uptake and effects of microplastics on cells and tissue of the blue mussel *Mytilus edulis* L. after an experimental exposure*. Environ Sci Technol, 2012. **46**(20): p. 11327-35.
70. Lu, Y., et al., *Uptake and Accumulation of Polystyrene Microplastics in Zebrafish (*Danio rerio*) and Toxic Effects in Liver*. Environ Sci Technol, 2016. **50**(7): p. 4054-60.
71. Sussarellu, R., et al., *Oyster reproduction is affected by exposure to polystyrene microplastics*. Proceedings of the national academy of sciences, 2016. **113**(9): p. 2430-2435.
72. Smith, M., et al., *Microplastics in Seafood and the Implications for Human Health*. Curr Environ Health Rep, 2018. **5**(3): p. 375-386.
73. Machado, A.A.D., et al., *Microplastics as an emerging threat to terrestrial ecosystems*. Global Change Biology, 2018. **24**(4): p. 1405-1416.
74. Prata, J.C., et al., *Environmental exposure to microplastics: An overview on possible human health effects*. Science of the Total Environment, 2020. **702**.
75. Wright, S.L. and F.J. Kelly, *Plastic and Human Health: A Micro Issue?* Environ Sci Technol, 2017. **51**(12): p. 6634-6647.
76. Ragusa, A., et al., *Plasticenta: First evidence of microplastics in human placenta*. Environ Int, 2021. **146**: p. 106274.
77. Ragusa, A., et al., *Raman Microspectroscopy Detection and Characterisation of Microplastics in Human Breastmilk*. Polymers (Basel), 2022. **14**(13).
78. Sabiha-Javied, M. Tufail, and S. Khalid, *Heavy metal pollution from medical waste incineration at Islamabad and Rawalpindi, Pakistan*. Microchemical Journal, 2008. **90**(1): p. 77-81.
79. Sharma, R., et al., *The impact of incinerators on human health and environment*. Rev Environ Health, 2013. **28**(1): p. 67-72.
80. da Silva, F.J.G. and R.M. Gouveia, *Cleaner Production Tools and Environmental Management Practices*, in *Cleaner Production*. 2020, Springer. p. 153-245.
81. Kuczenski, B. and R. Geyer, *PET bottle reverse logistics—environmental performance of California's CRV program*. The International Journal of Life Cycle Assessment, 2013. **18**(2): p. 456-471.
82. Gu, J., *Microbial corrosion of metals and deterioration of polymeric materials*. Journal of Materials Engineering, 1999.
83. Restrepo-Florez, J.M., A. Bassi, and M.R. Thompson, *Microbial degradation and deterioration of polyethylene - A review*. International Biodeterioration & Biodegradation, 2014. **88**: p. 83-90.
84. Yang, J., et al., *Evidence of polyethylene biodegradation by bacterial strains from the guts of plastic-eating waxworms*. Environ Sci Technol, 2014. **48**(23): p. 13776-84.
85. Yoshida, S., et al., *A bacterium that degrades and assimilates poly(ethylene terephthalate)*. Science, 2016. **351**(6278): p. 1196-1199.
86. Knott, B.C., et al., *Characterization and engineering of a two-enzyme system for plastics depolymerization*. Proc Natl Acad Sci U S A, 2020. **117**(41): p. 25476-25485.
87. Dvorak, P., et al., *Bioremediation 3.0: Engineering pollutant-removing bacteria in the times of systemic biology*. Biotechnology Advances, 2017. **35**(7): p. 845-866.
88. Wei, R. and W. Zimmermann, *Biocatalysis as a green route for recycling the recalcitrant plastic polyethylene terephthalate*. Microbial Biotechnology, 2017. **10**(6): p. 1302-1307.

89. Wei, R. and W. Zimmermann, *Microbial enzymes for the recycling of recalcitrant petroleum-based plastics: how far are we?* Microbial Biotechnology, 2017. **10**(6): p. 1308-1322.
90. Schwibbert, K., et al., *Bacterial Adhesion on Femtosecond Laser-Modified Polyethylene*. Materials (Basel), 2019. **12**(19).
91. Tribedi, P., A.D. Gupta, and A.K. Sil, *Adaptation of Pseudomonas sp. AKS2 in biofilm on low-density polyethylene surface: an effective strategy for efficient survival and polymer degradation*. Bioresources and Bioprocessing, 2015. **2**(1): p. 1-10.
92. Gu, J.D., *Microbiological deterioration and degradation of synthetic polymeric materials: recent research advances*. International Biodeterioration & Biodegradation, 2003. **52**(2): p. 69-91.
93. Bonhomme, S., et al., *Environmental biodegradation of polyethylene*. Polymer Degradation and Stability, 2003. **81**(3): p. 441-452.
94. Hakkarainen, M., S. Karlsson, and A.C. Albertsson, *Rapid (bio)degradation of polylactide by mixed culture of compost microorganisms - low molecular weight products and matrix changes*. Polymer, 2000. **41**(7): p. 2331-2338.
95. Jennings, D.H. and G. Lysek, *Fungal Biology: Understanding the Fungal Lifestyle*. 1999: betaIOS Scientific.
96. Otsuka, Y., et al., *Detection and characterization of a novel extracellular fungal enzyme that catalyzes the specific and hydrolytic cleavage of lignin guaiacylglycerol beta-aryl ether linkages*. European Journal of Biochemistry, 2003. **270**(11): p. 2353-2362.
97. Ho, B.T., T.K. Roberts, and S. Lucas, *An overview on biodegradation of polystyrene and modified polystyrene: the microbial approach*. Critical Reviews in Biotechnology, 2018. **38**(2): p. 308-320.
98. Williams, S. and O. Peoples, *Biodegradable plastics from plants*. Chemtech, 1996. **26**(9): p. 38-44.
99. De Wilde, B., *5. International and national norms on biodegradability and certification procedures*, in *Handbook of biodegradable polymers*. 2020, De Gruyter. p. 115-146.
100. Lebreton, L., et al., *Evidence that the Great Pacific Garbage Patch is rapidly accumulating plastic*. Scientific reports, 2018. **8**(1): p. 1-15.
101. Huerta Lwanga, E., et al., *Microplastics in the Terrestrial Ecosystem: Implications for Lumbricus terrestris (Oligochaeta, Lumbricidae)*. Environ Sci Technol, 2016. **50**(5): p. 2685-91.
102. Fotopoulou, K.N. and H.K. Karapanagioti, *Degradation of various plastics in the environment*. Hazardous chemicals associated with plastics in the marine environment, 2017: p. 71-92.
103. Singh, B. and N. Sharma, *Mechanistic implications of plastic degradation*. Polymer degradation and stability, 2008. **93**(3): p. 561-584.
104. Padsalgikar, A., *Biological properties of plastics*. Plastics in medical devices for cardiovascular applications. William Andrew Publishing, Cambridge, MA, 2017: p. 83-102.
105. Jenkins, M. and K. Harrison, *The effect of crystalline morphology on the degradation of polycaprolactone in a solution of phosphate buffer and lipase*. polymers for Advanced Technologies, 2008. **19**(12): p. 1901-1906.
106. Kitamoto, H.K., et al., *Phyllosphere yeasts rapidly break down biodegradable plastics*. AMB Express, 2011. **1**: p. 44.
107. Krueger, M.C., et al., *Degradation of polystyrene and selected analogues by biological Fenton chemistry approaches: Opportunities and limitations*. Chemosphere, 2017. **173**: p. 520-528.
108. Conradie, W., et al., *Investigating the Physicochemical Property Changes of Plastic Packaging Exposed to UV Irradiation and Different Aqueous Environments*. Microplastics, 2022. **1**(3): p. 456-476.
109. Gricajeva, A., A.K. Nadda, and R. Gudiukaite, *Insights into polyester plastic biodegradation by carboxyl ester hydrolases*. Journal of Chemical Technology & Biotechnology, 2022. **97**(2): p. 359-380.

110. Alcántara, A.R., M.J. Hernaiz, and J.V. Sinisterra, *3.28 - Biocatalyzed Production of Fine Chemicals*, in *Comprehensive Biotechnology (Second Edition)*, M. Moo-Young, Editor. 2011, Academic Press: Burlington. p. 309-331.
111. Athiappan, M., et al., *Chapter 13 - Rhizosphere engineering through pesticides-degrading beneficial bacteria*, in *Rhizosphere Engineering*, R.C. Dubey and P. Kumar, Editors. 2022, Academic Press. p. 239-257.
112. Ramli, A.N.M., et al., *Chapter 25 - An overview of enzyme technology used in food industry*, in *Value-Addition in Food Products and Processing Through Enzyme Technology*, M. Kuddus and C.N. Aguilar, Editors. 2022, Academic Press. p. 333-345.
113. Manubolu, M., et al., *Chapter 16 - Enzymes as direct decontaminating agents—mycotoxins*, in *Enzymes in Human and Animal Nutrition*, C.S. Nunes and V. Kumar, Editors. 2018, Academic Press. p. 313-330.
114. Jaiswal, S., B. Sharma, and P. Shukla, *Integrated approaches in microbial degradation of plastics*. Environmental Technology & Innovation, 2020. **17**: p. 100567.
115. Ghosh, S.K., S. Pal, and S. Ray, *Study of microbes having potentiality for biodegradation of plastics*. Environmental Science and Pollution Research, 2013. **20**(7): p. 4339-4355.
116. Mohanan, N., et al., *Microbial and Enzymatic Degradation of Synthetic Plastics*. Front Microbiol, 2020. **11**: p. 580709.
117. Gambarini, V., et al., *Phylogenetic Distribution of Plastic-Degrading Microorganisms*. mSystems, 2021. **6**(1).
118. Liu, J., et al., *Biodegradation and up-cycling of polyurethanes: Progress, challenges, and prospects*. Biotechnol Adv, 2021. **48**: p. 107730.
119. Taghavi, N., et al., *Challenges in biodegradation of non-degradable thermoplastic waste: From environmental impact to operational readiness*. Biotechnol Adv, 2021. **49**: p. 107731.
120. Ghatge, S., et al., *Biodegradation of polyethylene: a brief review*. Applied Biological Chemistry, 2020. **63**(1): p. 1-14.
121. Santo, M., R. Weitsman, and A. Sivan, *The role of the copper-binding enzyme – laccase – in the biodegradation of polyethylene by the actinomycete Rhodococcus ruber*. International Biodeterioration & Biodegradation, 2013. **84**: p. 204-210.
122. Yoon, M.G., H.J. Jeon, and M.N. Kim, *Biodegradation of polyethylene by a soil bacterium and AlkB cloned recombinant cell*. J Bioremed Biodegrad, 2012. **3**(4): p. 1-8.
123. Kong, D., et al., *Chemical-biological degradation of polyethylene combining Baeyer–Villiger oxidation and hydrolysis reaction of cutinase*. Green Chemistry, 2022. **24**(5): p. 2203-2211.
124. Zadjelovic, V., et al., *A mechanistic understanding of polyethylene biodegradation by the marine bacterium Alcanivorax*. Journal of Hazardous Materials, 2022. **436**: p. 129278.
125. Molitor, R., et al., *Agar plate-based screening methods for the identification of polyester hydrolysis by Pseudomonas species*. Microb Biotechnol, 2020. **13**(1): p. 274-284.
126. Park, S.L., et al., *Isolation of Microbulbifer sp. SOL66 with High Polyhydroxyalkanoate-Degrading Activity from the Marine Environment*. Polymers, 2021. **13**(23): p. 4257.
127. Schneider, C.A., W.S. Rasband, and K.W. Eliceiri, *NIH Image to ImageJ: 25 years of image analysis*. Nature Methods, 2012. **9**(7): p. 671-675.
128. Delacuvellerie, A., et al., *The plastisphere in marine ecosystem hosts potential specific microbial degraders including Alcanivorax borkumensis as a key player for the low-density polyethylene degradation*. J Hazard Mater, 2019. **380**: p. 120899.
129. Rose, R.S., et al., *Microbial Degradation of Plastic in Aqueous Solutions Demonstrated by CO₂ Evolution and Quantification*. Int J Mol Sci, 2020. **21**(4).
130. Kyaw, B.M., et al., *Biodegradation of Low Density Polythene (LDPE) by Pseudomonas Species*. Indian J Microbiol, 2012. **52**(3): p. 411-9.

