# Development of different optimized strategies and characterization of a Defined Mixed Consortia (DMC) for enhanced LDPE bacterial biodegradation

Master thesis, Sustainable biotechnology section


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## List of Abbreviations

| | |
|---|---|
| OD | Opitcal Density |
| LDPE | Light-Density Polyethylene |
| PE | Polyethylene |
| PP | Polypropylene |
| PVC | Polyvinyl-chloride |
| PET | Polyethylene terephthalate |
| PS | Polystyrene |
| PA | Polyamides |
| PUR | Polyurethane |
| M9 | Mineral Minimal Media M9 |
| MRD | Maximum Recovery Diluent |
| CMC | Critical Micelle Concentration |
| MEL | Mannosylerythritol Lipids |
| BCA | Bicinchoninic Acid |
| BSA | Bovine Serum Albumina |
| PHB | Polyhydroxybutyrate |
| UV | Ultraviolet |
| CFU | Colony-Forming Unit |
| LB | Lysogeny Broth |
| FTIR | Fourier-Transform Infrared Spectroscopy |
| MnP | Manganese Peroxidase |
| LiP | Lignin Peroxidase |
| PAH | Polycyclic Aromatic Hydrocarbons |
| HPLC | High-performance liquid chromatography |
| MIC | Microbial Inhibitory Concentration |
| DyP | Dye-decolorizing Peroxidase |
| GRAS | Generally Recognize as Safe |
| EPS | Extracellular Polymeric Substances |

## Abstract

Different strategies were proposed as enhancers of the inner biodegradability of polyethylene, in a sustainable approach: the supplement of a biosurfactant (MEL) or a co-substrate, and the use of a defined mixed consortia (DMC). Bacteria used for the DMC were isolated from a 3-month enrichment and a microtiter culture methodology was developed for high-throughput screening. FTIR analysis confirmed a weak oxidation in C=C bonds due to biodegradation within the most successful consortia. Furthermore, the genome of the strains was sequenced and a putative study of the presence of different enzymes was carried out. Laccases and Alkane hydroxylases were found within the genome of some of the isolates, which confirmed its putative capacity for polymer oxidation. Dye-decolourizing peroxidase was proposed to be a promising LDPE degrading enzyme or a facilitator enzyme within the consortia.

# 1 Overview of the project

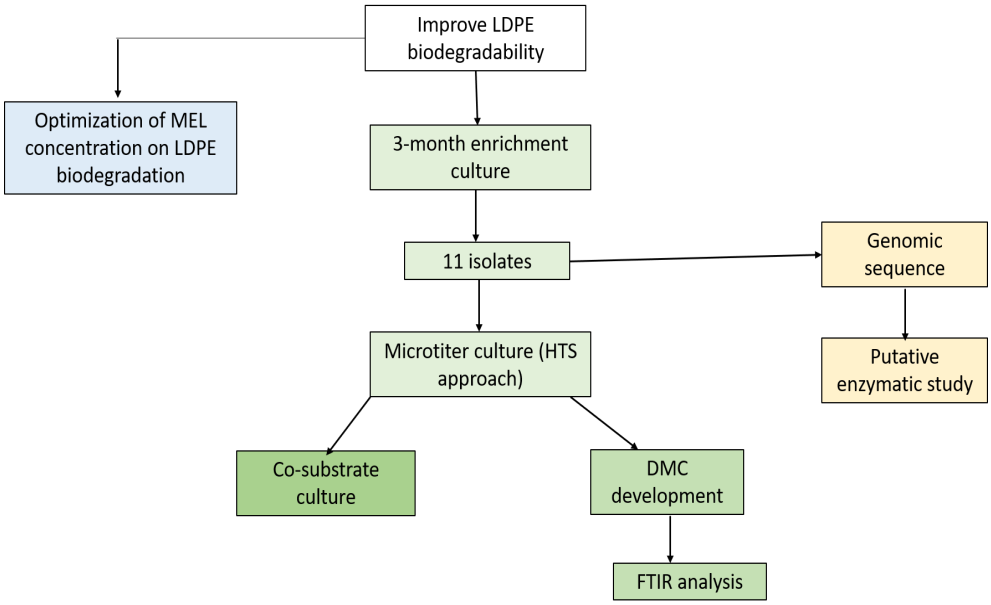


Figure 1: Scheme of the project.

# 2 Introduction

## 2.1 The plastic problem

Plastics production has increased over the past 50 years, from 15 million tonnes in 1964 to almost 350 million tons in 2017 and is expected to double again over the next 20 years, as plastics come to serve increasingly many applications. [2]

Of course, this quality is what makes them so appealing. Synthetic plastics from fossil fuels were invented in 1907, quickly plastic products out-compete traditional materials in almost all the industries, one of the reasons is their long life; they would outlast any other. Nowadays, despite their long lives 33% of plastics these days are used only once, and then thrown away with only 8% being recycled and converted to a low-value product that most of the time cannot be recycled again [3].

This low rate of re-use and recycling has brought us to the point where in just over 100 years synthetic plastics have gone from being essentially non-existent to one of the biggest problems facing the planet today.

Traditional methods of plastic waste disposal suffer from leakages of plastic to the environment, it is estimated that the 32% of the annual production of plastics it is lost by leakage into the environment [3].

Another traditional common fate for plastic, is to be burned out in an open space, producing carbon monoxide (CO) and carbon dioxide ($CO_2$) [4]. In addition to that, plastics, most of the times, are

synthesize with chemical additives that improve their performance. These chemical additives can also migrate to the environment being hazardous for human and ecosystem health [4].

Despite the rising of concern, and the willing of societies to change towards a more "circular" economy, traditional ways of plastic recycling can also lead to some side pollution. For example, it has been found in some children's toys made of recycled plastic, high levels of brominated flame retardants (a chemical additive commonly used on plastics) [4]. Brominated flame retardants are bioaccumulated at human tissue acting as endocrine disrupters [5], their direct contact with children is very alarming and a more meticulous regulation and quality controls of recycled plastic need to be assessed.

Even though, recycling of plastic is a safer alternative in terms of plastic depolymerization and sustainability of the process, it is little known about the effect of the chemical additives on the degraders. However, some studies are already pointing out the toxicity of some degradation products of the most common plasticizers (plastic chemical additives) [6] [2].

Baring in mind that plastic chemical additives are ubiquitous in our environments, it is an unexpected concern, either in recycling or biodegradation strategies, the releasing of these toxic products in our environments and the future effects on ourselves and our surroundings.

## 2.2 Classification of plastics

The term 'plastic' is one used liberally to describe an extremely broad group of different polymers with a high molecular weight [7]. While plastics themselves come in all shapes and sizes, they have some major characteristics in common; namely, that they often take an extraordinarily long time to degrade.

Plastics are usually classified by their capacity to be melted in two categories [8]:

### 2.2.1 Thermoset polymers:

Referring to polymers that undergo a chemical change when heated, creating a three-dimensional network. Once they are shaped, they are unable to be re-shaped, reformed or re-melted. Their structure is highly cross-linked and their main chain is made of hetero-atoms which makes them potentially more susceptible to be degraded by more easily to hydrolyze functional bonds such as ester bonds or amide bonds. Some examples are: Polyurethane (PUR), Unsaturated polyester, Epoxy resins, Melamine resin and silicone [8].

### 2.2.2 Thermoplastics:

Referring to polymers whose structure can be modified both ways; by heating or cooling it repeatedly. This characteristic is because the atoms and molecules are joined end-to-end into a series of long, sole carbon chains independent of the others. Some of the most used polymers are within this category. Also, this structure with a highly carbon backbone makes thermoplastics resistant

to degradation or hydrolytic cleavage of chemical bonds. Some examples of thermoplastics are: Polyethylene (PE), polypropylene (PP) Polyvinyl-chloride (PVC) Polyethylene Terephthalate (PET), Polystyrene (PS) and Polyamides (PA) [8].

According to a report from plastics Europe [2] the fifth categories of most produced plastics in Europe during 2018 in order of importance are: PP, PE, PVC, PUR and PET.

### 2.2.3 Low-Density Polyethylene (LDPE):

This project is focused on assessing the biodegradation of LDPE. As stated before, it is a thermoplastic, widely used as a packaging material because of its excellent mechanical and barrier properties.

The structure of LDPE is long chain branched (on about 2% of the carbon atoms) polymer of ethylene . It is a repetition of covalently bonded carbon atoms. Its inertness is due to its high molecular weight, hydrophobicity and lack of functional groups that can be recognised by microbial enzymatic systems. It has between 50-60% of crystalline degree, that means having properties very desirable for industry such as opacity, tensile strength, rigidity and chemical resistance [9].

There are two different types of LDPE: linear and branched. Both differ on the branching percentage, crystallinity and the functional groups availability on the surface of the polymer [9].

### 2.2.4 Current de-polymerisation strategies:

Other current examples of strategies apply on plastic recycling proposed are the recycling of polyolefins (e.g. Low density polyethylene (LDPE)) using a method named thermal-cracking. Cracking is the process of breaking down long hydrocarbon chains into shorter ones, its products depend on the temperature and the hydrocarbon used as substrate. Even though this strategy is gaining more attention, advanced thermo-chemical recycling of polyolefins still lacks the proper set up and kinetics background to overcome its economics drawbacks [10].

Two of the most recent EU projects researching towards a more cost-effective and sustainable strategy to recycle plastic are: DEPOTEC and DEMETEO.

DEPOTEC was an Irish and EU de-polymerisation project focusing on the de-polymerisation of rubber tyres in an attempt to raise their value once becoming waste. Rubber tyres are durable and heat resistance, making them very difficult to degrade and release harmful toxins when burnt or chemically degraded. The project aimed to use the recycled tyres to substitute carbon filler materials in the rubber manufacturing process as most carbon filler material at the time was produced with high emissions and no profit [11].

DEMETO within the horizon 2020 project researching the De-polymerisation of Polyethylene terephthalate (PET) by microwave technology. DEMETO aims to recycle PET chemically, meaning breaking down PET products into the most basic monomers and recycling those parts in what is described as “intensification” of the natural hydrolysis reaction. The microwave technology works through rotational excitement of molecular groups within the plastics, breaking the polymers into monomers. DEMETO state that their microwave de-polymerisation techniques yield on average

98-99% virgin grade products (ethylene glycol and terepthalic acid; the building blocks of PET) with only 1-2 % of the input PET becoming waste. Further, the only additions that the process needs are energy and water, no chemicals or other toxic materials are involved in the system [12].

HERE

## 2.3 Environmental Biotechnology

Environmental biotechnology is defined as "a system of sciences and engineering knowledge related to the use of microorganisms and their products in the prevention, treatment, and monitoring of environmental pollution through solid, liquid, and gaseous wastes biotreatment, bioremediation of polluted environments, and biomonitoring of environmental and treatment processes" [13].

This discipline is gaining more attention due to the fact that resources on earth are limited, together with climate change and the human societies impact. There is a need of trying to move forward to recycle the waste that it is produced, not only recycle but give it a value within a sustainable process. It is known that in nature, any resource becomes a product for another organisms. Sustainable biotechnology uses this natural capacities combined with technological advanced tools to cope with some of the challenges that our ecosystem is facing.

In this project, environmental biotechnology is used in order to give more insight into one of the most important problems that our ecosystem is facing nowadays, "The plastic problem".

As it can be seen, all the fifth most produced plastics are thermoplastics. As per definition, it is a category that it is not considered degradable. However, in the last two decades, more reports about organisms capable of attacking these substrates [8] are found. Consequently, several studies have been carried assessing their biodegradability and their natural mechanisms.

## 2.4 Plastic biodegradation

Plastic biodegradation is defined as the degradation and assimilation of the polymer by living microorganisms (such as bacteria, fungi and algae) to produce degradation products such as $CO_2$, $H_2$, $CH_4$ and biomass [14].

Enzymatic degradation of plastic materials is also considered biodegradation, and the term "enzymatic biodegradation" is widely used [15]. It is evident from the above discussion that microbial and enzymatic biodegradation of plastic are achieved under relatively milder environmental conditions of pH, temperature, and pressure [16]. In this paper, "biodegradation" refers to both microbial and enzymatic degradation of plastic.

The rising concern on plastic pollution is arising numerous alternatives to crude oil plastic. Nevertheless, the use of the terms "biodegradable" and "bio-based" can be misleading as bio- based materials need not be biodegradable, while biodegradable materials need not be necessarily bio-based. Whilst both biodegradable and bio-based plastics are a big step in the correct direction with regards to producing more sustainable plastic alternatives, this project will focus on the degradation of the problematic petroleum based, non-degradable plastics, in concrete, LDPE.

The hope would be that plastics such as LDPE can be moved into the 'biodegradable' section and perhaps even use the products of the degradation to produce bio-plastics or other valuable materials.

LDPE biodegradability is defined by many factors. Generally, polymers with side chains are more easily assimilated than those without. The molecular weight of plastics also contributes to their biodegradability. Morphology also has a great impact upon their biodegradability; polymers with highly crystalline structures such as polyethylene tend to be more resistant than polymers with more amorphous regions such as poly-carbonate (another type of plastic).

The amorphous regions of polymers are of great importance because the biodegradation enzymes mainly attack these domains as the molecules are less densely packed, thus, the more amorphous a polymer, the more areas there are for the enzymes to have access to and the faster the degradation can proceed [9].

Several strategies are proposed in order to boost LDPE biodegradability, they are discussed further on section 2.7.

### 2.4.1 Organism evolution towards plastic biodegradation

Baring in mind that plastic has been around earth for 100 years the capacity to degrade this polymer emerged relatively recent. Also plastic's raw material, crude oil, has been for millions of years stored underneath the earth's layers, which is an extreme environment for organisms due to the high pressure and temperature.

Most of the plastic degraders that are known show similar patterns of biodegradation as complex natural bio-polymers do. Even though eukaryotic organisms have lower mutation rates, which makes them slower in the evolution race, there are several reports where worms that naturally feed on wax are also able to use polyethylene as carbon source [17], in some species this capacity is given by bacteria composing their microflora [18].

Beeswax is composed of a highly diverse mixture of lipid compounds, including alkanes, alkenes, fatty acids and esters [19]. The most frequent hydrocarbon bond is the $CH_2$-$CH_2$, as in PE. Although the molecular details of wax biodegradation are not clear, so neither are the PE biodegradation, it seems likely that C-C single bond of these alipahtic compounds is one of the targets of wax worm's digestion [19].

HERE Chitin is also a bio-polymer that its resemblances with PE are high. Oil-degrading organisms.

Another complex bio-polymer that has a tight relationship with plastic biodegradation is lignin. Lignin is a recalcitrant polymer made of different aromatic units that form together with hemicellulose and cellulose the structural polymer of plants. Lignin degradation has been studied for many decades due to its abundance and its potential application in the field of environmental biotechnology.

White rot fungi is one of the most efficient organism that degrades lignin, it has also been tested on PE and discovered that the principal enzyme responsible for PE degradation is manganese

peroxidase (MnP), a well-known lignolytic enzyme [20]. Lacasses produced by these fungi have been also proved to have a role on the PE hydrocarbon backbone oxidation [1].

Not only fungi are able to degrade lignin, there are also lignolytic bacteria that are capable of degrade lignin and also PE. Mechanisms are very alike to the fungal ones. Dye-decolourizing perocidase ( DyP) together with lacasses are the main enzymatic complex shared in both substrates and both organisms [21].

### 2.4.2 General mechanism of PE biodegradation:

Generally in nature, due to its highly hydrophobic structure, PE is mainly susceptible to be biodegradable when a surface erosion occurred due to environmental oxidation agents (section 2.7), that opens up the polymer structure catalysing the formation of free radicals that can be recognized by the enzymes. Otherwise, it is hardly accessible for the hydrolytic enzymes [22] [23] [24].

Firstly, the polymer is hydrolysed or oxidised by enzymes to create functional groups for improvement of hydrophobicity, then the main chains of polymer are degraded resulting in polymer of low molecular weight and weak mechanical properties, thus, making it more accessible for further microbial assimilation [25] [21].

Such organisms can release the hydrolytic/oxidising enzymes or maintain them on the cell body, many opt for a combination to maximise degradation efficiency. Usually, the cell adheres to the substrate and directly lyses the monomers of the substrate with membrane bound enzymes. At the same time, free enzymes are released from the cell and independently degrade the substrate into smaller fragments that the membrane bound enzymes can further degrade [7].

Plastics biodegradation by certain enzymatic complexes also lead to breaking of the polymer into oligomers and monomers, which can be later absorbed by the cell, or further converted to organic intermediates like acids, alcohols and ketones [26]. These water soluble cleaved products are absorbed by the microbial cells and metabolized. Generally, the end products of PE metabolism are carbon dioxide and water, formed after aerobic metabolism [27], while anaerobic metabolism results in carbon dioxide, water and methane [28].

The products of PE biodegradation can vary depending on the organism as it can be detailed seen in table 1. Consequently, this diversity of products is what makes PE biodegradation hard to be monitored through techniques such as High-performance liquid chromatography (HPLC).

The finished products on PE are not directly toxic. However PE, as stated on section 2.2, usually come with chemical additives in order to improve its qualities. Chemical additives and its degradation products can be harmful for the ecosystem and human health [5] [6] [2].

Even though PE degradation products are not toxic, its degradation can produce some effects in the ecosystem in *in situ* biodegradation.

Toxicity studies of biodegraded polyethylene in contact with seeds of different types of plants was carried observing the effect on seed germination. It was recorded that seed germination decreased

when biodegraded polyethylene was present [29].

In addition, *P. aeruginosa, A. niger, Rhizopus spp.* and *Streptomyces spp.* were used for degradation of polythlyene bags and plastic cups. Toxicity levels of the biodegraded product were studied following the growth of *Vigna radiata* (plant). It was observed that the addition of biotreated polyethylene granules reduced soil pores size, which could have a negative effect on the nutrient uptake by the root of the plant [30].

On the other side, in case of larvae (*Chironomus spp.*) no toxicity was detected in terms of mortality rate [24]. while in case of larvae (Chironomous spp.) no toxicity was detected in terms of increases in mortality rate.

| Organims | Products | Reference |
|---|---|---|
| *C.globosum* (fungi) | Carboxylic acids, aldehydes, aromatics, alcohols, phenols, esters, ethers, alkyl halides and alkenes were formed at different frequencies | [31] |
| *Bacillus sp., Pseudomonas sp.* (bacteria) *Aspergillus sp., Penicillium sp., .* (fungi) | Octadecadienoic acid, Octadecatrienoic acid, Benzene Dicarboxylic acid, Cyclopropanebutanoic | [32] |
| *P. aeruginosa* PAO1 (bacteria) | Benzene, methyl; Docosane; Tetrachloroethylene; 3- Chloropropionic acid, heptadecyl ester; Benzene, 1,3- dimethyl; Tricosane; Octadecanoic acid, butyl ester;7,9- Di-tert-butyl-1-oxaspiro(4,5) deca-6,9-diene-2,8-dione; Alkanes (Octadecane, Tetracosane, Pentacosane, Hexacosane); Fatty acids (Hexadecanoic acid, Octanoic acid); Hexadecanoic acid, ethyl ester; 1-Nonadecene; 1,2 Benxenedicarboxylic acid, diisoostyl ester; Eicosane | [33] |
| *S. marcescens 724, B. cereus, P. aeruginosa , S. aureus B-324, M. lylae B-429* (bacteria) *P. chrysosporiu, P. ostretus, A. niger and A. glaucus* (fungi) | Ergosta-5,22-dien-3-ol, acetate (3, 22 E), 1-Monanalinoeoglycerol trimethylsilyl ether, Betamethasone acetate, Azafrin, 9, 12, 15-Octadecatrienoic acid, 2,3-bis [(trimetylsilyl) oxy] propyl ester, (Z,Z,Z)-C27H52O4Si2) | [29] |

Table 1: Detailed list of the different products found after polyethyelene degradation in different organisms. Table adapted from [1]

## 2.5 Enzymes involved in PE degradation

There are several enzymes that act on PE biodegradation, in nature, they usually act as a synergistic complex to achieve an improved activity. Fungal and bacterial enzymes use similar mechanisms.

It is known that in order to occur PE biodegradation, firstly the molecular weight of the polymer need to be reduced. The reduction of the polymer needs to be done for two main reasons: to enable transport through the cell membrane and to be recognized by enzymatic systems [34]

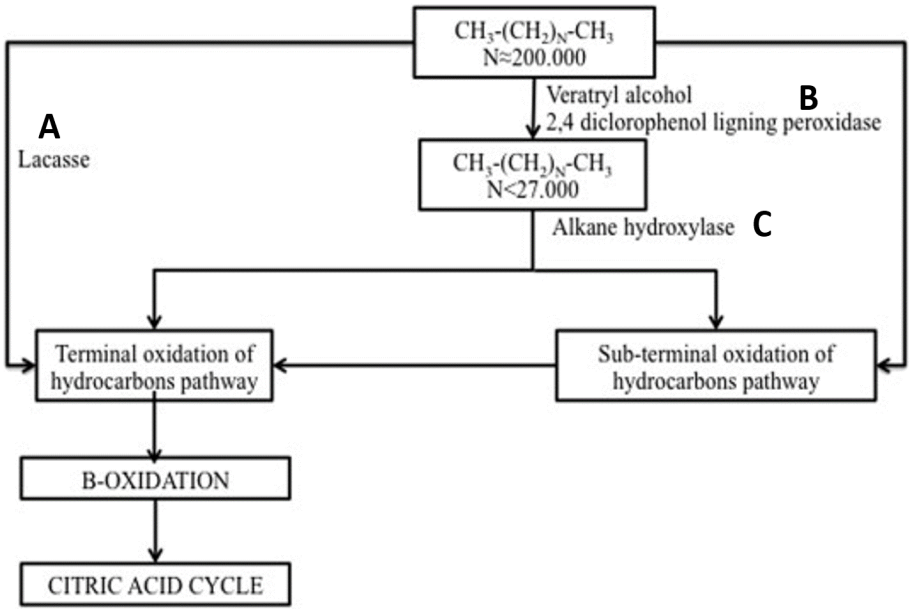


Figure 2: Scheme of a hypothetical PE enzymatic degradation path and its principal important enzymes. Extracted from [34].

In figure 2 a pathway of PE degradation is proposed, where two main enzymes are capable of breaking down the polymer: laccase (**A**) (copper-containing phenol oxidase) and lignin peroxidase (**B**) (LiP). Alkane hydroxylase (alkane 1-monooxygenase) (**C**) performs oxidation of the polymers that weight up to 27,000 Da [35], being this family of enzymes (AlkB) leading the first oxidation on hydrocarbons biodegradation [34].

**Laccase (Copper-containing phenol oxidase)**

Laccases (phenol oxidases) are a part of a larger group of enzymes designated as multi-copper enzymes. Such enzymes include three spectroscopically distinct copper ions [36]. Laccases are ubiquitous in fungi, plant and bacteria and its function it is not yet fully understood. Its oxidising activity is due to a four copper-binding sites [37].

They have been found implicated in highly diverse processes such as sporulation, pigment production, rhizoma formation and lignin degradation [38].

Fungal [20] and bacterial laccases play an important role on hydrocarbon backbone PE oxidation, its activity is highly up-regulated by the presence of copper [39].

**Heme peroxidase: Manganese peroxidase (MnP), lignin peroxidase (LiP) and Dye-decolorzing peroxidase (DyP)**

Manganese peroxidase (MnP) is the most common enzyme within the group of lignin-modifying peroxidases. It is produced by almost all lignolytic organisms [40].

It is a hycosylated heme protein that preferentially oxidizes manganese(II) ions ($Mn^{+2}$), which are always present in wood and soils, into highly reactive manganese(III) ions ($Mn^{+3}$) stabilized by chelators such as oxalic acid. Chelated $Mn^{+3}$ acts as diffusible redox-mediator that attacks phenolic lignin structures resulting in products as unstable free radicals that tend to disintegrate spontaneously. MnP is capable of oxidising and depolymerising natural and synthetic lignin as well as entire lignocelluloses [40]. Also, it is a key enzyme in fungal PE biodegradation [20].

Lignin peroxidase (LiP) was the first lignolytic enzyme to be isolated. It is a heme protein that is competent to oxidize high potential sites, such as aromatic rings [41]. Its mechanism is similar as with MnP, the difference is that LiP has a $H_2O_2$-dependant oxidation.

LiP together with MnP have been found in pre-oxidised PE fungal degradation [42].

Dye-decolorizing peroxidase (DyP) belongs to a small family of heme peroxidases. They contain a distal aspartat residue instead of a histidine [43].

They usually have been found to be responsible for the extracellular oxidation and decomposition of dyes and other aromatic xenobiotics in fungi [44].

They are separated to four different classes, being Dyp class B the responsible for bacterial lignin degradation of aromatic and non-aromatic compounds [45]. Also found this activity in fungal lignin biodegradation studies [46]. Although, any report was found for direct plastic biodegradation, its similarity with the other heme peroxidases make it a potential subject of study.

**Alkane hydroxylase (alkane 1-monooxygenase)**

This bacteria enzymatic systems consist of three components: alkane hydroxylase (AlkB), rubredoxin (AlkG) and rubredoxin reductase (AlkT).