131. Lee, H.M., et al., *Evaluation of the Biodegradation Efficiency of Four Various Types of Plastics by Pseudomonas aeruginosa Isolated from the Gut Extract of Superworms*. Microorganisms, 2020. **8**(9).
132. Pettinari, M.J. and D.E. Egoburo, *Chapter 23 - Manipulation of global regulators in Escherichia coli for the synthesis of biotechnologically relevant products*, in *Microbial Cell Factories Engineering for Production of Biomolecules*, V. Singh, Editor. 2021, Academic Press. p. 437-453.
133. Matavuly, M. and H.P. Molitoris, *In vitro degradation of poly [(r)-3-hydroxybutyrate] and BIOPOL™ by marine-derived fungi*. Biologia Serbica, 2016. **37**(1-2).
134. Gonda, K., D. Jendrosseck, and H.-P. Molitoris, *Fungal degradation of the thermoplastic polymer poly-β-hydroxybutyric acid (PHB) under simulated deep sea pressure*, in *Life at Interfaces and Under Extreme Conditions*. 2000, Springer. p. 173-183.
135. Mihreteab, M., B.A. Stubblefield, and E.S. Gilbert, *Microbial bioconversion of thermally depolymerized polypropylene by Yarrowia lipolytica for fatty acid production*. Appl Microbiol Biotechnol, 2019. **103**(18): p. 7729-7740.
136. Manilla - Pérez, E., et al., *Neutral lipid production in Alcanivorax borkumensis SK2 and other marine hydrocarbonoclastic bacteria*. European journal of lipid science and technology, 2011. **113**(1): p. 8-17.
137. Heinz, J., V. Rambags, and D. Schulze-Makuch, *Physicochemical Parameters Limiting Growth of Debaryomyces hansenii in Solutions of Hygroscopic Compounds and Their Effects on the Habitability of Martian Brines*. Life, 2021. **11**(11): p. 1194.
138. SadrAzodi, S.M., et al., *Biodegradation of long chain alkanes in halophilic conditions by Alcanivorax sp. strain Est-02 isolated from saline soil*. 3 Biotech, 2019. **9**(4): p. 141.
139. Sekova, V.Y., et al., *Soluble Sugar and Lipid Readjustments in the Yarrowia lipolytica Yeast at Various Temperatures and pH*. Metabolites, 2019. **9**(12): p. 307.
140. Barbier, M., et al., *From the Environment to the Host: Re-Wiring of the Transcriptome of Pseudomonas aeruginosa from 22°C to 37°C*. PLOS ONE, 2014. **9**(2): p. e89941.
141. Zhang, J., et al., *Selection and evaluation of microorganisms for biodegradation of agricultural plastic film*. 3 Biotech, 2018. **8**(7): p. 308.
142. Urbanek, A.K., M.C. Strzelecki, and A.M. Mirończuk, *The potential of cold-adapted microorganisms for biodegradation of bioplastics*. Waste Management, 2021. **119**: p. 72-81.
143. Vogel, F.A., et al., *Comparative Genomics of Marine Bacteria from a Historically Defined Plastic Biodegradation Consortium with the Capacity to Biodegrade Polyhydroxyalkanoates*. Microorganisms, 2021. **9**(1).
144. Doble, M. and A. Kumar, *Chapter 9, Biotreatment of Industrial Effluents*. 2005, USA: Butterworth-Heinemann, Elsevier.
145. Wu, H.F., et al., *Biodegradation of polyvinyl alcohol by different dominant degrading bacterial strains in a baffled anaerobic bioreactor*. Water Sci Technol, 2019. **79**(10): p. 2005-2012.
146. Larking, D.M., et al., *Enhanced degradation of polyvinyl alcohol by Pycnoporus cinnabarinus after pretreatment with Fenton's reagent*. Appl Environ Microbiol, 1999. **65**(4): p. 1798-800.
147. Sakai, K., et al., *Purification and characterization of an esterase involved in poly(vinyl alcohol) degradation by Pseudomonas vesicularis PD*. Biosci Biotechnol Biochem, 1998. **62**(10): p. 2000-7.
148. Suhaimi, N., et al., *Biodegradation of PEGs: A review*. Journal of Biochemistry, Microbiology and Biotechnology, 2021. **9**(1): p. 8-18.
149. Frings, J., E. Schramm, and B. Schink, *Enzymes Involved in Anaerobic Polyethylene Glycol Degradation by Pelobacter venetianus and Bacteroides Strain PG1*. Appl Environ Microbiol, 1992. **58**(7): p. 2164-7.

150. Rana, K. and N. Rana, *Isolation and Screening of Plastic Degrading Bacteria from Dumping Sites of Solid Waste*. Int. J. Curr. Microbiol. App. Sci, 2020. **9**(7): p. 2611-2618.
151. Roberts, C., et al., *Environmental Consortium Containing Pseudomonas and Bacillus Species Synergistically Degrades Polyethylene Terephthalate Plastic*. mSphere, 2020. **5**(6).
152. Nair, N., et al., *Biodegradation of biopolymers*, in *Current developments in biotechnology and bioengineering*. 2017, Elsevier. p. 739-755.
153. Murphy, C.A., et al., *Fusarium polycaprolactone depolymerase is cutinase*. Applied and environmental microbiology, 1996. **62**(2): p. 456-460.
154. Murphy, C., et al., *A second polycaprolactone depolymerase from Fusarium, a lipase distinct from cutinase*. Applied microbiology and biotechnology, 1998. **50**(6): p. 692-696.
155. Tokiwa, Y., et al., *Biodegradability of plastics*. International journal of molecular sciences, 2009. **10**(9): p. 3722-3742.
156. Richert, A. and G.B. Dąbrowska, *Enzymatic degradation and biofilm formation during biodegradation of polylactide and polycaprolactone polymers in various environments*. International Journal of Biological Macromolecules, 2021. **176**: p. 226-232.
157. Burg, K. and S. Shalaby, *PES and PEEK*. Encyclopedia of materials: Science and Technology, 2011: p. 6837-6839.
158. Chen, Y., B. Lin, and Y. Qiu, *Modification of polysulfone and the biomedical application of modified polysulfone*. International Journal of Polymeric Materials and Polymeric Biomaterials, 2021: p. 1-19.
159. Yadav, P., et al., *Assessment of the environmental impact of polymeric membrane production*. Journal of Membrane Science, 2021. **622**: p. 118987.
160. Naether, D.J., et al., *Adaptation of the hydrocarbonoclastic bacterium Alcanivorax borkumensis SK2 to alkanes and toxic organic compounds: a physiological and transcriptomic approach*. Appl Environ Microbiol, 2013. **79**(14): p. 4282-93.
161. Aguirre-Ramírez, M., et al., *Surfactants: physicochemical interactions with biological macromolecules*. Biotechnology Letters, 2021. **43**(3): p. 523-535.
162. Falk, N.A., *Surfactants as antimicrobials: a brief overview of microbial interfacial chemistry and surfactant antimicrobial activity*. Journal of Surfactants and Detergents, 2019. **22**(5): p. 1119-1127.
163. Ashok, A., A. Kumar, and F. Tarlochan, *10 - Colloidal metal oxide nanocrystals in catalysis*, in *Colloidal Metal Oxide Nanoparticles*, S. Thomas, A. Tresa Sunny, and P. Velayudhan, Editors. 2020, Elsevier. p. 247-288.
164. Nakama, Y., *Chapter 15 - Surfactants*, in *Cosmetic Science and Technology*, K. Sakamoto, et al., Editors. 2017, Elsevier: Amsterdam. p. 231-244.
165. Lackie, J.M., *S*, in *The Dictionary of Cell & Molecular Biology (Fifth Edition)*, J.M. Lackie, Editor. 2013, Academic Press: Boston. p. 581-644.
166. Thibeault, J., et al., *Sarkosyl: A milder detergent than SDS for identifying proteins with moderately high hyperstability using gel electrophoresis*. Analytical Biochemistry, 2019. **571**: p. 21-24.
167. Urbanek, A.K., et al., *Isolation and characterization of Arctic microorganisms decomposing bioplastics*. AMB Express, 2017. **7**(1): p. 148.
168. Shahbazi, R., et al., *Screening of SDS-degrading bacteria from car wash wastewater and study of the alkylsulfatase enzyme activity*. Iran J Microbiol, 2013. **5**(2): p. 153-8.
169. Lopes, M., et al., *The use of olive mill wastewater by wild type Yarrowia lipolytica strains: medium supplementation and surfactant presence effect*. Journal of Chemical Technology & Biotechnology: International Research in Process, Environmental & Clean Technology, 2009. **84**(4): p. 533-537.

170. Dhiman, S. and S.S. Chapadgaonkar, *Optimization of lipase production medium for a bacterial isolate*. Inter J Chem Technol Res, 2013. **5**: p. 2837-2843.
171. Vague, M., et al., *Pseudomonas isolates degrade and form biofilms on polyethylene terephthalate (PET) plastic*. BioRxiv, 2019: p. 647321.
172. Muthezhilan, R., et al., *Production and purification of biosurfactant from marine yeast isolated from kelambakkam salterns*. Biosci. Biotechnol. Res. Asia, 2014. **11**: p. 59-67.
173. Fickers, P., et al., *Hydrophobic substrate utilisation by the yeast Yarrowia lipolytica, and its potential applications*. FEMS Yeast Res, 2005. **5**(6-7): p. 527-43.
174. Zinjarde, S.S., et al., *Morphogenetic behavior of tropical marine yeast Yarrowia lipolytica in response to hydrophobic substrates*. Journal of microbiology and biotechnology, 2008. **18**(9): p. 1522-1528.
175. Bharathiraja, B., et al., *Biodegradation of Poly (vinyl alcohol) using Pseudomonas alcaligenes*. Asian Journal of Chemistry, 2013. **25**(15): p. 8663.
176. Obradors, N. and J. Aguilar, *Efficient biodegradation of high-molecular-weight polyethylene glycols by pure cultures of Pseudomonas stutzeri*. Appl Environ Microbiol, 1991. **57**(8): p. 2383-8.
177. Kaskatepe, B. and S. Yildiz, *Rhamnolipid biosurfactants produced by Pseudomonas species*. Brazilian Archives of Biology and Technology, 2016. **59**.
178. Samykannu, M. and A. Achary, *Utilization of Agro-Industry Residue for Rhamnolipid Production by P. aeruginosa AMB AS7 and Its Application in Chromium Removal*. Appl Biochem Biotechnol, 2017. **183**(1): p. 70-90.
179. Mohanan, N., et al., *Characterization of Polymer Degrading Lipases, LIP1 and LIP2 From Pseudomonas chlororaphis PA23*. Front Bioeng Biotechnol, 2022. **10**: p. 854298.
180. Jaeger, K.E., A. Steinbuchel, and D. Jendrosseck, *Substrate specificities of bacterial polyhydroxyalkanoate depolymerases and lipases: bacterial lipases hydrolyze poly(omega-hydroxyalkanoates)*. Appl Environ Microbiol, 1995. **61**(8): p. 3113-8.
181. Sivan, A., M. Szanto, and V. Pavlov, *Biofilm development of the polyethylene-degrading bacterium Rhodococcus ruber*. Appl Microbiol Biotechnol, 2006. **72**(2): p. 346-52.
182. Han, Y.N., et al., *Greater Biofilm Formation and Increased Biodegradation of Polyethylene Film by a Microbial Consortium of Arthrobacter sp. and Streptomyces sp.* Microorganisms, 2020. **8**(12).
183. Aggarwal, S., P.S. Stewart, and R.M. Hozalski, *Biofilm cohesive strength as a basis for biofilm recalcitrance: are bacterial biofilms overdesigned?* Microbiology Insights, 2015. **8**: p. MBI. S31444.
184. Hall-Stoodley, L., J.W. Costerton, and P. Stoodley, *Bacterial biofilms: from the natural environment to infectious diseases*. Nat Rev Microbiol, 2004. **2**(2): p. 95-108.
185. López, D., H. Vlamakis, and R. Kolter, *Biofilms*. Cold Spring Harbor perspectives in biology, 2010. **2**(7): p. a000398.
186. Shukla, S.K. and T.S. Rao, *An improved crystal violet assay for biofilm quantification in 96-well microtitre plate*. Biorxiv, 2017: p. 100214.
187. Veloz, J.J., M. Alvear, and L.A. Salazar, *Evaluation of Alternative Methods to Assess the Biological Properties of Propolis on Metabolic Activity and Biofilm Formation in Streptococcus mutans*. Evid Based Complement Alternat Med, 2019. **2019**: p. 1524195.
188. Muhonja, C.N., et al., *Biodegradability of polyethylene by bacteria and fungi from Dandora dumpsite Nairobi-Kenya*. PloS one, 2018. **13**(7): p. e0198446.
189. Usha, R., T. Sangeetha, and M. Palaniswamy, *Screening of polyethylene degrading microorganisms from garbage soil*. Libyan Agric Res Cent J Int, 2011. **2**(4): p. 200-4.
190. Zhang, Y., et al., *Biodegradation of polyethylene and polystyrene: From microbial deterioration to enzyme discovery*. Biotechnology Advances, 2022. **60**: p. 107991.