AlkB is a non-heme iron integral membrane protein which catalyse the hydroxylation reaction. It is grouped within the family of Cytochrome P450. Rubredoxin transfers electrons from the NADH-dependent flavoprotein rubredoxin reductase to the AlkB [47].

Large number of these genes were found in bacterial populations living in oil-contaminated sites [48]. This system has been studied as the main enzymatic complex degrading polyethylene in *Pseudomonas putida* and *Pseudomonas aeruginosa* [35] [49].

## 2.6 Understanding the bacterial ecology on the polymer: biofilms

A biofilm is a construction by a community of organisms that are embedded in a self-produced matrix of extracellular polymeric substances (EPS). Importantly, bacteria in a biofilm show some "emergent properties" that differ substantially from free-living bacterial cells [50]. Biofilms are one of the most successful living forms on earth [51].

The matrix of a biofilm plays an extremely important role in terms of the viability of the cells. It confers to the biofilm a higher "sorption" capacity. Meaning that it absorbs the water and adsorbs the bio-polymers, giving a constant source of nutrients to the organisms living there. On top of that, the matrix can lend antimicrobial properties due to the sorption of cytotoxic metals. Also, the biofilm matrix can confer antibiotic tolerance to the organisms, through a sort of dilution effect; moreover, when an antibiotic substance is diffused through it, chelation and enzymatic degradation within EPS have been found to occur [52] [53].

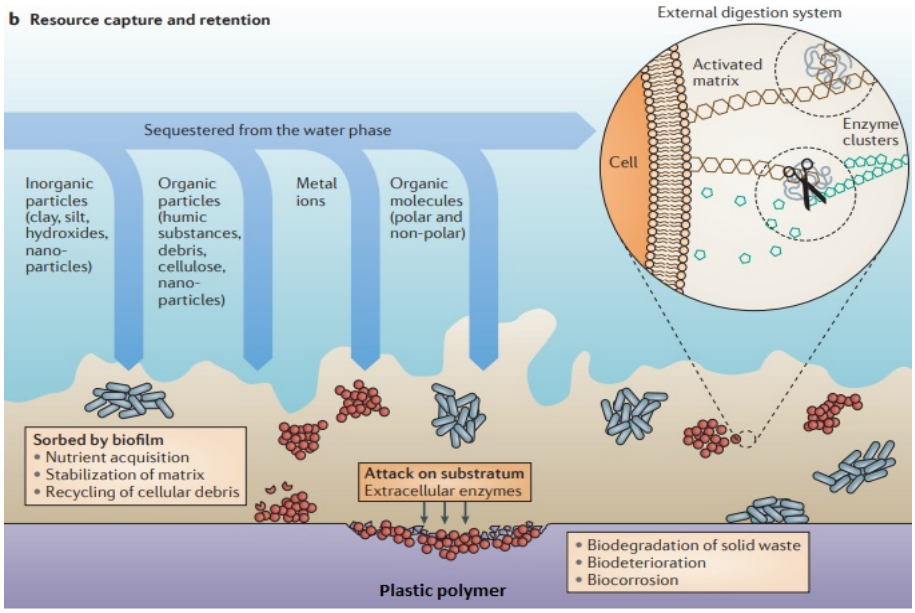


Figure 3: Scheme of a hypothetical biofilm growing on a polyethylene surface. Adapted from [51].

Figure 3 shows an examples of a biofilm growing on a polyethylene surface and its strategies to capture and retain resources from the external media. As it can be seen, the internal layer facing the substrate (plastic polymer) is used for excreting extracellular enzymes, whereas the external layer of the biofilm is used for sequestering the minerals and organic particles from the aqueous media.

Another fundamental characteristic of the biofilm is the communication within it. Inside of the biofilm different micro-environments can be found due to the gradient of different substances (e.g. pH or oxygen) [51]. As a result, there is a high heterogeneity within the biofilm that leads to adaptation to the different micro-environments by different phenotypes being expressed along the biofilm community [54].

Biofilms are not necessarily formed by the same species. The community can be composed of several different species that interact with each other in synergy and competition. The most common mechanisms of interaction and communication between cells is called "quorum sensing". Quorum sensing allows a large bacterial population to work together in a coordinated way to carry out different metabolic activities and withstand environmental stress conditions that an individual (free floating) bacterium cannot [55].

It consists of creating a concentration gradient of an extracellular signaling molecule. For example,

if a high number of bacteria are excreting the same signaling molecule, this molecule will be in high concentration in the media and will create a metabolic transduction signal, promoting the expression of certain genes. It has a huge impact during biofilm formation, because it can regulate (among other things) gene expression to produce EPS [56].

Whilst the most observed form of biofilm is the bacterial one, the concept also exists in fungal and yeast communities. Equally, they act as a sole "super-organism" attached to a surface and become more resistant to external threats [57].

Orr et al. studied the colonization of a PE surface by different enriched bacteria. They found that *Rhodococcus ruber* was the most successful microbe colonizing PE surface as well as the strain that showed highest levels of hydrophobicity in its cells [58]. Thus, indicating that polyethyelen's hydrophobicity is a limiting step for microbial colonization. Attempts to lower PE hydrophobicity resulted in more successful colonization for Psuedomonas aeruginosa [59].

## 2.7 Enhancers of the biodegradation

In nature, plastic biodegradation is not an isolated phenomenon happening between microorganism and polymer, but rather a complex multi-parameter event. Abiotic factors play an important role when it comes to erosion the polymer surface, leaving it more disposable for the biotic factors to access to the structure.

### 2.7.1 Boosting Biodegradability of Polymers:

Plastics can be made more biodegradable by improving their hydrophilicity, reducing the polymer chain length, lowering their number of crystalline regions, promoting more available branching or presence of more breakable bonds such as ester or amide against carbon-to-carbon bonds [26].

Different strategies have been tested to try to improve the biodegradability of synthetic plastic polymers.

**Pretreatment of the polymer**

Pretreatment is the prerequisite process applied to a substrate in order to facilitate the following degradation. It is widely used in *in vitro* studies to shorten the action time frame.

In nature, abiotic factors can be considered responsible for the "pretreatment" of plastic. In the laboratory, the most common plastic pretreatment would be thermal pretreatment (by using hot air oven), UV-light radiation exposure (photo-oxydation), microwave exposure and chemical treatment.

**Polymer blends**

Blending plastic with a more easily degraded and sustainable-sourced polymers such as starch or cellulose, results in an increase in polymer biodegradability [26].

On the other hand, this requires the combination with a pro-oxidant substance in order to make the whole process feasible. A similar effect is produced when blending the plastic with synthetic polymers, including poly-lactic acid or poly-caprolactum, as these act as natural enhancers of polymer biodegradability [60] [61].

### 2.7.2 Boosting Biodegradation Capabilities of Microorganisms:

#### Biosurfactants

An important strategy in the field of biodegradation optimization is the use of surfactants. It has been already tested its efficacy on polyethylene bacterial biodegradation [62] [59].

In nature, when bacteria are colonizing the surface of PE, production of biosurfactant occurs in order to lower the hydrophobicity [63] [64].

It is an emerging field, and some mechanisms still need to be understood regarding biosurfactant activity over plastic biodegradation.

Surfactants are molecules that lower the superficial tension between a liquid and another compound. These events take place thanks to the amphipathic (referring to a molecule that has both polar groups: a hydrophilic group and hydrophobic group) characteristic of surfactants.

Surface active agents (surfactants) at low concentrations are present in emulsions (the dispersion of one liquid in another) as single molecules and they bring changes in the interfacial tension (tension between a liquid-liquid) [65].

As a surfactant concentration increased, a critical concentration is reached beyond which no change in interfacial properties is noticed. Beyond this critical concentration, surfactant molecules aggregate to form micelles, colloidal particles where the hydrophobic fraction is positioned inside the micelle towards a central oil droplet, and the hydrophilic fraction face out towards a water phase [66].

Biosurfactants are usually extracellular, produced by wide range of microrganisms from various substrates. It is a hot topic because they are considered to have "green" properties such as low toxicity and functionality under extreme conditions. Their applications are also very wide: from cosmetics, to detergents, oil mining, agriculture and even in food industry [67].

There are several biosurfactants produced as secondary metabolites by bacteria, yeast and fungi. They are produced during the stationary phase of microbial growth. They are classified according to their biochemical composition as glycolipids, lipoproteins, phospholipids, fatty acids and particulate surfactants [68].

Biosurfactants can act on different levels; either allowing the microorganisms to attach to the recalcitrant polymer surface, by solubilizing it or helping to bioassimilate rapidly the oligomers, by coating them.

There are several microorganisms known to produce surfactants and have plastic biodegradation activity. One of the most well-studied examples is *Pseudomonas spp.*, which produces rhamnolipids.

Rhamnolipids are glycolipids that are composed of one or two l-rhamnose molecules coupled to a mono or dimer of beta-hydroxy fatty acids. Other studies have looked at the use of actinomycetes for degrading LDPE and its biosurfactant production [69].

Although the majority of biosurfactants have been reported in bacteria, the pathogenic nature of some producers restricts their use. In the last decade, yeast biosurfactant production have gained more popularity, not only for their majority status of GRAS (generally safe organisms), but also given their importance in the biotechnology field [70].

An interesting biosurfactant produced by different yeast strains, such as *Candida antarctica* is Mannosylerythritol lipids (MEL) [70]. This molecule act as storage molecule in yeast, similar to triacylglycerol. Its biochemical composition consists in 4-O-$\beta$-d-mannopyranosyl-meso-erythritol as the hydrophilic group and a fatty acid and/or an acetyl group as the hydrophobic moiety. It is produced in high amounts (165 g/L), although it has a relatively high associated production costs in comparison with regular surfactants [26].

In this study, the application of MEL for facilitating LDPE microbial biodegradation is tested as sustainable alternative to synthetic surfactants.

**Co-substrate culture**

Co-substrate culture, it is a methodology that consist on adding an additional carbon source to your culture in order to stimulate the metabolic activity within it. There are several reports stating that a combination of two different substrates that stimulate the same or similar pathways can lead to an increase of the metabolic activity by the degraders [71] [72] [73] [74] [75].

Co-substrate culture has been already proposed for PE degradation, due to it can palliate the possible weak enzymatic inducing effect that the substrate itself can have. Also, PE has a low rate of carbon and energy deliver, which a co-substrate can also improve [76].

In this project, lignin was used as its additional substrate due to its similarity in terms of metabolic pathways. The possible effect that the combination of both substrates that stimulate the same enzymatic pathways could have on the LDPE biodegradation efficiency is tested in this study.

Furthermore, a report where PE and PP-lignin blends biodegradability using a white-rot fungus was tested, finding that the presence of lignin on the blends, facilitate the attack of the fungus to PP and PE structures [77].

## 2.8 BRISK2 funding

BRISK2's main activity is to fund researchers to access biological and thermal biomass conversion facilities across Europe. Funded by European Horizon 2020, BRISK2 aims to improve the success of biofuels implementation.

A proposal of assembling a consortia that is able to degrade complex polymers, as for instance, lignin, was presented.

## 2.9 Aim and approach of the project

The main aim of this project is to develop and understand different strategies that could improve LDPE biodegradability under an enviornmental biotechnology approach.

In order to achieve that, different experiments will be carried out; testing the effect of a biosurfactant on a mix LDPE-degrading culture or study the effect of a co-substrate approach.

Thanks to the BRISK2 funding, defining a mixed culture using high-throughput screening and compare it with each single strain culture. Also, genomic sequence of the strains will be performed which enables to study the putative enzymes present and their possible relation with LDPE oxidation.

# 3 Materials and methods

## 3.1 Sampling

All the samples were taken from the first 5 cm of soil next to one of the piles from a plastic dump named AV Miljø, located in Hvidovre municipality, Denmark. The plastic was about 10 years old.

The samples were extracted using an ordinary non-sterile spade. Approximately 500 g were taken from three different spots randomly chosen.

## 3.2 Processing of samples

The soil was stored at 4°C. The processing of the samples was done in two different methods, depending which would be the enrichment strategy that follows.

### 3.2.1 Classical enrichment strategy

The processing of samples consisted of diluting 100g of soil in 1l of liquid Mineral Media M9 (section 3.3.2). The mixture was done in an 1l Erlenmeyer flask with a stirring magnet and let it stirring for 10 min.

Three aliquots of 50 ml each were taken while the solution was stirring, with a pipette and an open tip, so big particles were taken as part of the enrichment starting culture. They were stored at 4°C waiting for processing.