191. Hasan, F., et al., *Synergistic effect of photo and chemical treatment on the rate of biodegradation of low density polyethylene by Fusarium sp. AF4*. Journal of applied polymer science, 2007. **105**(3): p. 1466-1470.
192. Yamada-Onodera, K., et al., *Degradation of polyethylene by a fungus, Penicillium simplicissimum YK*. Polymer degradation and stability, 2001. **72**(2): p. 323-327.
193. Abrusci, C., et al., *Biodegradation of photo-degraded mulching films based on polyethylenes and stearates of calcium and iron as pro-oxidant additives*. International Biodeterioration & Biodegradation, 2011. **65**(3): p. 451-459.
194. Das, M.P. and S. Kumar, *An approach to low-density polyethylene biodegradation by Bacillus amyloliquefaciens*. 3 Biotech, 2015. **5**(1): p. 81-86.
195. Gajendiran, A., S. Krishnamoorthy, and J. Abraham, *Microbial degradation of low-density polyethylene (LDPE) by Aspergillus clavatus strain JASK1 isolated from landfill soil*. 3 Biotech, 2016. **6**(1): p. 52.
196. Ojha, N., et al., *Evaluation of HDPE and LDPE degradation by fungus, implemented by statistical optimization*. Sci Rep, 2017. **7**: p. 39515.
197. Abraham, J., et al., *Microbial degradation of low density polyethylene*. Environmental Progress & Sustainable Energy, 2017. **36**(1): p. 147-154.
198. Skariyachan, S., et al., *Enhanced polymer degradation of polyethylene and polypropylene by novel thermophilic consortia of Brevibacillus sps. and Aneurinibacillus sp. screened from waste management landfills and sewage treatment plants*. Polymer Degradation and Stability, 2018. **149**: p. 52-68.
199. Samanta, S., D. Datta, and G. Halder, *Biodegradation efficacy of soil inherent novel sp. Bacillus tropicus (MK318648) onto low density polyethylene matrix*. Journal of Polymer Research, 2020. **27**(10): p. 324.
200. Gupta, K.K. and D. Devi, *Characteristics investigation on biofilm formation and biodegradation activities of Pseudomonas aeruginosa strain ISJ14 colonizing low density polyethylene (LDPE) surface*. Heliyon, 2020. **6**(7): p. e04398.
201. Biki, S.P., et al., *Polyethylene degradation by Ralstonia sp. strain SKM2 and Bacillus sp. strain SM1 isolated from land fill soil site*. Environmental Technology & Innovation, 2021. **22**: p. 101495.
202. Ren, L., et al., *Biodegradation of Polyethylene by Enterobacter sp. D1 from the Guts of Wax Moth Galleria mellonella*. Int J Environ Res Public Health, 2019. **16**(11).
203. Hadad, D., S. Geresh, and A. Sivan, *Biodegradation of polyethylene by the thermophilic bacterium Brevibacillus borstelensis*. J Appl Microbiol, 2005. **98**(5): p. 1093-100.
204. Veerasingam, S., et al., *Contributions of Fourier transform infrared spectroscopy in microplastic pollution research: A review*. Critical Reviews in Environmental Science and Technology, 2021. **51**(22): p. 2681-2743.
205. Nadeem, H., et al., *Isolation and identification of low-density polyethylene degrading novel bacterial strains*. Archives of Microbiology, 2021. **203**(9): p. 5417-5423.
206. Leja, K. and G. Lewandowicz, *Polymer biodegradation and biodegradable polymers-a review*. Polish Journal of Environmental Studies, 2010. **19**(2).
207. Kowalczyk, A., et al., *Achromobacter xylosoxidans as a new microorganism strain colonizing high-density polyethylene as a key step to its biodegradation*. Environmental Science and Pollution Research, 2016. **23**(11): p. 11349-11356.
208. Balasubramanian, V., et al., *High - density polyethylene (HDPE) - degrading potential bacteria from marine ecosystem of Gulf of Mannar, India*. Letters in applied microbiology, 2010. **51**(2): p. 205-211.

209. Agamuthu, P. and P.N. Faizura, *Biodegradability of degradable plastic waste*. Waste Manag Res, 2005. **23**(2): p. 95-100.
210. Raddadi, N. and F. Fava, *Biodegradation of oil-based plastics in the environment: Existing knowledge and needs of research and innovation*. Sci Total Environ, 2019. **679**: p. 148-158.
211. Volke - Sepúlveda, T., et al., *Thermally treated low density polyethylene biodegradation by *Penicillium pinophilum* and *Aspergillus niger**. Journal of applied polymer science, 2002. **83**(2): p. 305-314.
212. Temporiti, M.E.E., et al., *Fungal Enzymes Involved in Plastics Biodegradation*. Microorganisms, 2022. **10**(6).
213. Khruengsai, S., T. Sripahco, and P. Pripdeevech, *Low-Density Polyethylene Film Biodegradation Potential by Fungal Species from Thailand*. J Fungi (Basel), 2021. **7**(8).
214. Koutny, M., et al., *Acquired biodegradability of polyethylenes containing pro-oxidant additives*. Polymer Degradation and Stability, 2006. **91**(7): p. 1495-1503.

7. Appendixes

Appendix 1

Table S1. 10 % acrylamide gel used in SDS-PAGE

Reagents	Running gel	Stacking gel
Acrylamide/Bis-acrylamide (40:2)	1.75 mL	0.28 mL
pH 8.8 buffer (0.75 M Tris-HCl, 0.2 % SDS)	2.8 mL	-
pH 6.8 buffer (0.25 M Tris-HCl, 0.2 % SDS)	-	1.1 mL
H₂O	1.0 mL	0.9 mL
10 % APS	30.0 µL	12.5 µL
TEMED	5.0 µL	4.0 µL

Loading buffer (SDS-PAGE, 5x concentrated)

10 % SDS; 40 % Glycerol; 0.2 M Tris-HCl; 50 mM β-mercaptoethanol; 0.1 % Bromophenol Blue

Appendix 2

Protocol for silver nitrate gel staining

- 1- Stir the gel for 20 min. in solution 1 (50 % methanol and 5 % glacial acetic acid).
- 2- Stir for 10 min. in solution 2 (50 % methanol).
- 3- Stir for 10 min. in deionized water (diH₂O).
- 4- Stir for 1 min. in solution 3 (0.02 % sodium thiosulphate).
- 5- Wash the gel 2 times with diH₂O, 1 min. each wash.
- 6- Stir for 20 min. in solution 4 (0.1 % silver nitrate) at 4 °C.

7- Put a little of solution 5 (3 % sodium carbonate with 50 μ L formaldehyde) and dispose of the liquid. Add solution 5 again and shake it until the bands are properly revealed.

8- To stop the revelation use solution 6 (5 % glacial acetic acid).

9- Leave the gel in diH₂O.

Appendix 3

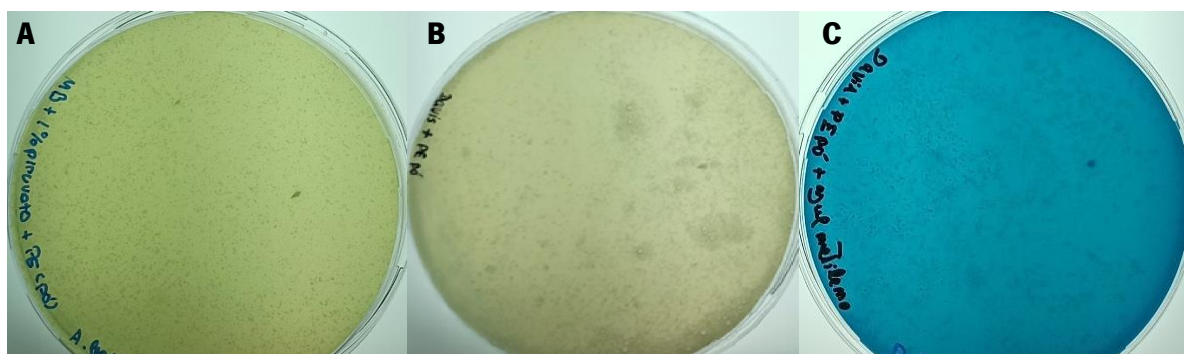


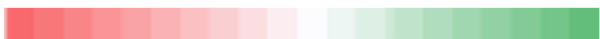
Figure S1. Solid medium supplemented with PE powder. A) MBP medium; B) Davis; C) Davis + blue.

Appendix 4

Tables containing the growth values of colonies for the first day of incubation:

Table S2. Mean size of the colonies after 1 day of growth in MBP, Davis and Davis + blue, supplemented with PEG, PVA or coconut oil (C.O.) using three different inoculation methods: non-perforation (non-perf.), perforation (perf.) and drop. Results are expressed in millimetres.

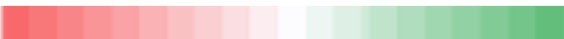
Medium Microorganism		MBP			Davis			Davis + blue		
		PEG	PVA	C.O.	PEG	PVA	C.O.	PEG	PVA	C.O.
<i>Y. lipolytica</i>	Non-perf.	-0.28	1.18	1.84	-0.81	-0.53	0.55	n.a.	n.a.	n.a.
	Perf.	0.48	3.47	2.15	-3.02	-2.23	-2.52	n.a.	n.a.	n.a.
	Drop	-1.11	3.08	1.56	n.a.	1.10	-1.00	n.a.	n.a.	0.82
<i>D. hansenii</i>	Non-perf.	0.30	-0.22	1.03	-0.57	-0.51	-0.24	1.59	0.46	2.44
	Perf.	-0.24	0.49	0.54	1.38	1.18	1.05	0.77	0.67	2.63
	Drop	-0.06	0.51	0.60	n.a.	n.a.	n.a.	n.a.	-0.35	n.a.
<i>P. aeruginosa</i>	Non-perf.	1.52	0.30	2.50	0.15	0.04	1.53	3.70	5.12	0.64
	Perf.	0.91	1.07	2.16	-2.04	-1.63	0.32	3.17	4.42	-1.51
	Drop	-0.06	0.18	0.84	-0.35	1.91	0.23	0.35	0.95	0.12
<i>A. borkumensis</i>	Non-perf.	-0.12	n.a.	0.23	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Perf.	-0.10	n.a.	-1.33	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Drop	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

- Growth  + Growth

n.a. indicates that no microbial growth was detected. For the non-perforation (non-perf.), a sterile toothpick was used to remove the biomass of a single microbial colony and transferred to the surface of the solid medium to be inoculated. For the perforation (perf.), a toothpick was used to remove the biomass of a single microbial colony and used to perforate the solid culture medium up to approximately 2-thirds. For the drop method, a pre-inoculum was made and its OD was adjusted to 0.6. Then, using a micropipette, a 3 μ L drop was placed onto the surface of the plate.

Table S3. Mean size of the colonies after 1 day of growth in MBP, Davis and Davis + blue in the presence of the surfactants SDS and Sarkosyl at a concentration of 0.35 % or 0.01 %, using three different inoculation methods: non-perforation (non-perf.), perforation (perf.) and drop. Results are expressed in millimetres.