### 3.2.2 Use of Maximum Recovery Diluent (MRD) strategy

100 g of soil were dissolved in 1L of MRD solution. MRD was a protective and isotonic diluent for maximal recovery of micro-organisms containing (per liter): 1g of peptone and 8.5 g of sodium chloride in an 1l Erlenmeyer flask. Once mixed the soil with the diluent, let it settle all the solid particles from the mixture on the bottom for 30 minutes [78].

Three aliquots of 50 ml each were taken from the supernatant and stored it at 4°C waiting for processing.

## 3.3 Enrichment

### 3.3.1 Light-Density Polyethylene (LDPE)

LDPE powder without any additives and plasticizers was used.

LDPE powder was pretreated for 1 month under an ultraviolet (UV) irradiation lamps (W, nm) in a dark and closed chamber to photooxydise the substrate and facilitate microbial degradation.

Once pretreated, the granules were submerged into liquid nitrogen to be more easily grounded. The grinding was manually performed using a marble mortar, until the powder had a dusty appearance. After, the "plastic dust" was carefully rinsed with 70% ethanol, and let the ethanol evaporate at room temperature overnight.

### 3.3.2 Mineral Minimal Media (M9)

The minimal media used for this enrichment had all the minimal requirements for the microorganisms to synthesise proteins, sugars and nucleic acids.

It was used thoroughly in other researches of enriching plastic-degraders [78], [79], [80]. The media was composed (all amounts calculated per a liter) by a phosphate buffer 3 g $K_2HPO_4$ and 7.5 g $NaH_2PO_4$; 0.5 g $NH_4Cl$ was added as a nitrogen source; 0.25 g $MgSO_4$, 0.5 g NaCl, and 0.05 g $CaCl_2$ were added as essential mineral salts. In addition, the medium contained 10 mL mixed trace element solution

A 1000X trace elements solution containing $CuSO_4$, 0.04 g/L; KI, 0.1 g/L; $ZnSO_4$, 0.4 g/L; $Na_2MoO_4$, 0.2 g/L; $FeCl_3$, 0.2 g/L; and $H_3BO_3$, 0.5 g/L and filter sterilized was also used.

### 3.3.3 Biosurfactant

The biosurfactant agent used is a Mannosylerythritol lipid (MEL) produced by *Candida Antarctica* PYCC5048 using rapeseed oil as carbon source. It was an unknown mixture with predominance of MEL-A.

As a reference, the commercial surfactant Tween 80 has a CMC of 13 mg/l [81], MEL's CMC was assumed to be around 3 mg/l [82].

MEL biosurfactant's Critical Micelle Concentration (CMC), was approximately ten times less than the commercial surfactant Tween 80, therefore, as Tween 80 concentration used for plastic enrichment was 1 ml MEL per liter of culture [78], in the enrichment was used 0.1 ml of MEL per liter of culture.

### 3.3.4 Metabolic enhancer: glucose

Initially, all the flasks were added 0.5g/l of glucose as a metabolic enhancer and as an available carbon source that allowed bacteria to build-up enough biomass to synthesise the enzymatic machinery needed to metabolize LDPE.

### 3.3.5 Classic enrichment strategy set up

6 Erlenmeyer flasks of 100 ml were used with 20 ml as working volume. Two different conditions were cultured in triplicates: presence of biosurfactant and absence of biosurfactant.

Every week, 10% of the volume of each flasks was transferred to a another sterile flasks with fresh media and LDPE as sole carbon source for almost 3 month.

Also, a gradual decrease of enhancer (glucose) was added until the 4th week: the first week 0.5g/L of glucose was added, the second week 0.25g/L and after the 3rd week onwards, no glucose was added at all.

They were culture under aerobic conditions, 150 rpm and 30°C.

Sampling was performed once per week, extracting 2 ml of each flasks. 1 ml was used for analyses and the rest was froze down at -20°C. Refilling of the flasks with fresh media when was required (evaporation losses).

### 3.3.6 MDR enrichment set up

6 Erlenmeyer flasks of 150 ml were used with 50 ml as working volume (to prevent evaporation losses). Two different conditions were cultured in triplicates: presence of biosurfactant and absence of biosurfactant.

As inoculum MDR solution was used, also 10% of the working volume, the rest was filled with the M9 media and LDPE as sole carbon source. They were culture under aerobic conditions, 150 rpm and 30°C.

Sampling was performed once per week, extracting 2 ml of each flasks, 1 ml was used for analyses and the rest was frozen down at -20°C. Refilling of the flasks with fresh media when was required (evaporation losses).

### 3.3.7 Previous enrichment: summertime 2018

Some samples of isolates from an enrichment starting on the summertime 2018 were used as participants of the consortium, as well as the inoculum for the microtiter culture experiment carried on in Aalborg University's lab (Copenhagen).

This enrichment was performed as the classic enrichment strategy (section 3.3.5) with the only difference that the carbon source used was LDPE non-pretreated, and the enrichment last for 6 month instead of 3 months.

Also, different sampling sources were used in this enrichment:

1. PP: *Pseudomonas putida* from Aalborg University lab collection.
2. L: Plastic samples taken from a landfill, the plastic samples were washed and brushed with an isotonic solution and a sterlie tooth brush, the solution resulted from that was used as the inoculum for the enrichment.
3. S: Samples taken from the soil of the landfill, the procedure is the same as in the section 3.2.1.

4. SW: Plastic samples taken from the canals in Copenhagen. The samples were processed as with the landfill (L) samples, but with a marine saline synthetic solution instead.

## 3.4 Analyses of the enrichment

### 3.4.1 Extracellular protein quantification

The protein quantification was made with a commercial kit: Pierce$^{TM}$ BCA Protein Assay Kit by Thermo scientific. The microtiter plate, 96 wells, total volume of 200$\mu$l each, method was used, in triplicates and taking 25$\mu$l as inoculum with 200$\mu$l as working volume, media with LDPE and with/without glucose were taken as blanks.

Once the mix was in the microtiter plate (25$\mu$ of inoculum with reagent A and B), a shacking step of 30 seconds followed by an incubation of 30 minutes at 37°C was carried on the microtiter plate reader.

Once the incubation was completed, a step of 15 minutes to cool down at room temperature before reading the absorbance of all the samples at 562nm.

As blank, media with the reagent with and without LDPE was used, no difference between with/without LDPE were found, and the blank was taken off from each measurement as the unwanted reactions of the reagents with the media.

The background of this technique consist of the presence of bicinchoninic acid (BCA) for the colorimetric detection and quantification of total protein. This method combines the well-known reduction of $Cu^{+2}$ to $Cu^{+1}$ by protein in an alkaline medium (the biuret reaction) with the highly sensitive and selective colorimetric detection of the cuprous cation ($Cu^{+1}$) using a unique reagent containing bicinchoninic acid.

The purple-colored reaction product of this assay is formed by the chelation of two molecules of BCA with one cuprous ion. This water-soluble complex exhibits a strong absorbance at 562nm that is nearly linear with increasing protein concentrations over a broad working range (20-2000$\mu$g/mL).

Standard curve elaborated with BSA as described in protocol is attached in the appendix section.

### 3.4.2 Optical Density (OD) measurements

Optical density was measured at 600nm in a microtiter plate reader. Each sample was measured in triplicates.

Each sample was filtered in a sterile 0.2$\mu$m pore filter and sterile syringe, in order to only take into consideration the cells' turbidity.

As blanks, samples from each enrichment strategy and condition (with/without biosurfactant) were taken. These values were taken off from the rest of the values to standardize the absorbance.

## 3.5 Optimization of several factors on plastic degradation studies in two single strains and two enriched bacterial mixtures on microtiter culture:

An attempt to work with a higher number of samples was made. This experiment was started in parallel with the enrichment.

O.D. measurements were recorded every hour for a period of 130 hours.

### Inoculum

The inoculum used for this experiment were 3 isolates of the enriched culture and 1 commercial strain. The samples used were the following ones:

1. PP0: *Pseudomonas putida* not enriched. Pre-activated on LB.
2. PP1: *Pseudomonas putida* 6 month enriched
3. L2.1: Unkown mix sampled from a landfill, 6 month enriched
4. SW1.1: Unknown mix samples from plastic floating in the canals, 6 month enriched
5. Control: No inoculum

### Variables tested

Seven different variables were tested with each of the inoculums. The culture media was M9 for all the samples.

The longest period that the machine can be programmed for incubation was 72 hours, therefore, the whole period of 130 hours was achieved thanks to a manual re-set of the incubation period in the middle of the experiment.

In table 2 all the variables are listed with a more detailed explanation.

| Culture set-up abbreviation and code | Culture detailed composition |
|---|---|
| PE pret (PE1) | M9 media with addition of 1% sterile LDPE pretreaded for 1 month under UV-light |
| PE non-pret (PE2) | M9 media with addition of 1% sterile LDPE non-pretreaded |
| PE pret + T80 (T80) | M9 media with addition of 1% sterile LDPE pretreaded for 1 month under UV-light and used of 1ml/l of a commercial surfactant agent, Tween 80. |
| PE pret + MEL (BIOS) | M9 media with addition of 1% sterile LDPE pretreaded for 1 month under UV-light and used of 0.1ml/l of a biosurfactant agent, MEL. |
| PE pret + Glu (G) | M9 media with addition of 1% sterile LDPE pretreaded for 1 month under UV-light and used of 0.5g/L of glucose as metabolic enhancer. |
| PE pret + PHB (PHB) | M9 media with addition of 1% sterile LDPE pretreaded for 1 month under UV-light and used of 0.38g/L of polyhydroxybutyrate as metabolic enhancer. |
| PE pret + MEL + PHB (COMB) | M9 media with addition of 1% sterile LDPE pretreaded for 1 month under UV-light and used of 0.38g/L of polyhydroxybutyrate as metabolic enhancer and also 0.1ml/l of biosurfactant. |

Table 2: a summary of all the different variables tested in the experiment. The codes in the brackets are matching with the matrix attached in the appendix section.

## 3.6 Biosurfactant concentration optimization for LDPE degradation with an enriched mixed culture

The composition of the MEL given was not determined, as well as its CMC. Therefore, the values can be related to similar production process found on literature but not exactly determined for the biosurfactant used. As a result, an experiment was set up in order to determine the optimal concentration of biosurfactant for this project purpose.

As a positive control, a well known commercial surfactant Tween 80 was used. It is also known that MEL's CMC is lower than Tween 80. Tween 80 has a CMC of 13 mg/l [81], MEL's CMC was

assumed to be around 3 mg/l when its produced from vegetable oil [82].

MEL was prepared in an ethanol solution (1:1). Its high viscosity makes it hard to be manipulated in small quantities. The necessary amount of the mixture was taken, before adding it to the culture media, an extra step of ethanol evaporation was performed with nitrogen gas. Different times of exposure to the nitrogen were used, according to the amount of ethanol present.

One minute was flushed to the samples of the smaller volume, 52 $\mu$l. Two minutes were used for the 104 $\mu$l and five minutes for the 520 $\mu$l. Taking into account that half of the volume need to be evaporated.

Density of both surfactants (biosurfactant and tween 80) was considered close to 1 g/l.

The different relative concentrations of surfactant agents are shown in table 3.

| According to MEL CMC (BS CMC) | According to Tween 80 CMC (T80 CMC) | Amount of biosurfactant added per liter |
|---|---|---|
| $\frac{1}{2}$ x BS CMC | $\frac{1}{20}$ x T80 CMC | 0.65 ml |
| 1 x BS CMC | $\frac{1}{10}$ x T80 CMC | 1.3 ml |
| 5 x BS CMC | $\frac{1}{5}$ x T80 CMC | 6.5 ml |
| 10 x BS CMC | 1 x T80 CMC | 13 ml |

Table 3: Table showing the CMC proportion in each flasks for the biosurfactant and the surfactant.

The experiment was carried in duplicates, total working volume was 20 ml per flasks, and a total of 6 different conditions were set up. Table 3 shows the different conditions and the samples' code name that were set up in order to optimize the concentration of the biosurfactant for LDPE degradation in a model mixed culture taken from the enrichment.

All the Erlenmeyer flasks (100 ml total volume) were inoculated with 2 ml of the enrichment sample SM1, and 18 ml of M9 media without any carbon source. The rest of the compounds can be seen in table 3 and 4.

Sampling was performed once per week during two weeks, 2 ml of each flasks were taken. Extracellular protein content quantification, together with Colony-Forming Unit (CFU) counting were performed in all the samples.

All the different batch compositions of the experiment are shown in table 4.

| Sample code | Substrate (LDPE pretreated) | Surfactant: biosurfactant (MEL) or tween 80 (T80) |
|---|---|---|
| BS01, BS02 | 0.2 g | None |
| BS11, BS12 | 0.2 g | 26 $\mu$l BS |
| BS21, BS22 | 0.2 g | 52 $\mu$l BS |
| BS31, BS32 | 0.2 g | 260 $\mu$l BS |
| BS41, BS42 | None | 52 $\mu$l BS |
| BS51, BS52 | 0.2 g | 520 $\mu$l T80 |

Table 4: Set up composition in all 6 flasks. All the amounts are referred for the total working volume of 20 ml per flasks.

### 3.6.1 Extracellular protein quantification

The procedure used was exactly the same as in section 3.4.1.

### 3.6.2 Biomass estimation: CFU counting.

In order to have an idea of the biomass generated in the different conditions, they were estimated through a microbiological technique that consisted in using a unit that estimates the number of viable cells in a sample. It is a culture dependent technique.

Firstly, different serial 10 fold dilutions were made. In our case, the volume for inoculation was only 20 $\mu$l, therefore the dilutions were preformed in a 2 ml eppendorf, 100 $\mu$l were extracted from each culture in 3 different times: right just before starting the incubation, after 7 days and after 15 days. this 100 $\mu$l of culture were mixed with 900 $\mu$l of sterile saline solution composed with 0.9% NaCl dissolved in distilled water and autoclaved for 20 minutes.

From this first solution, which was $10^{-1}$ concentrated from the original one, 100 $\mu$l were taken and introduced it to another eppendorf with 900 $\mu$l of saline solution. Once mixed, the dilution was $10^{-2}$ diluted regrading the original culture, serial dilutions were repeated till reach $10^{-6}$ dilution.

The method used to culture the samples in Lysogeny Broth (LB) agar media. Its composition was (per liter): 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, 15 g of bacto agar.

As it can be seen in figure 6, in each petri dish, three serial dilutions were inoculated in triplicates. The inoculum consisted in a 20 $\mu$l drop, that was deposited in the petri dish. A total of 2 petri dishes per experiment sample were used. One contained the first three serial dilutions, and the other contained the three last dilutions.

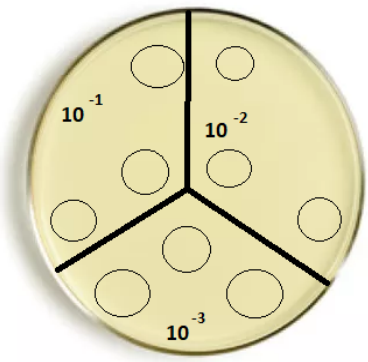


Figure 4: represents the distribution of the drops with the inoculum and triplicates (black circles) along the petri dish, as well as the distribution of the three different dilutions ($10^{-1}$, $10^{-2}$, $10^{-3}$).

The counting of CFUs assumes that every colony is separate and funded by a single viable microbial cell. The total number of colonies counted in a plate together with the dilution factor used can be combined to count the CFUs per ml. The standard accepted ranges are the plates that have between 30 to 300 CFUs [83]. Each of the drops were counted for CFUs, when possible.

## 3.7 Isolation

Once the enrichment was on going for 3 month, the isolation step started. The isolation was carried using the streak plate technique in LB agar media, the recipe is the same as in the previous section.

Firstly, 100 $\mu$l of each flask (12 flasks in total) was inoculated to an LB plate, and spread it with an L-shape spatula to all the plate surface in order to have a plate starting culture where the single colonies can be picked and transfer to a another plate (section **A** from figure 16).

After 24 hours of incubating them at 30°C, were carefully picked 5 to 9 morphologically different colonies with a 1 $\mu$l diameter inoculating sterile loop and transferred to a half of a LB plate. The first streak was on the first quarter of the half, the streaking lines were more joined, so the majority of the cells will be left in that quarter, whereas in the following quarter, just a streaking line is dragged to there and the lines are more separate, so a lower cell concentration is assumed. On that surface was where the single colonies were expected (section **B** from figure 16).

After 24 hours of growth at 30°C, the same procedure was repeated, picking a colony and transferring to another half of a petri dish. A total number of 3 transfers were performed before considering them "isolates".

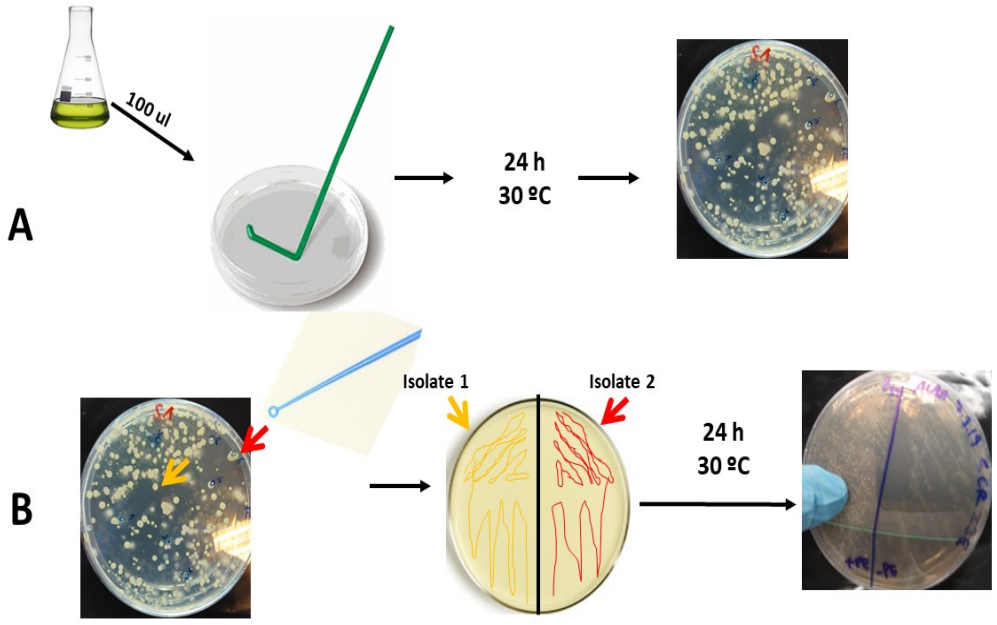


Figure 5: Represent a graphic scheme of the isolation step.°textbfA is the initial step.**B** is the following step.

## 3.8 Screening of the isolates for the fastest degraders

A high number of potential isolates were found. Therefore, a screening for the best degraders was done.

In this step different experiments were combine: samples from the summertime enrichment, from the 3-month enrichment and also one isolate from an external enrichment where malathion was its only carbon source for 3-month enrichment.

In the screening all the isolates were transferred from an LB agar plate, to a M9 agar plate. The M9 agar plate was divided in two half and each half in two quarters. There were two isolates per M9 petri dish, and in one quarter there was LDPE pretreated as the only carbon source, whereas in the other quarter there was no carbon source. The inoculation of each isolate was done in each half.

They were incubated at 30°C for 1 week.

## 3.9 Isolates -80°C Library

Once the isolates were chose to continue with the next experiments, a -80°C library was done with 11 isolates together with 3 already known strains.

A total of 14 strains were stored at -80°C with the following sample code of the enrichment and a new number that will be used for the further experiments in SINTEF:

To prepare the library, all the selected isolates were transferred from the screening plate to a new plate with LB. In this case, it is needed to transfer one isolate per plate.

Afterwards, a 500 $\mu$l of a 15% of glycerol diluted in sterile water solution was deposited on the surface of each petri dish. Between 10-15 sterile glass beats of 3 mm diameter were thrown in each plate and vigorously shaken in order to embed them with the culture and the glycerol solution.

Once it was seen that the glass beats had embedded the mixed solution on their surface, with a sterile and flamed metal spatula, transferred to a cryotube. Each glass beat represented an inoculum and you can defrost them independently of the others, making sure that the rest of cells do not suffer from thawing effect.

In total, 14 cryotubes were deposited in an isopropanol recipient, Mr. Frosty™ freezing container from Thermo Scientific™. The recipient is store at -80°C freezer. The isopropanol helps to preserve the viability of the cells when freezing, due to it can achieve a rate of cooling very close to -1°C/minute which preserve the cells from the sudden freezing.

## 3.10 Transportation of the isolates to SINTEF (Trondheim, Norway)

After freezing them down at -80°C with Mr. Frosty™, they were taken and store them in a dry ice container which was further send to Trondheim by a courier.

## 3.11 High-throughput screening of artificial assembled consortia with the enriched isolates on plastic and lignin biodegradation studies

This part of the project was carried in Trondheim, supported and thanks to BRISK2 funding.

14 strains described at section 3.9 were used for these purposes.

### Defrosting: activation and normalization of the cultures

The activation media chosen was the regular LB used in this project, for *I. sakaiensis* M9 media with approximately 1% of pretreated sterilized LDPE was used, due to it is not able to uptake any carbon source from LB itself.

Under sterile conditions, 10 ml of LB media was poured into 13 falcon tubes of 50 ml, in one falcon tube, 10 ml of M9 media was poured with the addition of LDPE as carbon source.

All the strains shipped and stored at -80°C, were taken and from each of the 14 cryotubes, a glass beat was taken with the help of a 10 $\mu$l sterile inoculation loop, and introduced it in the correspondent labelled falcon (*I. sakaiensis* was activated in M9 and LDPE as carbon source).

Then all the falcon tubes were incubated for 48h, checking its O.D. every 24h, making sure that all have the same O.D. with an error of $\pm$ 0.4, if exceeded a diluting step will be ensured.

**Inoculum preparation**

Once all the 14 strains were equilibrate at around 1 of absorbance units, transferred 5 ml from each seed culture to another 50 ml falcon tube. In order to got rid of the media and any carbon source that could be in it, a centrifugation step at 20°C, for 6 minutes at 4000 rpm was needed, twice in all 14 newly transfer 5 ml culture.

The LB media was substituted for 5 ml of M9 media with no carbon source. This step was repeated twice.

A pre-incubation of the inoculum was performed in a master plate of 500 $\mu$l as working volume, using 10% of inoculum, in M9 mineral media and 0.2% of glucose as the only carbon source, at 30°C at 800 rpms with 85% of humidity in a microtiter plate incubator for 24 hours.

Before inoculation, the master plate was centrifuged for 6 minutes at 4000 rpms at 20°C and washed with M9 minimal media without any carbon source. This process was repeated twice in order to make sure that there was no carbon source in the inoculum.

**Single strain biodegradation test**

Test each single strain activity in five different substrates: M9 supplemented with 0.2% of glucose, M9 with approximately 10% of lignin, M9 with approximately 10% of LDPE, M9 with approximately 5% of LDPE and 5% of lignin together and LB media.

Solid substrates were added manually to each well with a manually calibrated sterile steel spoon, assuming an error per sample of approximately $\pm$ 0.005 mg.

The strains were cultured in a sterile 96-well microtiter black plate with transparent bottom and lid. A total working volume of 100 $\mu$l was used in each well which 10% consisted on the inoculum and the rest was filled with the different media.

They were cultured in triplicates, and three negative controls were included to see if there was any effect of the different compounds in the assays: distilled water, media without any carbon source and media with carbon source. A total of 5 microtiter plates were used.

Once cultured for 48 hours ATP quantification was performed.

### 3.11.1 Assessing the cell viability of the strains: BacTiter-Glo™

BacTiter-Glo™ Microbial Cell Viability Assay (Promega) is a homogeneous method for determining the number of viable bacterial cells in culture based on quantification of the ATP present. ATP is an indicator of metabolically active cells. The assay procedure involves adding a single reagent (BacTiter-Glo™ Reagent) directly to bacterial cells in medium and measuring luminescence.

The formulation of the reagent supports bacterial cell lysis and generation of a luminescent signal. The luminescent signal is proportional to the amount of ATP present, which is directly proportional to the number of cells in culture.

The BacTiter-Glo™ Reagent uses a thermostable luciferase (Ultra-Glo™ Recombinant Luciferase) and a method of extracting ATP from bacteria. The assay generates a "glow-type" luminescent signal, produced by the luciferase reaction which has a signal half-life generally over 30 minutes depending on the bacterium and medium.

It is a good candidate to measure viable cells in solid non-soluble polymer degradation due to ATPs can be extracted and determined from entire sample, which means simultaneously from both the biofilm on the material surface and the surrounding liquid medium, the assay is extremely sensitive and the response linear across several orders of magnitude. Also, already has been used in other biodegradation studies [76].

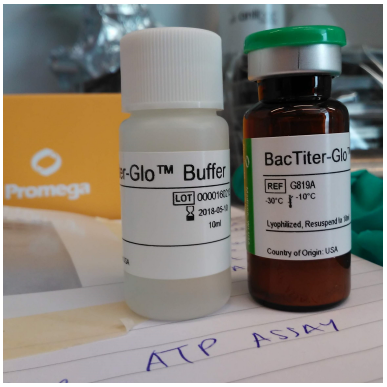


Figure 6: shows two reagents of BacTiter-Glo™, the buffer and the enzymatic cocktail.