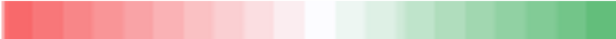
Medium Microorganism		MBP				Davis				Davis + blue			
		0.35 %		0.01%		0.35 %		0.01%		0.35 %		0.01%	
		SDS	Sarkosyl	SDS	Sarkosyl	SDS	Sarkosyl	SDS	Sarkosyl	SDS	Sarkosyl	SDS	Sarkosyl
<i>Y. lipolytica</i>	Non-perf.	n.a.	-0.10	-0.01	0.43	-0.49	n.a.	-0.56	-0.01	n.a.	n.a.	n.a.	n.a.
	Perf.	n.a.	0.92	2.12	2.31	-0.78	n.a.	-2.13	0.37	n.a.	n.a.	n.a.	n.a.
	Drop	n.a.	1.59	0.68	0.58	-0.03	n.a.	n.a.	1.41	n.a.	n.a.	n.a.	n.a.
<i>D. hansenii</i>	Non-perf.	n.a.	n.a.	0.33	0.79	n.a.	n.a.	-1.44	-1.10	n.a.	n.a.	n.a.	n.a.
	Perf.	n.a.	-2.15	0.17	-0.01	n.a.	n.a.	0.53	0.59	n.a.	n.a.	n.a.	n.a.
	Drop	n.a.	n.a.	0.43	0.60	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
<i>P. aeruginosa</i>	Non-perf.	0.01	1.05	1.02	0.88	0.52	0.29	0.88	0.63	0.22	0.20	0.07	-0.16
	Perf.	-0.25	0.59	1.05	0.78	-1.36	-1.44	-1.37	1.58	1.92	-0.27	-1.68	0.47
	Drop	0.56	1.60	0.22	0.69	-0.70	0.02	0.80	0.62	1.70	0.43	1.17	0.73
<i>A. borkumensis</i>	Non-perf.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Perf.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Drop	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

- Growth  + Growth

n.a. indicates that no microbial growth was detected. For the non-perforation (non-perf.), a sterile toothpick was used to remove the biomass of a single microbial colony and transferred to the surface of the solid medium to be inoculated. For the perforation (perf.), a toothpick was used to remove the biomass of a single microbial colony and used to perforate the solid culture medium up to approximately 2-thirds. For the drop method, a pre-inoculum was made and its OD was adjusted to 0.6. Then, using a micropipette, a 3 µL drop was placed onto the surface of the plate.

Table S4. Mean size of the colonies after 1 day of growth in MBP, Davis and Davis + blue with PCL emulsions by SDS and Sarkosyl surfactants at the concentration of 0.35 % and PSF (Sarkosyl 0.35%), using three different inoculation methods: non-perforation (non-perf.), perforation (perf.) and drop. Results are expressed in millimetres.

Medium Microorganism		MBP			Davis			Davis + blue		
		0.35%			0.35%			0.35%		
		PCL (SDS)	PCL (Sarkosyl)	PSF	PCL (SDS)	PCL (Sarkosyl)	PSF	PCL (SDS)	PCL (Sarkosyl)	PSF
<i>Y. lipolytica</i>	Non-perf.	n.a.	0.73	0.00	n.a.	n.a.	-0.03	n.a.	n.a.	n.a.
	Perf.	n.a.	0.38	-0.28	n.a.	n.a.	-0.30	n.a.	n.a.	n.a.
	Drop	n.a.	1.56	0.58	n.a.	n.a.	2.79	n.a.	n.a.	n.a.
<i>D. hansenii</i>	Non-perf.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	1.11	n.a.
	Perf.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	0.37	n.a.
	Drop	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
<i>P. aeruginosa</i>	Non-perf.	0.41	2.83	1.62	0.13	1.22	1.08	n.a.	1.37	0.89
	Perf.	-2.10	-0.60	0.94	1.25	0.71	0.76	-0.18	-1.77	-0.20
	Drop	0.11	3.36	0.94	0.48	0.85	1.98	0.69	-0.78	2.50
<i>A. borkumensis</i>	Non-perf.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Perf.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Drop	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

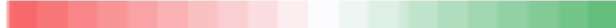
- Growth  + Growth

n.a. indicates that no microbial growth was detected. For the non-perforation (non-perf.), a sterile toothpick was used to remove the biomass of a single microbial colony and transferred to the surface of the solid medium to be inoculated. For the perforation (perf.), a toothpick was used to remove the biomass of a single microbial colony and used to perforate the solid culture medium up to approximately 2-thirds. For the drop method, a pre-inoculum was made and its OD was adjusted to 0.6. Then, using a micropipette, a 3 µL drop was placed onto the surface of the plate.

Tables containing the clear halos values of the colonies for the first day of incubation:

Table S5. Mean size of the clear halos around the colonies of *Y. lipolytica*, *D. hansenii*, *P. aeruginosa* and *A. borkumensis* after 1 day of growth in MBP, Davis and Davis + blue, with the substrates PEG, PVA or coconut oil (C.O.). Results are expressed in millimetres.

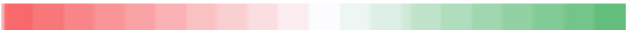
Medium Microorganism		MBP			Davis			Davis + blue		
		PEG	PVA	C.O.	PEG	PVA	C.O.	PEG	PVA	C.O.
<i>Y. lipolytica</i>	Non-perf.	0.03	0.03	n.a.	n.a.	0.00	n.a.	n.a.	n.a.	n.a.
	Perf.	-0.09	0.06	n.a.	n.a.	-0.09	n.a.	n.a.	n.a.	n.a.
	Drop	-0.11	-0.05	n.a.	n.a.	0.03	n.a.	n.a.	n.a.	n.a.
<i>D. hansenii</i>	Non-perf.	-0.03	0.06	n.a.	0.13	0.13	n.a.	n.a.	n.a.	n.a.
	Perf.	0.03	0.04	n.a.	-0.54	-0.19	n.a.	n.a.	n.a.	n.a.
	Drop	0.15	-0.23	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
<i>P. aeruginosa</i>	Non-perf.	-0.13	-0.07	n.a.	n.a.	n.a.	n.a.	0.26	n.a.	n.a.
	Perf.	0.00	-0.06	n.a.	n.a.	n.a.	n.a.	0.14	n.a.	n.a.
	Drop	-0.12	-0.09	n.a.	n.a.	n.a.	n.a.	-0.10	n.a.	n.a.
<i>A. borkumensis</i>	Non-perf.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Perf.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Drop	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Smaller clear halo  Larger clear halo

n.a. indicates that no clear halos were detected. For the non-perforation (non-perf.), a sterile toothpick was used to remove the biomass of a single microbial colony and transferred to the surface of the solid medium to be inoculated. For the perforation (perf.), a toothpick was used to remove the biomass of a single microbial colony and used to perforate the solid culture medium up to approximately 2-thirds. For the drop method, a pre-inoculum was made and its OD was adjusted to 0.6. Then, using a micropipette, a 3 μ L drop was placed onto the surface of the plate.

Table S6. Mean size of the clear halos around the colonies of *Y. lipolytica*, *D. hansenii*, *P. aeruginosa* and *A. borkumensis* after 1 day of growth in MBP, Davis and Davis + blue, with the substrates PCL and PSF both in emulsion with 0.35 % surfactant. Results are expressed in millimetres.

Microorganism \ Medium		MBP			Davis			Davis + blue		
		0.35%			0.35%			0.35%		
		PCL (SDS)	PCL (Sarkosyl)	PSF	PCL (SDS)	PCL (Sarkosyl)	PSF	PCL (SDS)	PCL (Sarkosyl)	PSF
<i>Y. lipolytica</i>	Non-perf.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Perf.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Drop	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
<i>D. hansenii</i>	Non-perf.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Perf.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Drop	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
<i>P. aeruginosa</i>	Non-perf.	n.a.	n.a.	n.a.	n.a.	0.21	n.a.	n.a.	n.a.	n.a.
	Perf.	n.a.	n.a.	n.a.	n.a.	0.00	n.a.	n.a.	n.a.	n.a.
	Drop	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
<i>A. borkumensis</i>	Non-perf.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Perf.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Drop	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Smaller clear halo  Larger clear halo

n.a. indicates that no clear halos were detected. For the non-perforation (non-perf.), a sterile toothpick was used to remove the biomass of a single microbial colony and transferred to the surface of the solid medium to be inoculated. For the perforation (perf.), a toothpick was used to remove the biomass of a single microbial colony and used to perforate the solid culture medium up to approximately 2-thirds. For the drop method, a pre-inoculum was made and its OD was adjusted to 0.6. Then, using a micropipette, a 3 µL drop was placed onto the surface of the plate.

Appendix 5

Tables containing colony growth values for the third day of incubation:

Table S7. Mean size of the colonies after 3 days of growth in MBP, Davis and Davis + blue, supplemented with PEG, PVA or coconut oil (C.O.) using three different inoculation methods: non-perforation (non-perf.), perforation (perf.) and drop. Results are expressed in millimetres.

Microorganism	Medium	MBP			Davis			Davis + blue		
		PEG	PVA	C.O.	PEG	PVA	C.O.	PEG	PVA	C.O.
<i>Y. lipolytica</i>	Non-perf.	-0.06	1.90	1.41	-1.41	-0.02	3.37	-0.11	-0.23	1.06
	Perf.	0.38	4.07	2.15	-3.15	-1.31	5.48	-1.87	-2.43	-1.71
	Drop	-0.56	4.16	2.46	0.26	1.37	7.14	n.a.	1.20	0.53
<i>D. hansenii</i>	Non-perf.	0.27	0.23	2.32	-0.57	-0.55	-0.20	1.47	0.17	1.03
	Perf.	-0.20	0.81	1.67	1.07	1.68	-0.29	0.38	1.04	0.00
	Drop	-0.07	0.20	0.45	0.04	-0.66	n.a.	n.a.	-1.45	n.a.
<i>P. aeruginosa</i>	Non-perf.	1.49	0.82	2.96	0.07	-0.18	1.45	3.71	5.60	0.48
	Perf.	0.97	1.83	3.09	-3.82	3.78	1.03	3.48	5.08	-0.71
	Drop	-0.16	0.85	1.15	1.14	2.77	0.94	0.73	1.74	-0.53
<i>A. borkumensis</i>	Non-perf.	0.26	0.52	1.20	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Perf.	-0.20	0.59	-0.33	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Drop	2.14	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

- Growth  + Growth

n.a. indicates that no microbial growth was detected. For the non-perforation (non-perf.), a sterile toothpick was used to remove the biomass of a single microbial colony and transferred to the surface of the solid medium to be inoculated. For the perforation (perf.), a toothpick was used to remove the biomass of a single microbial colony and used to perforate the solid culture medium up to approximately 2-thirds. For the drop method, a pre-inoculum was made and its OD was adjusted to 0.6. Then, using a micropipette, a 3 μ L drop was placed onto the surface of the plate.

Table S8. Mean size of the colonies after 3 days of growth in MBP, Davis and Davis + blue in the presence of the surfactants SDS and Sarkosyl at a concentration of 0.35 % or 0.01 %, using three different inoculation methods: non-perforation (non-perf.), perforation (perf.) and drop. Results are expressed in millimetres.

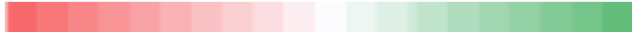
Medium Microorganism		MBP				Davis				Davis + blue			
		0.35 %		0.01%		0.35 %		0.35 %		0.01%		0.35 %	
		SDS	Sarkosyl	SDS	SDS	Sarkosyl	SDS	SDS	Sarkosyl	SDS	SDS	Sarkosyl	SDS
<i>Y. lipolytica</i>	Non-perf.	n.a.	-0.85	-0.12	0.13	-1.21	-0.83	0.28	-0.43	n.a.	n.a.	-0.16	0.17
	Perf.	n.a.	-0.12	1.81	1.75	-1.37	-0.81	0.93	0.24	n.a.	n.a.	-0.14	1.32
	Drop	n.a.	1.16	0.89	0.72	-0.10	0.73	1.24	1.52	n.a.	1.10	1.79	1.25
<i>D. hansenii</i>	Non-perf.	n.a.	-0.59	0.11	0.54	-1.50	-1.00	-0.64	-0.73	n.a.	n.a.	0.26	0.52
	Perf.	n.a.	-0.94	-0.11	0.00	-2.09	-0.11	1.94	-0.25	n.a.	n.a.	2.22	1.49
	Drop	n.a.	1.17	-0.04	0.15	-1.18	2.30	4.10	3.79	n.a.	n.a.	n.a.	n.a.
<i>P. aeruginosa</i>	Non-perf.	0.48	1.24	0.96	0.69	1.28	0.27	0.74	1.53	1.24	-0.33	0.07	-0.28
	Perf.	0.05	0.51	1.29	0.90	-0.34	0.34	1.00	1.56	0.77	-0.33	0.37	0.38
	Drop	0.95	1.51	0.00	0.82	1.66	1.61	1.95	2.39	2.13	0.48	0.83	0.37
<i>A. borkumensis</i>	Non-perf.	n.a.	n.a.	-0.15	-0.49	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Perf.	n.a.	n.a.	-0.88	0.26	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Drop	n.a.	n.a.	1.65	1.39	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

- Growth  + Growth

n.a. indicates that no microbial growth was detected. For the non-perforation (non-perf.), a sterile toothpick was used to remove the biomass of a single microbial colony and transferred to the surface of the solid medium to be inoculated. For the perforation (perf.), a toothpick was used to remove the biomass of a single microbial colony and used to perforate the solid culture medium up to approximately 2-thirds. For the drop method, a pre-inoculum was made and its OD was adjusted to 0.6. Then, using a micropipette, a 3 μ L drop was placed onto the surface of the plate.

Table S9. Mean size of the colonies after 3 days of growth in MBP, Davis and Davis + blue with PCL emulsions by SDS and Sarkosyl surfactants at the concentration of 0.35 % and PSF (Sarkosyl 0.35 %), using three different inoculation methods: non-perforation (non-perf.), perforation (perf.) and drop. Results are expressed in millimetres.

Medium Microorganism		MBP			Davis			Davis + blue		
		0.35%			0.35%			0.35%		
		PCL (SDS)	PCL (Sarkosyl)	PSF	PCL (SDS)	PCL (Sarkosyl)	PSF	PCL (SDS)	PCL (Sarkosyl)	PSF
<i>Y. lipolytica</i>	Non-perf.	n.a.	-0.38	n.a.	n.a.	0.19	-0.47	n.a.	n.a.	0.90
	Perf.	n.a.	0.19	0.74	-3.19	-2.67	-0.55	n.a.	n.a.	-0.02
	Drop	n.a.	1.53	1.02	-1.46	0.36	3.53	n.a.	n.a.	2.92
<i>D. hansenii</i>	Non-perf.	n.a.	n.a.	n.a.	n.a.	n.a.	-0.61	n.a.	1.52	n.a.
	Perf.	n.a.	n.a.	n.a.	n.a.	-1.11	-0.78	n.a.	-0.36	n.a.
	Drop	n.a.	n.a.	n.a.	n.a.	4.85	n.a.	n.a.	n.a.	n.a.
<i>P. aeruginosa</i>	Non-perf.	0.50	1.14	2.80	0.76	-0.04	1.29	-0.17	-0.13	1.04
	Perf.	0.38	-0.55	3.23	1.54	0.48	1.14	0.46	-2.52	0.94
	Drop	-0.10	2.84	2.12	2.38	1.73	2.85	2.41	1.99	2.75
<i>A. borkumensis</i>	Non-perf.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Perf.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Drop	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

- Growth  + Growth

n.a. indicates that no microbial growth was detected. For the non-perforation (non-perf.), a sterile toothpick was used to remove the biomass of a single microbial colony and transferred to the surface of the solid medium to be inoculated. For the perforation (perf.), a toothpick was used to remove the biomass of a single microbial colony and used to perforate the solid culture medium up to approximately 2-thirds. For the drop method, a pre-inoculum was made and its OD was adjusted to 0.6. Then, using a micropipette, a 3 µL drop was placed onto the surface of the plate.

Tables containing the clear halos values of the colonies for the third day of incubation:

Table S10. Mean size of the clear halos around the colonies of *Y. lipolytica*, *D. hansenii*, *P. aeruginosa* and *A. borkumensis* after 3 days of growth in MBP, Davis and Davis + blue, with the substrates PEG, PVA or coconut oil (C.O.). Results are expressed in millimetres

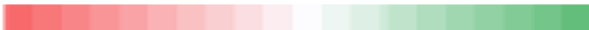
Medium Microorganism		MBP			Davis			Davis + blue		
		PEG	PVA	C.O.	PEG	PVA	C.O.	PEG	PVA	C.O.
<i>Y. lipolytica</i>	Non-perf.	-0.06	0.08	0.31	n.a.	-0.02	0.56	0.01	n.a.	1.39
	Perf.	0.00	0.06	0.73	n.a.	0.51	1.48	n.a.	-0.08	1.10
	Drop	-0.15	-0.05	0.73	n.a.	0.00	1.55	n.a.	n.a.	1.42
<i>D. hansenii</i>	Non-perf.	-0.10	-0.12	-0.02	-0.05	-0.22	-0.09	-0.15	0.07	-0.16
	Perf.	-0.06	-0.15	0.06	-0.32	n.a.	-0.34	0.12	0.26	-0.06
	Drop	-0.11	0.12	0.20	0.01	n.a.	n.a.	n.a.	n.a.	n.a.
<i>P. aeruginosa</i>	Non-perf.	-0.02	0.25	1.30	n.a.	-0.02	n.a.	0.48	1.94	2.26
	Perf.	0.32	0.39	1.17	-0.03	0.29	1.26	0.02	1.23	1.49
	Drop	0.10	0.11	1.35	-0.69	-1.06	0.40	-0.02	0.88	1.89
<i>A. borkumensis</i>	Non-perf.	-0.24	-0.31	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Perf.	0.07	0.12	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Drop	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Smaller clear halo  Larger clear halo

n.a. indicates that no clear halos were detected. For the non-perforation (non-perf.), a sterile toothpick was used to remove the biomass of a single microbial colony and transferred to the surface of the solid medium to be inoculated. For the perforation (perf.), a toothpick was used to remove the biomass of a single microbial colony and used to perforate the solid culture medium up to approximately 2-thirds. For the drop method, a pre-inoculum was made and its OD was adjusted to 0.6. Then, using a micropipette, a 3 µL drop was placed onto the surface of the plate.

Table S11. Mean size of the clear halos around the colonies of *Y. lipolytica*, *D. hansenii*, *P. aeruginosa* and *A. borkumensis* after 3 days of growth in MBP, Davis and Davis + blue, with the substrates PCL and PSF both in emulsion with 0.35 % surfactant. Results are expressed in millimetres.

Medium Microorganism		MBP			Davis			Davis + blue		
		0.35%			0.35%			0.35%		
		PCL (SDS)	PCL (Sarkosyl)	PSF	PCL (SDS)	PCL (Sarkosyl)	PSF	PCL (SDS)	PCL (Sarkosyl)	PSF
<i>Y. lipolytica</i>	Non-perf.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Perf.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Drop	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
<i>D. hansenii</i>	Non-perf.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Perf.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Drop	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
<i>P. aeruginosa</i>	Non-perf.	0.22	n.a.	n.a.	0.00	n.a.	n.a.	n.a.	n.a.	n.a.
	Perf.	0.66	n.a.	n.a.	0.14	n.a.	n.a.	4.74	n.a.	n.a.
	Drop	0.66	n.a.	n.a.	-1.32	n.a.	n.a.	1.81	n.a.	n.a.
<i>A. borkumensis</i>	Non-perf.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Perf.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
	Drop	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.

Smaller clear halo  Larger clear halo

n.a. indicates that no clear halos were detected. For the non-perforation (non-perf.), a sterile toothpick was used to remove the biomass of a single microbial colony and transferred to the surface of the solid medium to be inoculated. For the perforation (perf.), a toothpick was used to remove the biomass of a single microbial colony and used to perforate the solid culture medium up to approximately 2-thirds. For the drop method, a pre-inoculum was made and its OD was adjusted to 0.6. Then, using a micropipette, a 3 μ L drop was placed onto the surface of the plate.

Appendix 6

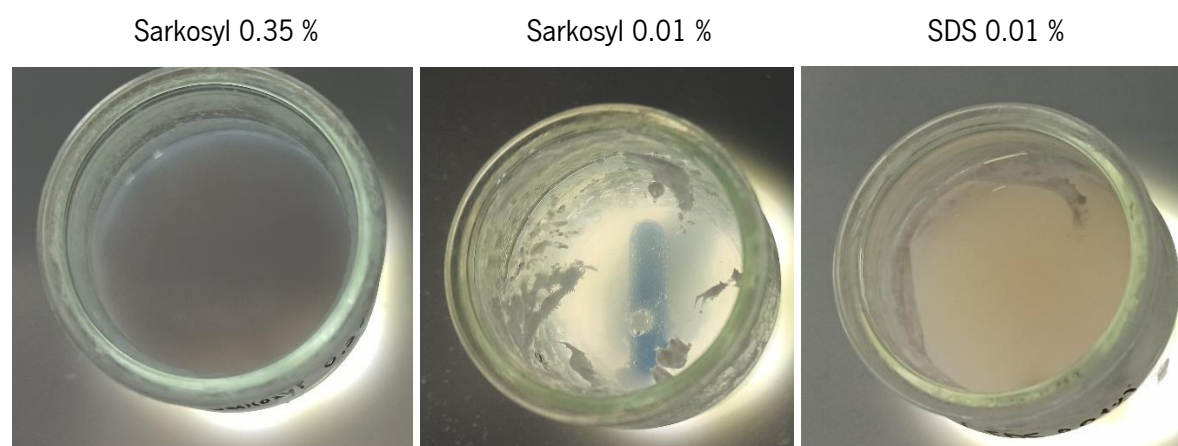


Figure S2. Comparison of PCL emulsions formed with surfactants (Sarkosyl and SDS) at 0.01 % with the selected one (Sarkosyl 0.35 %)

Appendix 7

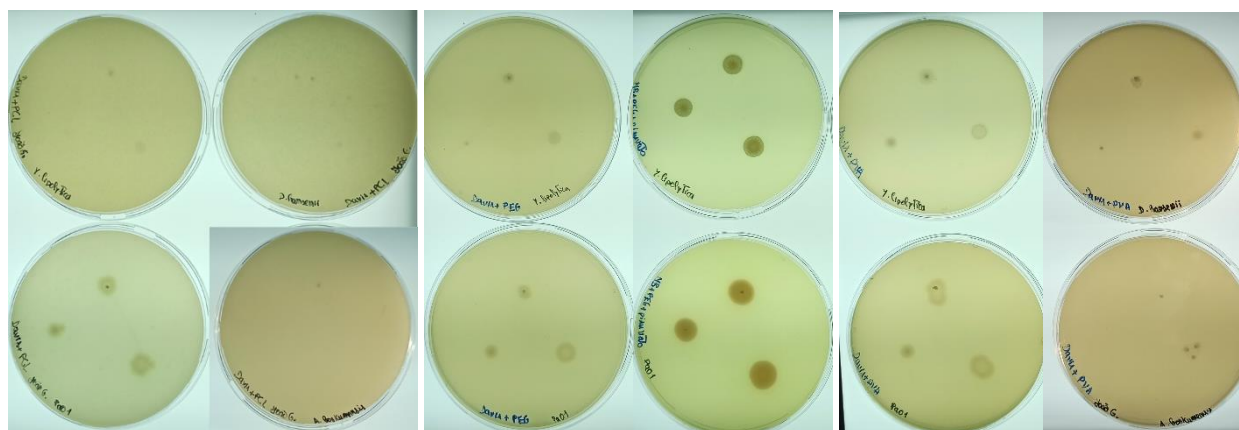


Figure S3. Examples of the solid medium obtained by no staining for the various substrates used and incubated with the microorganisms under study.