Always two negative control were included in the assay: media with the substrate and media without the substrate. If any background noise was recorded on them, the value would be taken off of the rest of the values.

100 $\mu$l of sample was analyzed in the same microtiter plate, 100 $\mu$l of BacTiter-Glo™ reagent was added to all the samples.

BacTiter-Glo™ reagent was prepared mixing both reagents equilibrated at room temperature. One of the reagents consisted on a buffer and the other consisted on a powder-like substrate mix. Transferred the 10 ml of the buffer to the bottle of the substrate, mix it and equilibrate the reagent for at least 15 minutes at room temperature.

After adding the mixed reagent with a multi-pipette channel to each of the microtiter plates, a shaking step at 800 rpms of approximately 1 minute was performed, followed by 5 minutes of incubation at room temperature.

After incubation, an extra 3 minute centrifugation step at 3000 rpms was performed to all the microtiter plates, in order to clear the luminescence background before being recorded.

The luminescence was recorded with a microplate reader whose model is SpectraMax Paradigm Multi-Mode.

**Using a Biomek i7 automated station for liquid handling.**

In order to perform in a more cost-effective way, a liquid handling robot (Biomek i7) was used to inoculate from the master plate to the different microtiter plates used. Also the M9 media was transfer to each well with the robot.

In each cultivation step of the consortia assembling experiment, a master microtiter plate with a high volume was used, the robot was taking from there the inoculum and distributing it to all the 16 microtiter plates that were used per round, afterwards it also distributed the M9 media. Substrates were distributed mannually as stated previously.

**High-throughput screening of artificial assembled consortia with the enriched isolates on plastic and lignin biodegradation studies**

The microtiter plates were the same and set up on the same way as in section 3.12.3..

Three different culture steps were performed in order to increase the number of bacterial strains forming the mix in each step, depending on the performance of the previous one. All of them were performed on duplicates for 48 hours at 30°C in a microtiter plate shaker incubator at 800 rpms and 85% humidity.

After the incubation the ATP was quantified, according to the protocol described at section 3.13.1.

1. Two strains bacterial mix

   Firstly, a culturing step of combining manually all the isolates (14) between themselves was performed. This results on a total of 91 combinations of 2 strains each.

2. Four strains bacterial mix

   Secondly, a culturing step of combining manually the 14 best performers of the first round, according to the ATP assay, between themselves, having a total of 4 strains in each of the 91 combinations.

3. Eight strains bacterial mix

   Lastly, a culturing step of combining manually the 14 best performers of the second round, according to the ATP assay, again between themselves, having a total of 4 strains in each of the 91 combinations.

## 3.12 Genomic sequencing of the unknown isolates

### 3.12.1 DNA extraction of the unknown isolates

The DNA extraction for the 11 unknown isolates was performed according to the protocol of the gram positive DNA purification kit MasterPure$^{TM}$ Cat. N°. MGP04100 from Epicenter.

The purified DNA was stored overnight in the fridge in order to allow the DNA to dissolve on the TE buffer.

### 3.12.2 DNA quality assessment

Three different methods for the DNA quality assessment were performed.

1. Nanodrop:

   1 $\mu$l of the samples is loaded in order to measure the absorbance with the nanodrop. As a blank the TE buffer was used.

2. Electrophoresis:

   Agarose gel 0.8% concentrated was used with DNA-binding dye GelRed®. 2 $\mu$l of DNA bladder as a references was used. In each load a total of 21 $\mu$l of volume was used whose composition was: 3$\mu$l of dye, 3$\mu$l of each sample and 15$\mu$l of water. The gel was running for an hour at 120V.

3. Quantification of the DNA (Qubit$^{TM}$ fluorometer):

   5 $\mu$l of each sample is mixed with the kit's dye that specifically binds with the DNA. Fluorescence is read with the fluorescence reader provided by the Qubit$^{TM}$ kit.

## 3.13 Assembly of the data

Sequencing libraries were prepared by the Nextera XT method (Illumina) and sequenced on an Illumina MiSeq sequencer in the 2*300bp Paired End read mode applying the MiSeq MiSeq Reagent Kit v3.

Sequencing data were demultiplexed in MiSeq reporter. Generated fastq files were then processed in CLC Genomics Workbench v. 12 (Qiagen). Raw reads were quality trimmed, removing adapters and read-through sequences using the Trim Reads 2.3 tool. Trimmed reads were then assembled into contigs by the De Novo Assembly 1.5 tool. Contigs were filtered by length and coverage , removing all contigs shorter than 500bp as well as reads shorter than 1kb with a coverage below 5 (or 10).

Final assemblies were taxonomically evaluated by the BLAST 1.0 tool (also within CLC Genomics workbench) searching against the 16S Microbial database and species/genus was assigned from these hit tables.

## 3.14 Polymer biodegradation: Fourier-Transform Infrared Spectroscopy

### 3.14.1 Culture

A culture was set up in a microtiter plate, the volume of the culture was 100 $\mu$l in a 400 $\mu$l microtiter plate. 7 different consortia were used as inoculum and culture in triplicate. The culture

was cultured at 150 rpm, 30°C for 17 days in total, having a sampling step at day 4.

The inoculum used was selected according to the consortia that had a higher ATP production in the ATP assays. The composition of the bacterial mixtures were the following:

1. *Brucella sp., P. stutzeri, B. cereus, P. putida*
2. *Brucella sp., P. stutzeri, I. sakaiensis*
3. *Brucella sp., I. sakaiensis, Pseudoxanthomonas sp., B.cereus*
4. *R. ruber, Pseudoxanthomonas sp., Amycolatopsis sp., B. cereus, O. cicer*
5. *B. cereus, R. ruber*
6. *Pseudoxanthomonas sp., P. stutzeri, B. cereus*
7. *O. ciceri, B.cereus*

### 3.14.2 Fourier transform infrared spectrophotometry (FTIR) analysis

There were two types of samples analysed:

1. Recovery of the LDPE from the culture (approx 0.001 mg)
2. Recovery of the mixture of LDPE and lignin from the culture (approx a total of 0.001 mg)

The substrate was recovered by a small steel spoon (approx. 1 mm of diameter) manually from each of the microtiter wells. Afterwards, they were washed in a separate 1.5 Eppendorf tube with deionitzate water. Once washed, using the same spoon, the substrate is again fished and deposited over a glass coverslip and let the water evaporate before analysing them.

They were measured before and after washed in order to detect if a subtle change of the polymer was being washed, due to the short exposure time to the organisms.

Prepared sample was determined at room temperature by a FTIR (Nicolet 6700/8700, Thermo Scientific, USA) in the spectral range of 4,000 to 400 $cm^1$ using 64 scans at 2 $cm^1$ resolution and the measurement time for each sample was around 60 s. The data is represented as the percentage of transmittance.Five different measurements were taken per sample.

# 4 Results

## 4.1 Monitoring the enrichment culture: O.D. and extracellular protein content.

The enrichment was sampled once per week and quantification of extracellular protein was assessed. Standard curve for protein content are shown in the Appendix section. In the first month of the enrichment, O.D. measurements also were monitored.

Results of the O.D. measurements and the quantification of the extracellular protein content are shown in figures 7 and 8 respectively. Both strategies results were plotted together in order to be compared.

Functions coloured in blue and orange represent the enrichment cultures with the classical set up, whereas the grey and yellow colors represent the enrichment with the MDR set up.

Sampling times were not constant in all the enrichment period. In figure 7 all measurements were taken once per week, during the first four weeks of the enrichment. While in figure 8 after the first four weeks, a pause of the sampling due to Christmas holidays left an interval between T4 and Tx of 2 weeks instead of 1 week as in the rest of the data.

However, O.D. results were not very representatives for the ecology that is expected to have the enrichment samples. Consequently, after transfer n$^{o}$4, it was decided to stop measuring the O.D.

Furthermore, it is important to remark that while MDR enrichment was supplied with 0.5 g/l of glucose as metabolic enhancer at T0, classical enrichment had a sequential decreased addition of glucose: 0.5 g/l at T0 and 0.25 g/l at T1.

In figure 7 O.D. values for the cultures that the MDR strategy was used (orange and blue), showed a slight net increase of the after four weeks (0.1 units), with a peak from the first to the second week and a more steady period in the last two transfers, where the samples that were not using MEL (blue) were overtaken the samples that did (orange).

Alternatively, the O.D. data for the culture that the classical strategy was used (grey and yellow), T1 and T2 were not plotted in order to avoid unrepresentative values due to the dirt instead of the bacterial growth turbidity. However, at T3 and T4 it can be seen a net increase of the O.D. value in both cases that can be attributed to the culture turbidity. When MEL was applied (yellow) much higher values were achieved, and it doubled the O.D. units within one week (T3 to T4).

If both strategies were compared, for the same time periods (T3 and T4) the classical strategy developed a better capacity to build-up bacterial biomass and showed turbidity in the O.D. analysis, taking into consideration that at transfer number 4 the amount of glucose would be almost nonexistent.

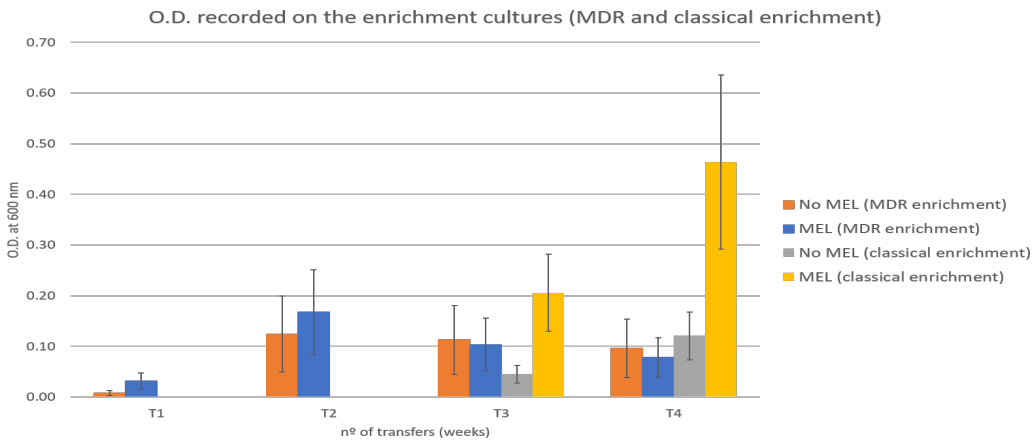


Figure 7: O.D. data evolution in the four first weeks of the enrichment culture following the MDR strategy. Standard deviation average of the triplicates are represented as error bars

The results of the total extracellular protein content are shown in figure 8. It reflects a rough coordination with the O.D. results in the first four weeks.

In the cultures that the MDR enrichment was used (orange and blue), it was detected a depletion of proteins from T3 to T4 and Tx to T5. Having a smooth rise in the last week, it leaves the total protein net content close to zero.

Even the addition of 0.5 g/l of glucose, the amount of proteins at T1 is relatively low (20 $\mu$g/ml) indicating that the glucose was already consumed after a week.

According to the protein content, samples where MEL was added (orange), at the beginning of the enrichment, had better production of extracellular proteins during almost all the enrichment period, being considerably constant the low values of protein content.

On the other hand, cultures where the classical enrichment was used (yellow and grey), higher values of proteint content than the enrichment with MDR were recorded til Tx. From Tx (after christmas) to T6 a sharp depletion of the protein content in both samples (with MEL and without MEL) was found.

For the classical enrichment, when transferring from T0 to T1, 0.25 g/l of glucose were added, thus, it could explain the reason why the higher amount of proteins were produced between T1 and T2, suffering T3 an important depletion because there was no addition of glucose when transferred. The following peak, from T3 to T4 could be explained because of the overtaken of activity of other bacterial community once the fastest growers were starving.

As a result, cultures that were set up with the classical enrichment suffered a net loss of their protein content not reaching any stability within the 9 weeks of enrichment, whereas in the MDR strategy, the protein content suffered a steady evolution along the different transfers having a more stable protein production along the enrichment.