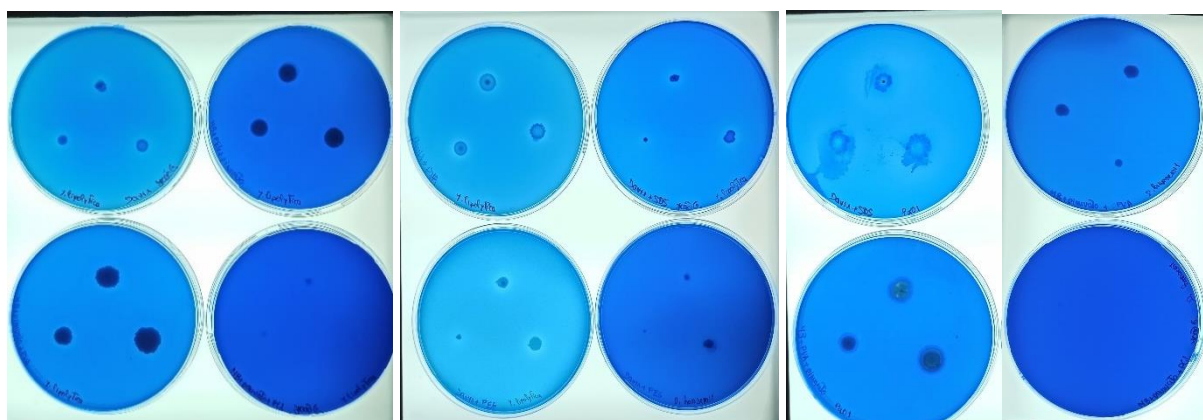


Figure S4. Examples of the solid medium obtained by Coomassie blue staining for the various substrates used and incubated with the microorganisms under study.

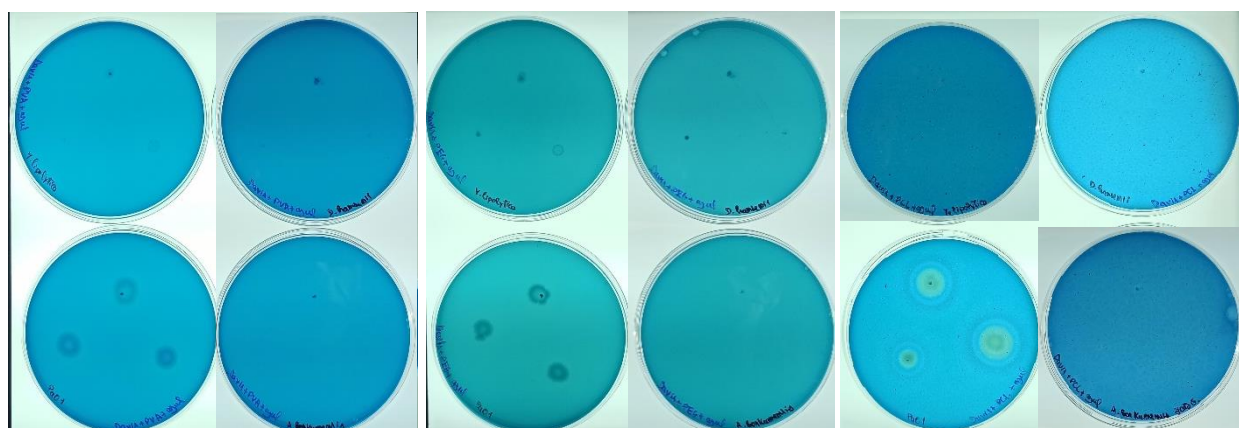


Figure S5. Examples of the solid medium obtained by methylene blue staining for the various substrates used and incubated with the microorganisms under study.

Appendix 8

FTIR spectra of the samples along the 90 days of incubation and of the 3 different parts of the PE film.

15 days of incubation:

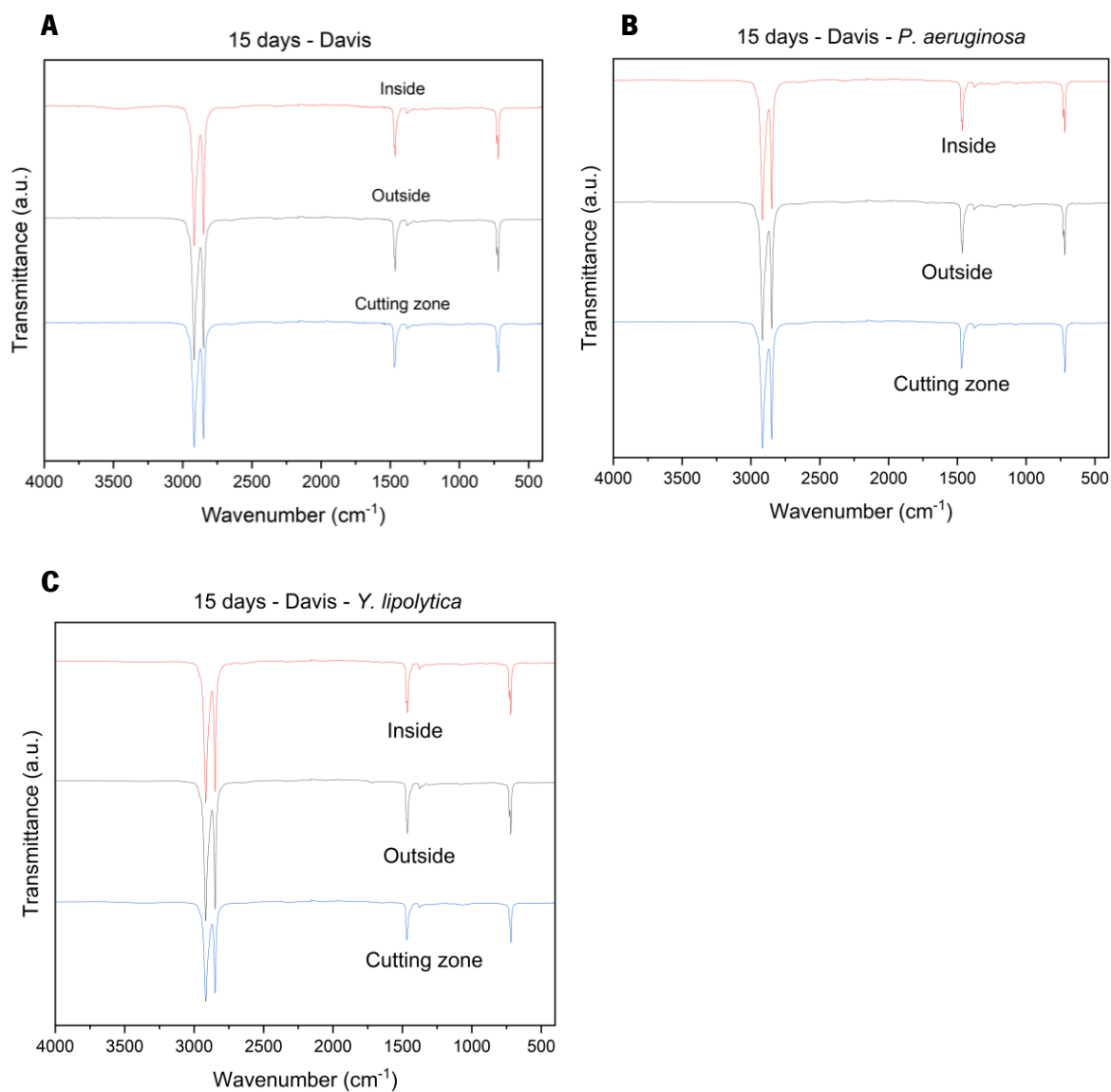


Figure S6. FTIR spectra of PE film incubated for 15 days in Davis with A) no microorganisms; B) *P. aeruginosa*; C) *Y. lipolytica*. For the three zones of the film: inside (red), outside (green) and cutting zone (blue).

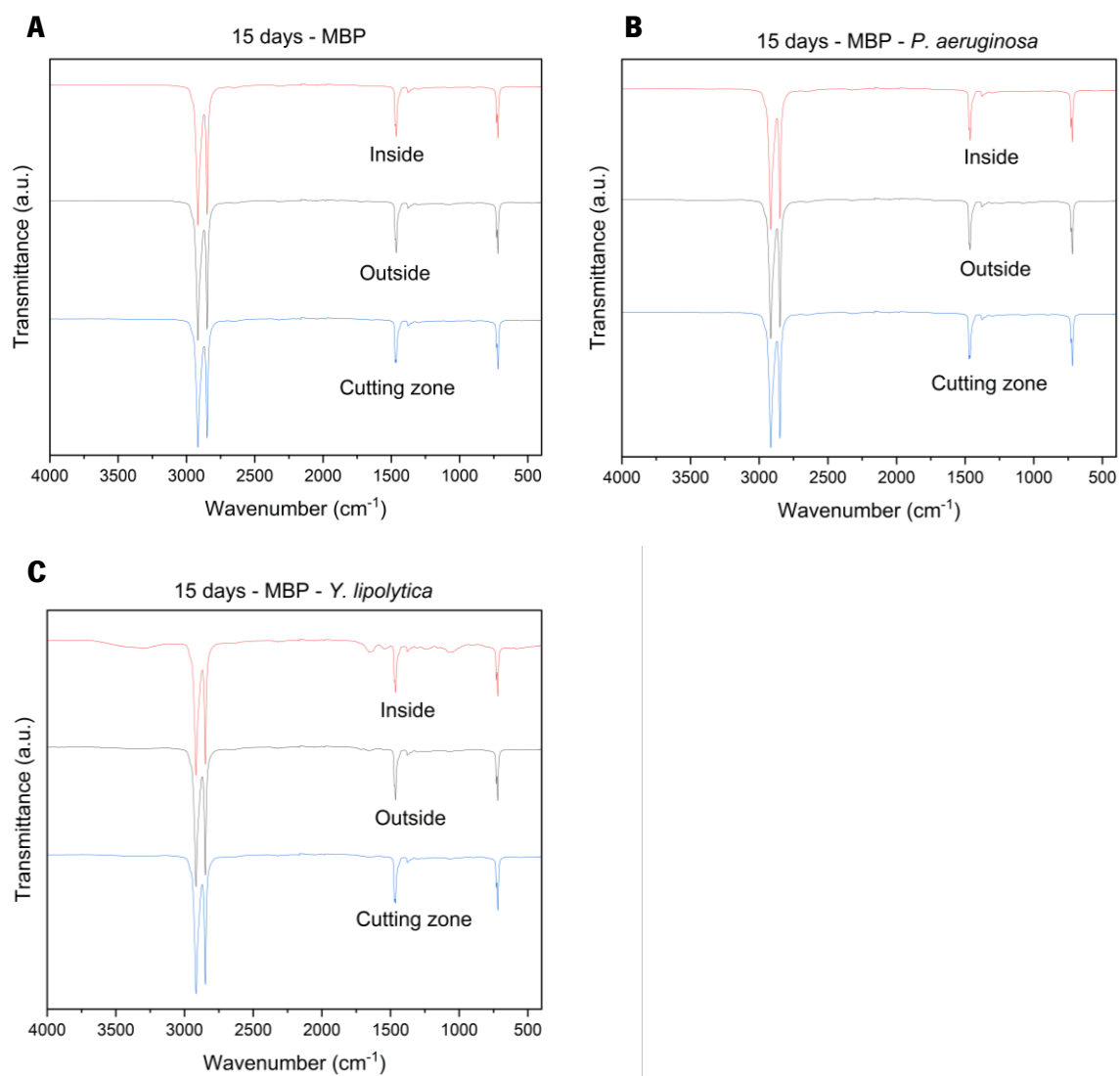


Figure S7. FTIR spectra of PE film incubated for 15 days in MBP with A) no microorganisms; B) *P. aeruginosa*; C) *Y. lipolytica*. For the three zones of the film: inside (red), outside (green) and cutting zone (blue).