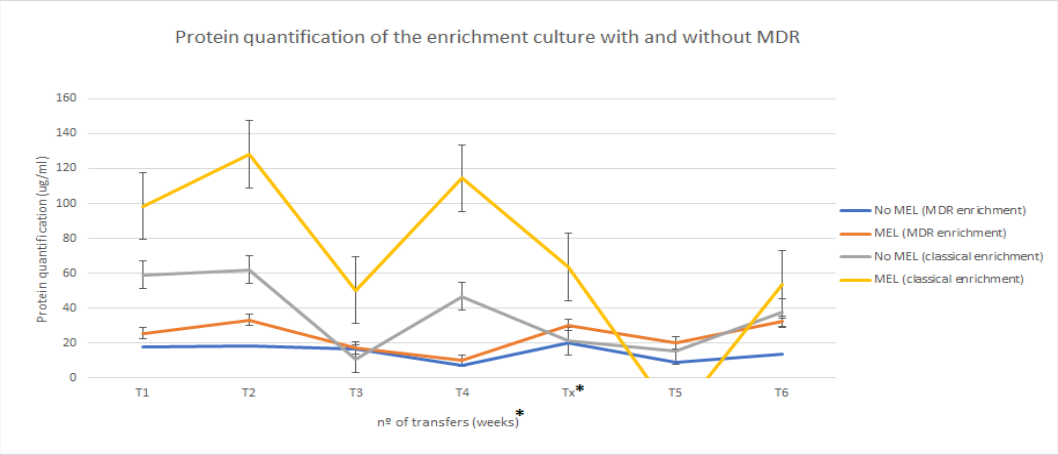


Figure 8: Extracellular protein data evolution from the beginning of the enrichment til the end, set up following the MDR strategy (blue and orange) and the classical strategy (yellow and grey). Standard deviation average of the triplicates are represented as error bars. The blue and grey lines represent the cultures that have not been used any biosurfactant (MEL), whereas in orange and yellow are represented the cultures that have been added MEL. ***Tx** *represents the measurements taken after Christmas (2 weeks period, instead of 1 week period as the rest of the transfers).*

## 4.2 Optimization of several factors on plastic degradation studies in two single strains and two enriched bacterial mixtures on microtiter culture:

An attempt of optimizing the degradation conditions was made in a microtiter plate, the incubation and the O.D. monitoring were carried by a microtiter incubator and plate reader that was recording every hour the O.D. data.

Seven different conditions and four different incoulum were used: two single strains: PP1 and PP0. PP1 is an enriched culture of *P.putida*, while PP0 is a fresh culture of *P.putida* that was pre-activated on LB and inoculated to the experiment afterwards. Alternatively, two other inoculum were tested: SW1 and L2. Both are a mix culture that have been enriched on plastic as the sole carbon source for six months.

In the appendix section each condition's O.D. is plotted against time (10 hours intervals) together with a table where all the coefficient of variance's percentage are shown per each sample at the beginning and at the end of the experiment.

In this section, two different bar graphs were made in order to summarize the important results and compare them. Figure 9 shows the overall net growth of all the different conditions tested, the values were calculated using the following equation:

O.D. net growth = [(sample O.D. at 130h)] - [(sample O.D. at 1h) - (control O.D. at 130 - control O.D. at 1h)]

As time zero the microtiter plate reader recorded the first O.D. value after 1 hour of incubation.

In addition, figure 10 showed the maximum growth in all the different conditions tested. The values

were calculated using the following equation:

O.D. maximum growth = [(sample O.D. max. value)] - [(sample O.D. at 1h) - (control O.D. max value - control O.D. at 1h)]

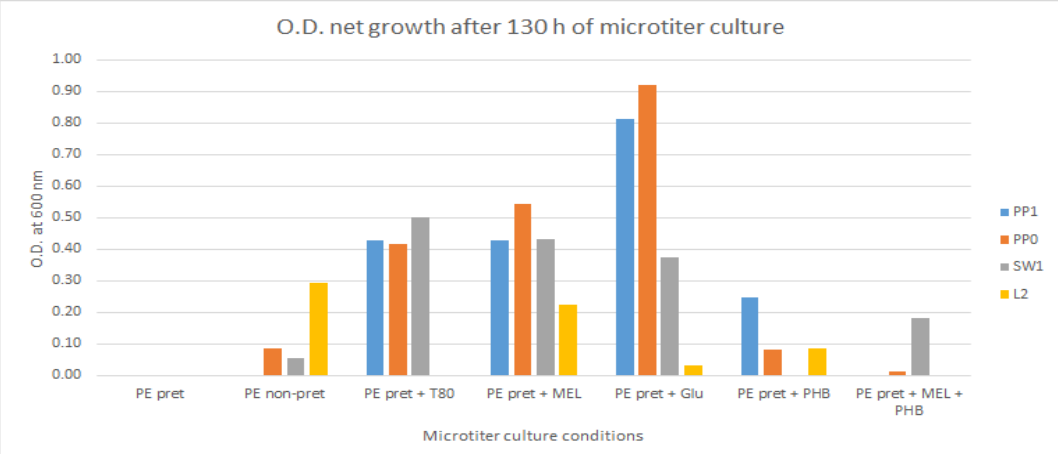


Figure 9: O.D. net growth of the overall culture in seven different conditions for each of the different inoculums.

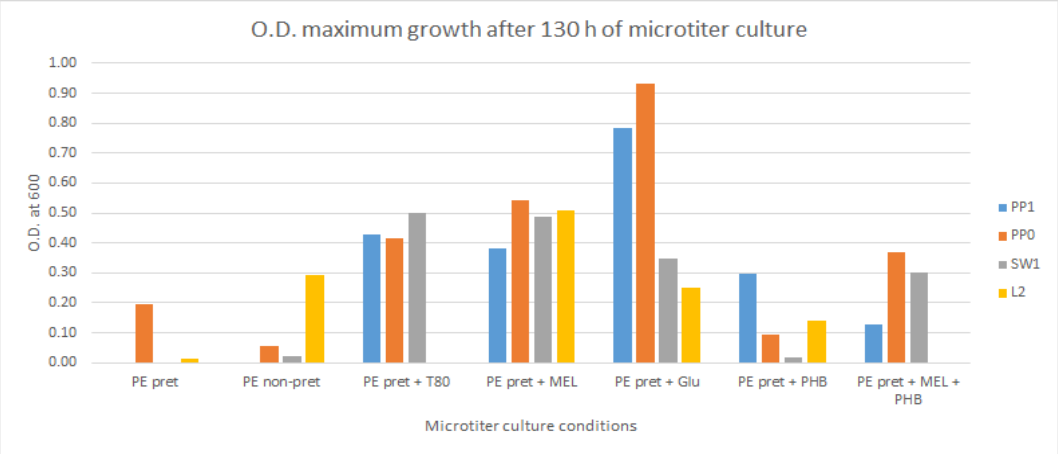


Figure 10: O.D. maximum growth of the overall culture in seven different conditions for each of the different inoculums.

**Pretreatment efficiency: LDPE pretreated and LDPE non-pretreated.**

It can be seen that the O.D. measurements taken under these two conditions are relatively low. Surprisingly, the highest O.D. values recorded were with LDPE non-pretreated as the sole carbon source, and L2 was the inoculum that scored the highest O.D. value (0.3) followed by the *P.putida* not enriched (PP0) under LDPE pretreated (0.2).*P.putida* enriched (PP1) did not record any significant O.D. and SW1 recorded a low O.D. value (0.05) which can not be considered significant either.

It can not be conclude that the UV-photoxidation pretreatment showed any significant difference

regarding the O.D. data in any of the cultures.

### Surfactant agent: tween 80 and MEL

It can be seen that both surfactant agents promoted higher O.D. measurements in all the cultures than when were not used. Both surfactants were in the same concentration as their respective CMC. They gave similar scores in all the different strains but not in the mix enriched culture L2, which only showed turbidity under the presence of MEL.

*P. putida* not enriched (PP0) showed slightly better performance under the presence of MEL than under the presence of tween 80 respect *P. putida* enriched (PP1).

According to these results, 1 CMC of MEL and Tween 80 provoked similar turbidity to all the different cultures but not in L2.

### Metabolism enhancer: glucose and PHB

Two different carbon metabolism enhancers were tested, the same carbon moles were added, so the amounts were adjusted accordingly. The concentration was 0.5 g/l for glucose, and 0.38 g/l of PHB.

When glucose was used as enhancer, very different growth patterns were shown: *P. putida* not enriched (PP0) performs better (almost reaching 1 as O.D. value) in terms of turbidity than the rest, followed by *P. putida* enriched (PP0) SW1 and finally L2.

The total O.D. values reached when glucose was used are the highest values for all the experiment for both *P.putida* strains, while the mix culture SW1 and L2 performed better when biosurfactant MEL or Tween 80 was added.

When PHB was used instead, none of them performed better than with glucose, the best perofrmer was *P.putida* not enriched that reach a maximum O.D. growth of 0.3.

130 hours were too little to show the long-term effects of both enhancer, being glucose more effective as a short term enhancer.

### Combination of surfactant agent and metabolism enhancer

When MEL and PHB were combined, it improved the O.D. growth of mix culture SW1 and *P.putida* not enriched (PP0) respect the PHB alone, but it lowered the growth of all of them respect the MEL alone.

## 4.3 Biosurfactant concentration optimization for LDPE degradation with an enriched mixed culture

Three different concentrations of MEL were tested and compared with a well-known surfactant used for LDPE biodegradation, tween 80 [78]. Colony-forming units and extracellular protein content were performed once per week during two weeks. Results are shown in figure 11.

In Appendix it can be found the results of CFU and extracellular protein content separately. Also their coefficient of variance for the triplicates of each measurement taken.

Colony-forming units counts and extracellular protein content were compared and both duplicates are shown (1 and 2) in figure 11.

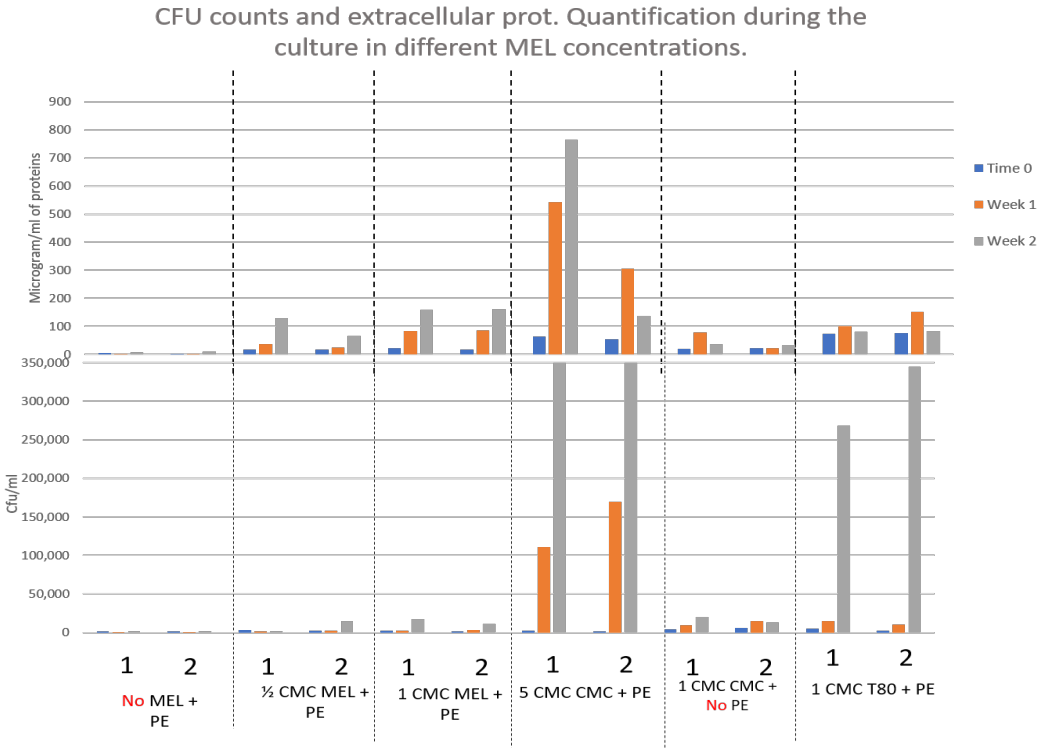


Figure 11: Extracellular protein content and colony-forming units counts represented in two bar graph for each of the duplicates (1 and 2) in six different culture conditions during 2 weeks. Sampling once per week. The blue bars represent the values at time 0, orange bars are the values obtained taken after a wekk and the grey bars are the last values taken, after two weeks.

Both duplicates showed a similar pattern of results. Also, both measurements methods indicate a similar pattern of growing and growing intensity in each of the duplicates.

In figure 11 the samples where no surfactant agent was added (BS01, BS02) which are represented as the first values in the graph (no MEL + PE) were the lowest, being either in protein content or in CFU counts almost non-significant in comparison with the rest of the conditions.

For the samples where a half of the MEL CMC concentration was added (BS12, BS12), their performance is slightly better, in terms of CFU, than the previous condition whereas in the protein

content, concentration found is the third lower within the experiment.

When the concentration of the theoretical CMC for MEL was applied to the culture (BS21, BS22), CFU counts were almost the same as when it halved. In terms of protein content, there was a small gradual increase along the weeks, being on week 2 the second best condition.