30 days of incubation:

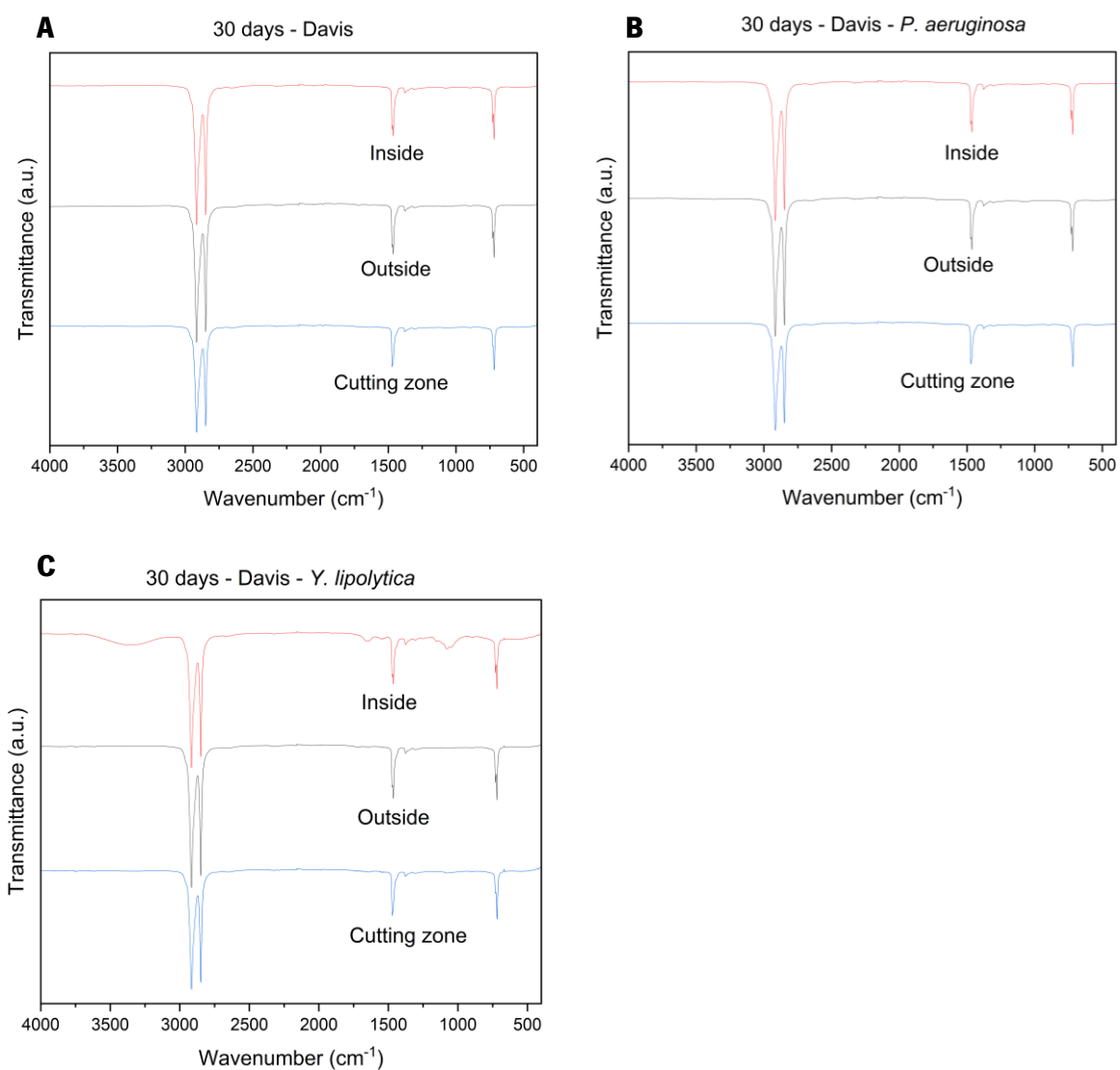


Figure S8. FTIR spectra of PE film incubated for 30 days in Davis with A) no microorganisms; B) *P. aeruginosa*; C) *Y. lipolytica*.

For the three zones of the film: inside (red), outside (green) and cutting zone (blue).

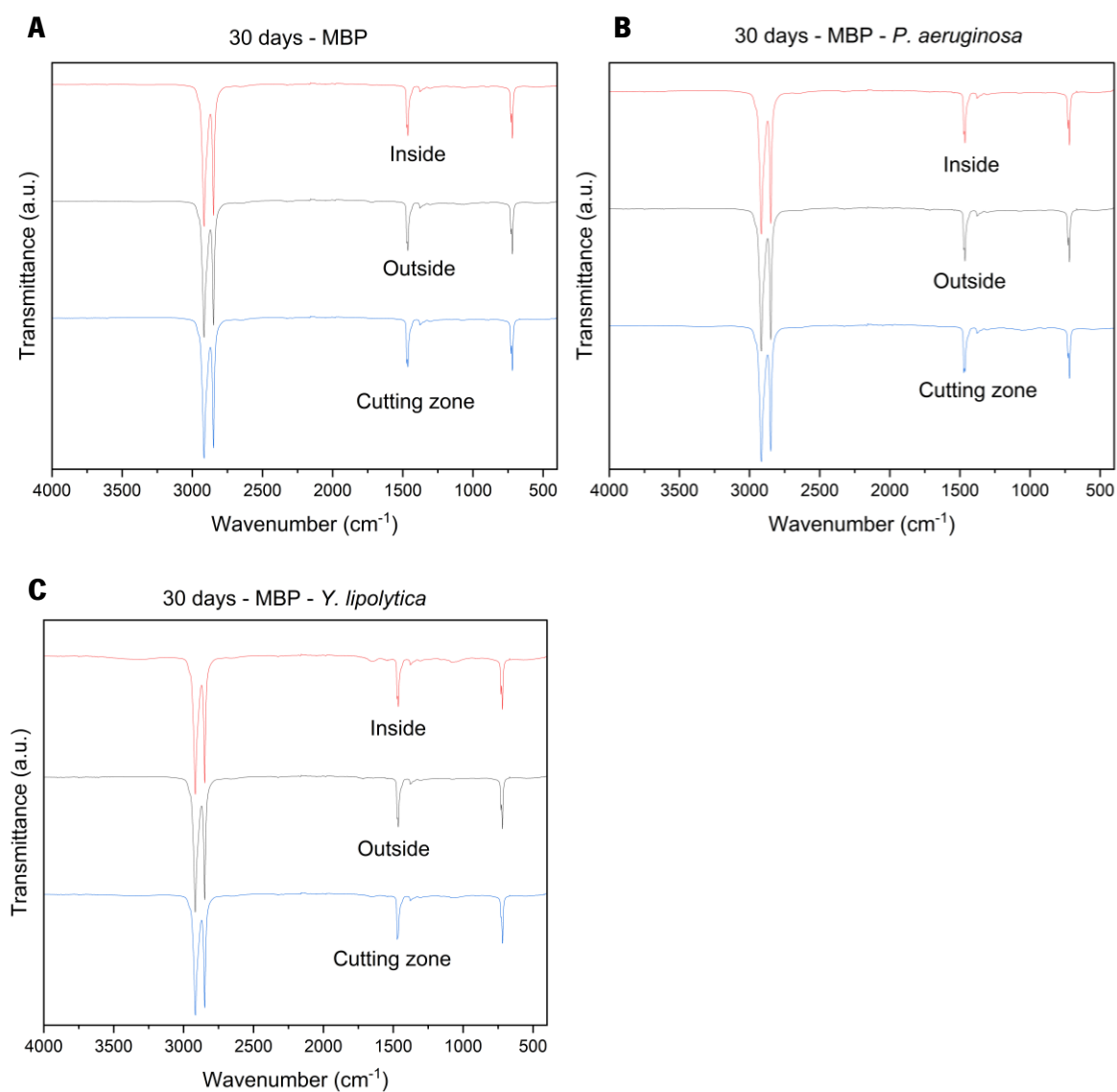


Figure S9. FTIR spectra of PE film incubated for 30 days in MBP with A) no microorganisms; B) *P. aeruginosa*; C) *Y. lipolytica*. For the three zones of the film: inside (red), outside (green) and cutting zone (blue).

60 days of incubation:

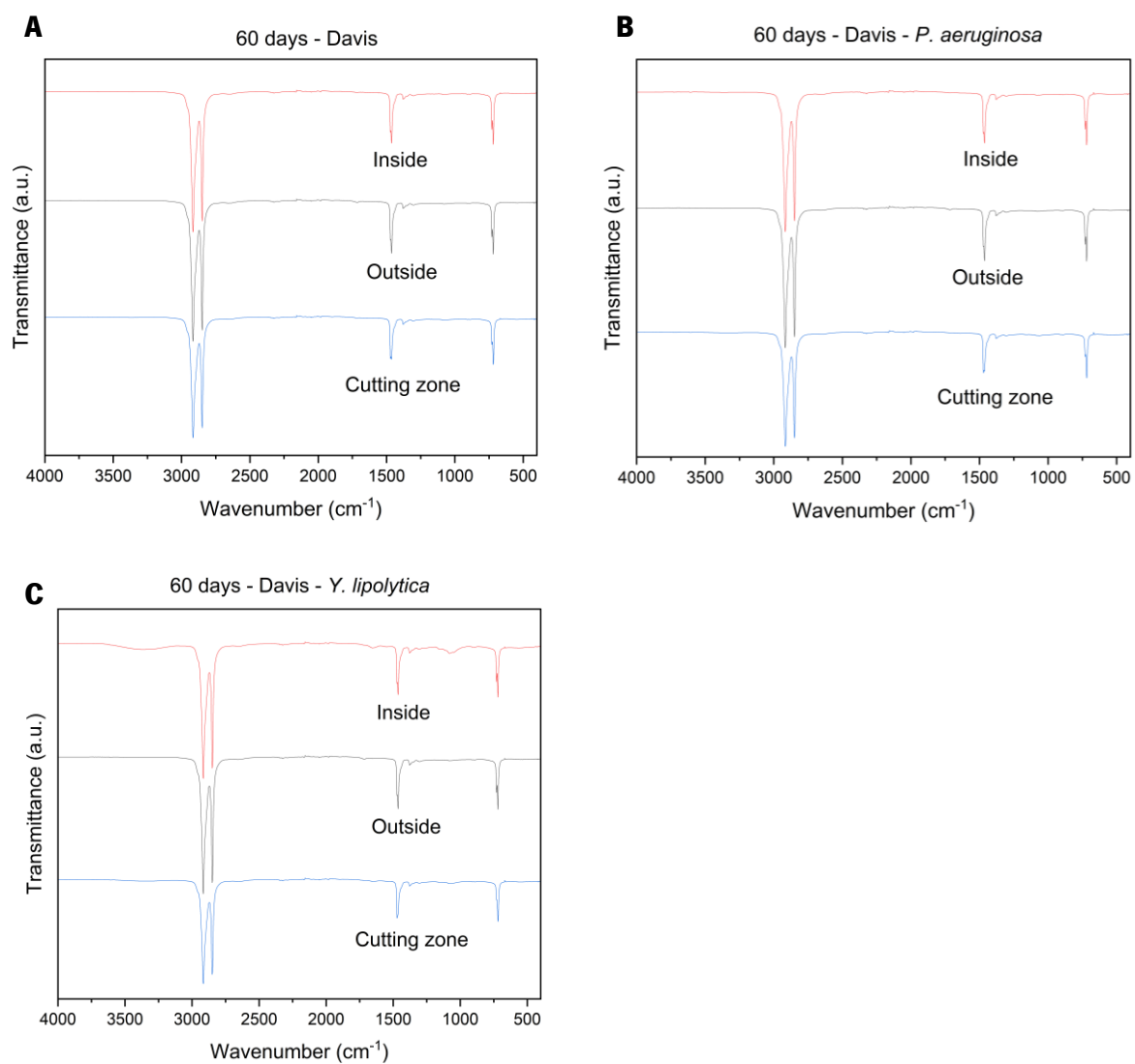


Figure S10. FTIR spectra of PE film incubated for 60 days in Davis with A) no microorganisms; B) *P. aeruginosa*; C) *Y. lipolytica*. For the three zones of the film: inside (red), outside (green) and cutting zone (blue).

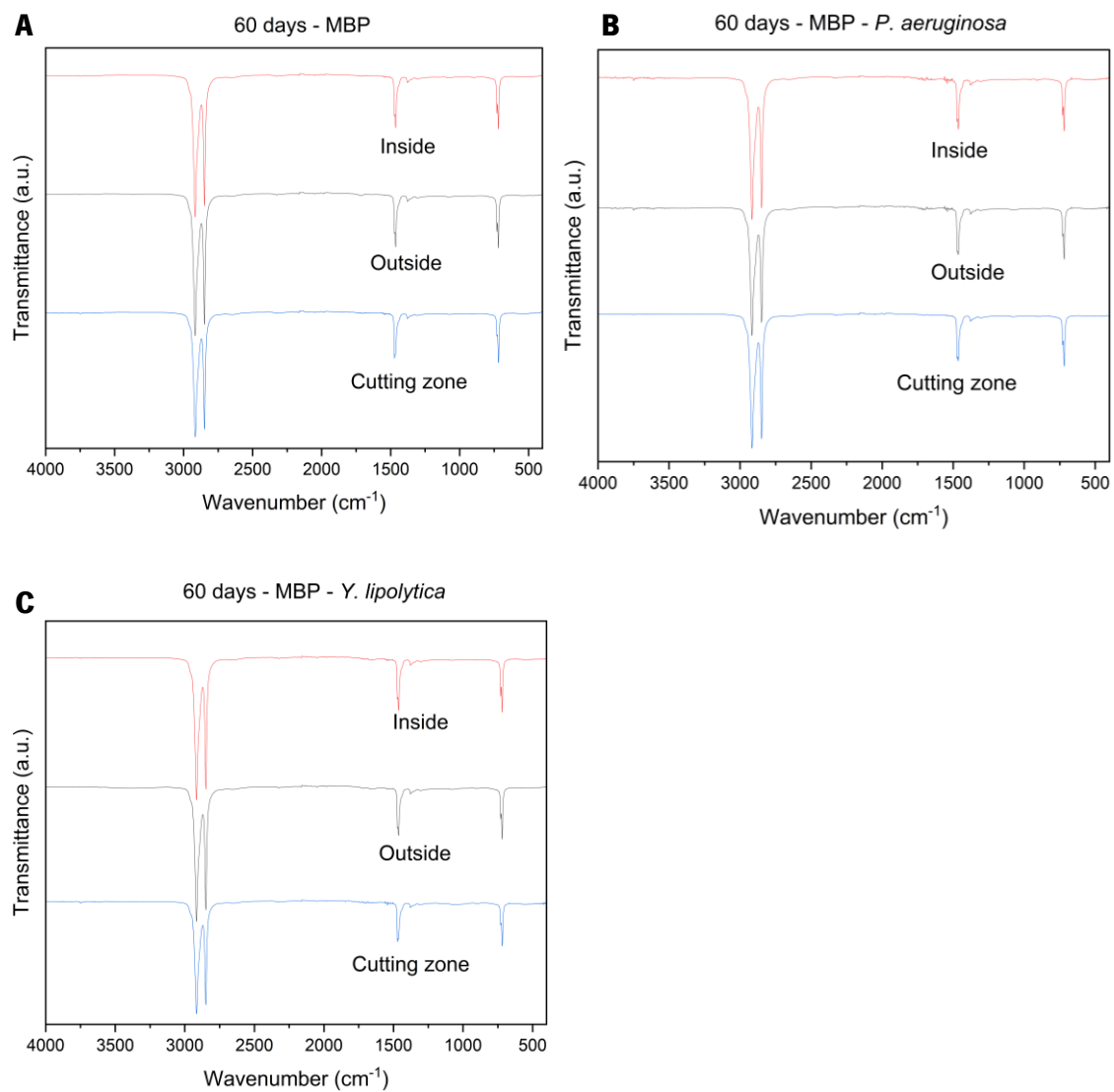


Figure S11. FTIR spectra of PE film incubated for 60 days in MBP with A) no microorganisms; B) *P. aeruginosa*; C) *Y. lipolytica*. For the three zones of the film: inside (red), outside (green) and cutting zone (blue).

90 days of incubation:

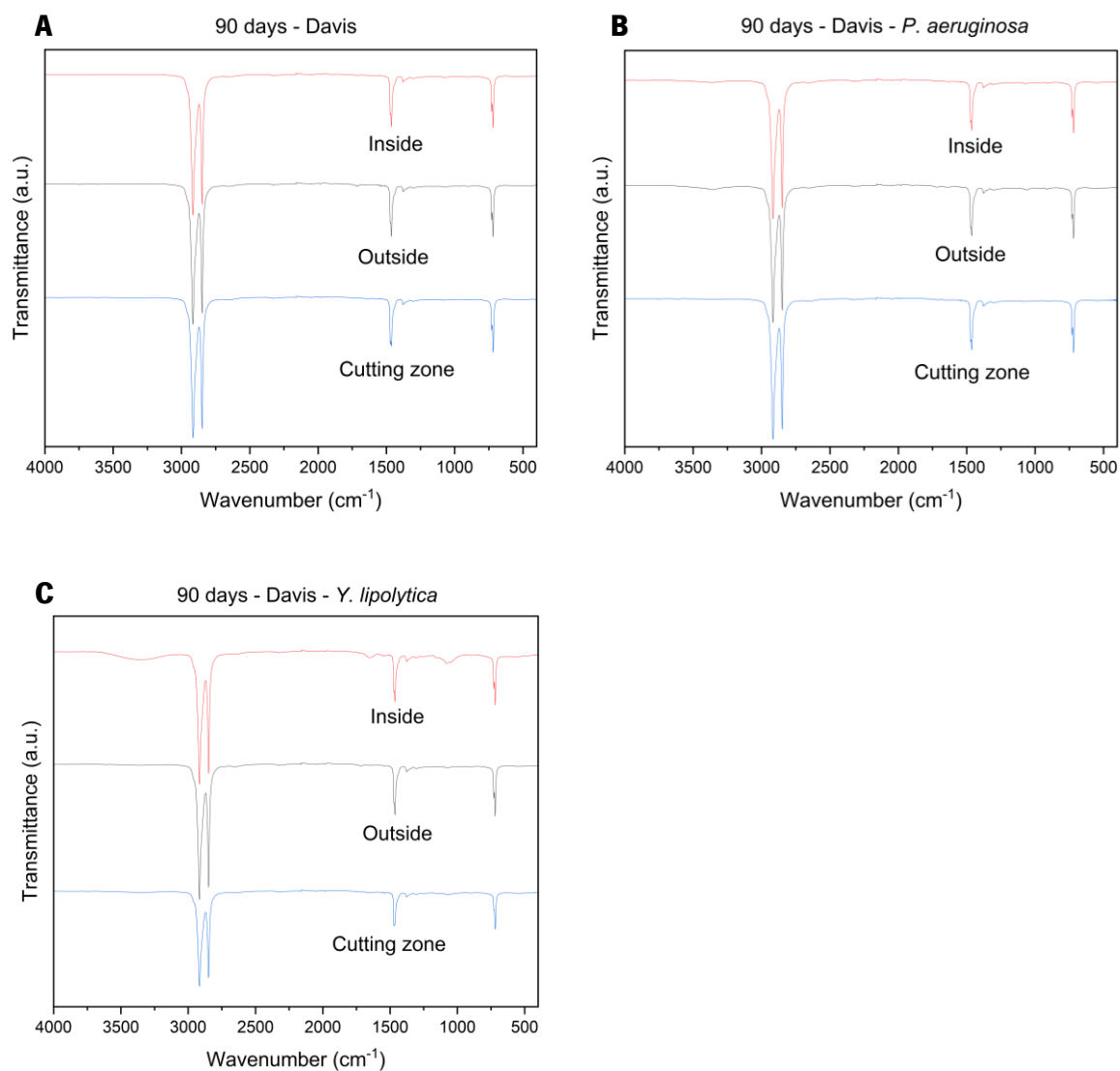


Figure S12. FTIR spectra of PE film incubated for 90 days in Davis with A) no microorganisms; B) *P. aeruginosa*; C) *Y. lipolytica*. For the three zones of the film: inside (red), outside (green) and cutting zone (blue).

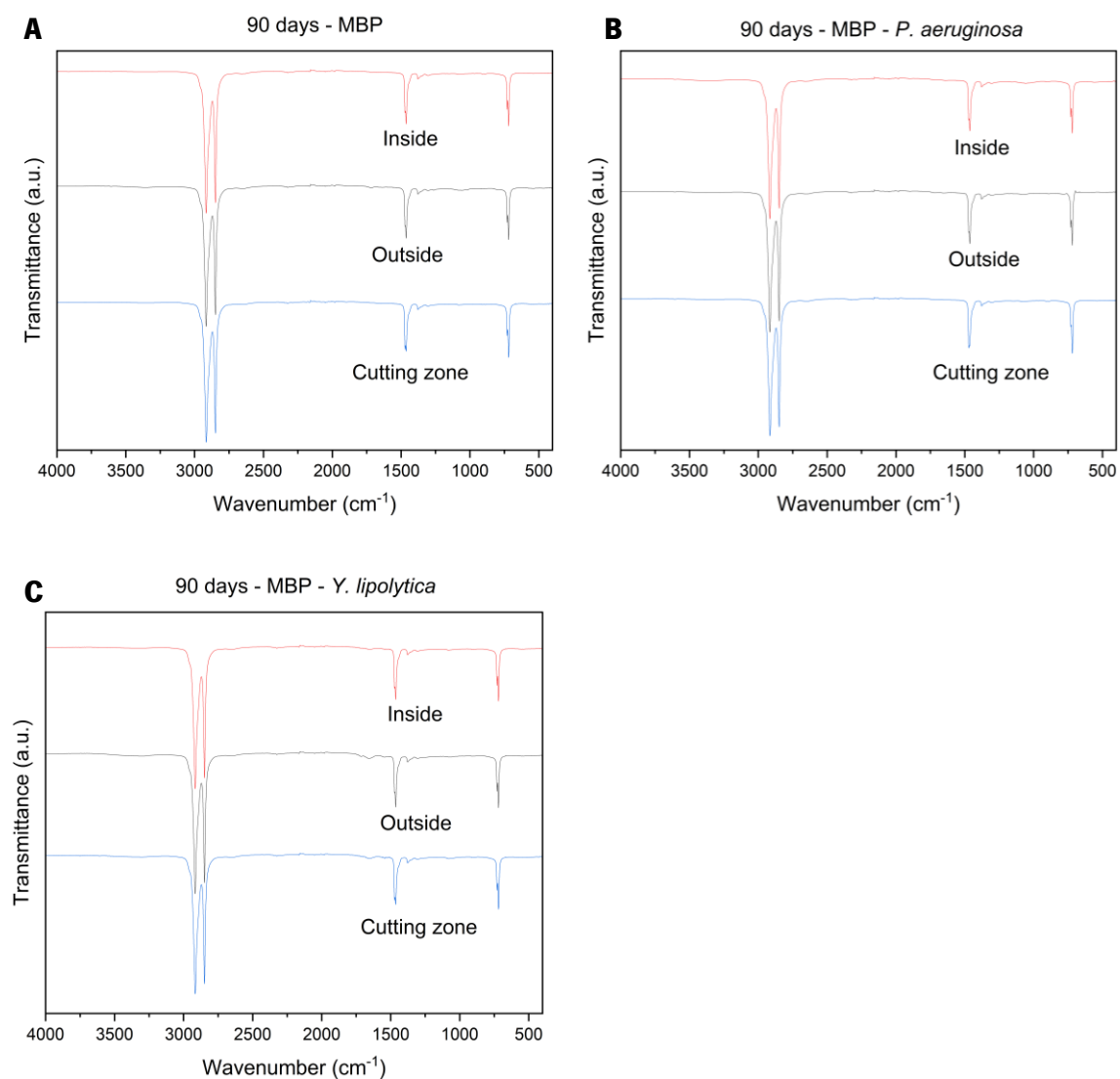


Figure S13. FTIR spectra of PE film incubated for 90 days in MBP with A) no microorganisms; B) *P. aeruginosa*; C) *Y. lipolytica*. For the three zones of the film: inside (red), outside (green) and cutting zone (blue).