Five times the MEL's CMC concentration was tested (BS31, BS32), a huge increase was achieved after 2 weeks of growth, in both assays: proteins and CFU counting. In terms of protein content, there was a difference between replicate 1 and 2. In the first replicate in week 2, 300 $\mu$g/ml more than replicate 1 were recorded, while in the last week, replicate number 1 rose up its protein content til almost 800 $\mu$g/ml and replicate number 2 lowered its protein content til almost 100 $\mu$g/ml.

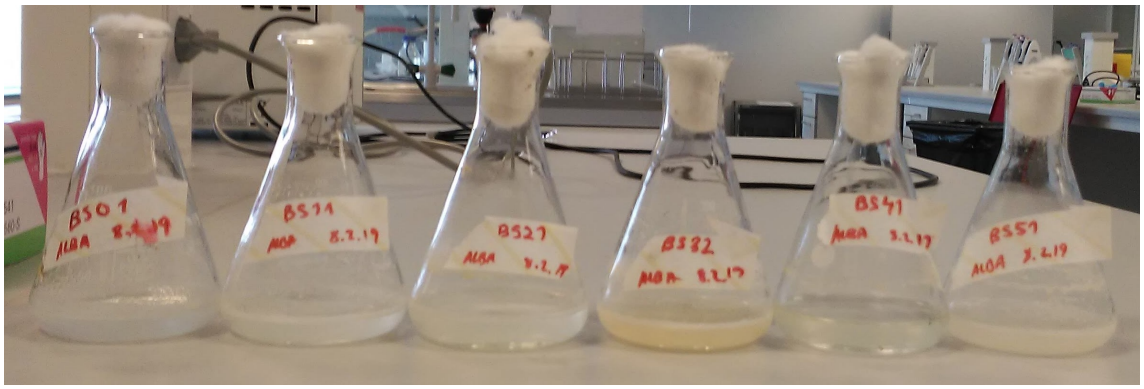


Figure 12: A picture of the different flasks containing the different conditions described. It can be seen the different turbidity depending on the surfactant agent concentration.

On the other hand, for the same conditions and time, CFU counts were more equal between duplicates and both showed also a growing tendency from week 0 to week 1, reaching around 200,000 cfu/ml. Finally, on the second week, duplicate number one showed a 6 million UFC/ml and 2.2 million UFC/ml which represented a 10 fold increase of biomass within one week.

A condition where the biosurfactant was the only carbon source was also set up (BS41, BS42), in the graph is represented as; 1 CMC BS + No PE, to proof that the organisms' growth was on LDPE and not on the biosurfactant. UFC counting showed higher results as in other conditions and also showed a slight growth from week 0 to week 2 reaching both duplicats a value of around 20,000 UFC/ml, which is 1000 times less than the value achieved when 5 times the CMC concentration of MEL was added.

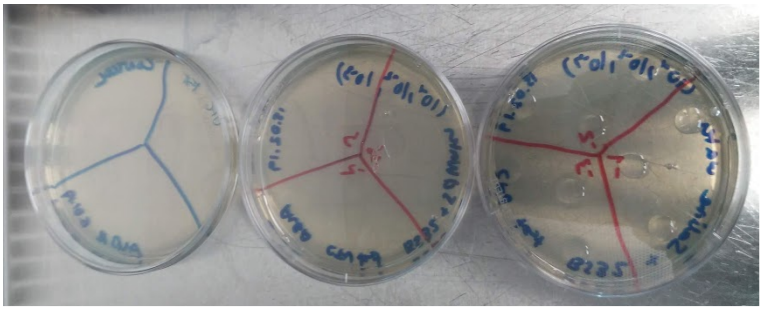

Figure 13: A picture were the UFC count methodology used is shown.

As protein content, it had lower results than when 1 CMC of MEL added and LDPE as carbon source, being the maximum protein content reached at around 30 $\mu$g/ml, which was 800 $\mu$g/ml less protein content than the maximum value recorded on the experiment. It showed that even there was a residual growth on the community, the highest growth values can be attributed to the main carbon source.

As the last condition tested, tween 80 was used in its CMC concentration. The protein content reached a maximum of 154 $\mu$g/ml at week 1, at week 2 there was a decrease of around 70 $\mu$g/ml, being approximately half of the content found at week 2 for 1 CMC of MEL. Alternatively, for UFC counts, values for 1 CMC of tween 80 increased 10 fold at week 2 respect the samples that 1 CMC of MEL were used. These results represented the second best values of UFC, 10 fold lower than the higher score (5 CMC of MEL).

To conclude, according to the experimental data, 1 CMC of Tween 80 promoted more UFC/ml and protein content within 2 weeks in the same consortia used, which indicated that either the CMC value for MEL it is higher than thought or the efficiency in plastic required to apply more than 1 CMC of MEL.

## 4.4 Bacteria selected for the artificial consortia assembling

A screening step was performed without any success due to impurities on the agar used. Bacteria was growing on the part were no carbon source was added at all, therefore these testes were not taken into consideration. Instead, a qualitative assessment of the most-colonizing PE surface was chosen as a screening parameter.

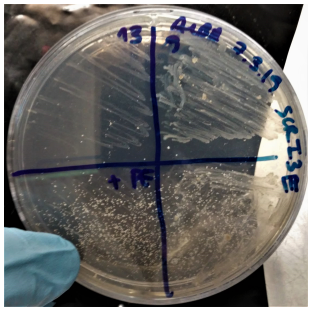

Figure 14: Screening of the best LDPE degraders isolates was performed, but the agar contained impurities which made dismiss the test.

As a result, the bacteria selected for the consortia assembling studies were the following:

1. S4.2
2. L2.2
3. L2.1
4. PP1
5. SM5.8
6. L2.1.1
7. MAL 1.1.2
8. SM2.3
9. SM6.2
10. BS7
11. S1.6
12. *Serratia marcescens*
13. *Pseudomonas putida*
14. *Ideonella sakaiensis*

*S. marcescens* and *I. sakaiensis* were taken from an LB seed culture that was used for other studies in AAU (Copenhagen).

The classical enrichment's isolates represented 3/14 (S4.2, S1.6, BS7) of the total bacteria selected, same as the MDR enrichment (SM5.8, SM2.3, SM6.2), the long enrichment represented a 4/14 (L2.2, L2.1, PP1, L2.1.1), *P. putida* was also enriched for 6 month before its use. Finally MAL 1.1.2 was taken from an external project. MAL 1.1.2 was enriched for three month with Malathion as the only carbon source and it was added to the isolates used for artificial consortia assembling to give more diversity to my mixtures.

## 4.5 High-throughput screening of artificial assembled consortia with the enriched isolates on plastic and lignin biodegradation studies

In order to assess the viability of the cells and its metabolic activity, a study where the ATP was quantified from each of the cultures was performed for all the single cultures and the different bacterial artificial assembled mixtures (up to 2 strains, up to 4 strains and up to 8 strains).

Standard curves for the ATP quantification and tables with the coefficient of variance are found inthe Appendix section (figure 41).

### Single strains culture ATP quantification after 48h in three different substrates: lignin, plastic (LDPE), lignin and plastic combined and glucose

It was studied that after 48 hours the growth of almost all the single strains on 0.2% glucose, lignin and LDPE was not reaching the stationary phase, and therefore this period could be set up to measure the cultures and compare between themselves, knowing that their metabolism is still active. All the graphs are shown in the Appendix section.

To compare each of the isolates with all the different substrates taken into consideration after 48 hours of culture, figure 14 was elaborated.

In figure 15 generally it can be seen that the carbon source where more ATP content was registered was in plastic, representing the 43% of the 14 isolates. Isolates that showed higher activity on lignin represented the 36% of the total. Plastic and lignin did show some activity in some isolates but not in the highest position. Lastly, glucose was the carbon source that promote the highest ATP content in the 21% of the isolates, being the favorite for the non-enriched bacteria (*I.sakaiensis* cannot uptake glucose as carbon source)

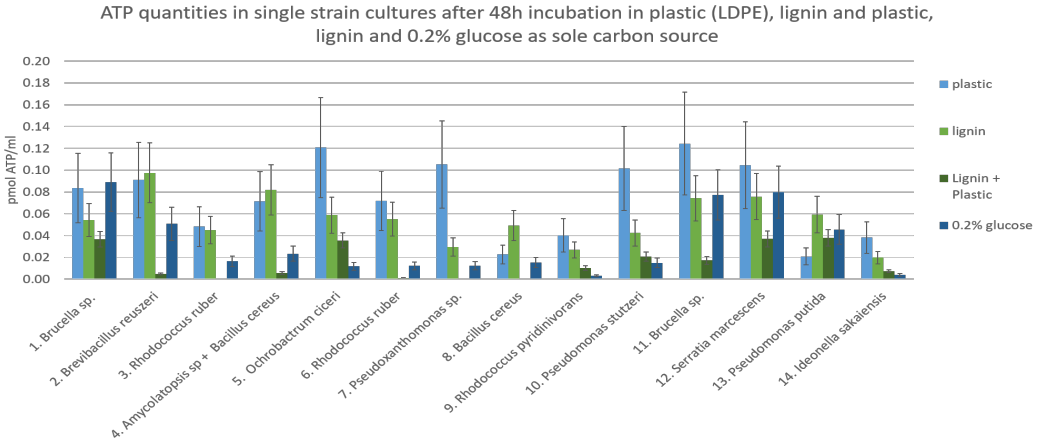


Figure 15: ATP content after 48 hours of each of the 14 isolates growing on plastic pretreated (LDPE) (light blue bars), lignin (light green bars), lignin and plastic pretreated (dark green bars) and glucose (dark blue bars) as sole carbon source. Average standard deviation per each of the substrates is represented as error lines in each of the bars.

**Results of the ATP quantification in the different bacterial combinations and substrates**

Once the isolates and the time were set up, the artificial consortia started to be assembled. To do so, three different dependent sequential cultures were set up. The first one, containing up to 2 strains, the 14 isolates were co-cultured amongst themselves in duplicates. 14 best combinations, in terms of ATP content, were selected from there and another round was started, having 4 strains in the mixtures. Again, the 14 best combinations were selected and they were cultured for another 48 hours in mixtures of 8 strains.

Due to the big amount of data generated per each of the cultures, the data was analysed taking into consideration the best 14 values in each of the mixtures. Once the genomic sequence was done, the isolates species' names were added to the results part.

To compare which of the methods resulted in a higher production of ATP within 48 hours, figure 17 represents the average value of the 14 higher values of ATP content.

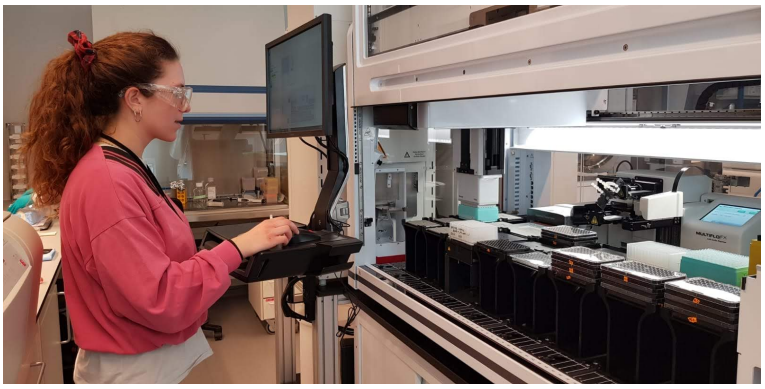

Figure 16: A image of the biomek i7 liquid transfer station for inoculating the different microtiter plates to test the different consortia.

It can be clearly seen that single strains (blue bars) happened to achieve the lowest ATP values for the non-enriched substrates as lignin and the combination of lignin and plastic. While in plastic, only the combination of 4 strains doubled the value of single strain culture.

It also can be seen, that the 4 strains mix (grey bars) it was the condition that achieved the best values in all the substrates. Being the 8 strains mix (yellow bars) the second best condition for lignin and lignin combined with plastic, the 2 strains mix (orange bars) the third best and the single strain the worst (not in plastic).

Concretely, lignin and lignin combined with plastic's best ATP content values were 0.5 pmol/ml higher than in plastic. Also, lignin seems to be the carbon source that gave the higher scores in ATP content in all the conditions, but reaching an average value close to plastic when the inoculum is a single strain.

During the performance of the ATP assay, one of the duplicates for all the substrates suffered an experimental mistake which make the results not being comparable. Consequently, the results of one of the duplicates were not taken into consideration for the data analysis.

It can be conclude that in all the substrates the ATP highest content its achieved by mixing different isolates rather than having pure strains cultured.

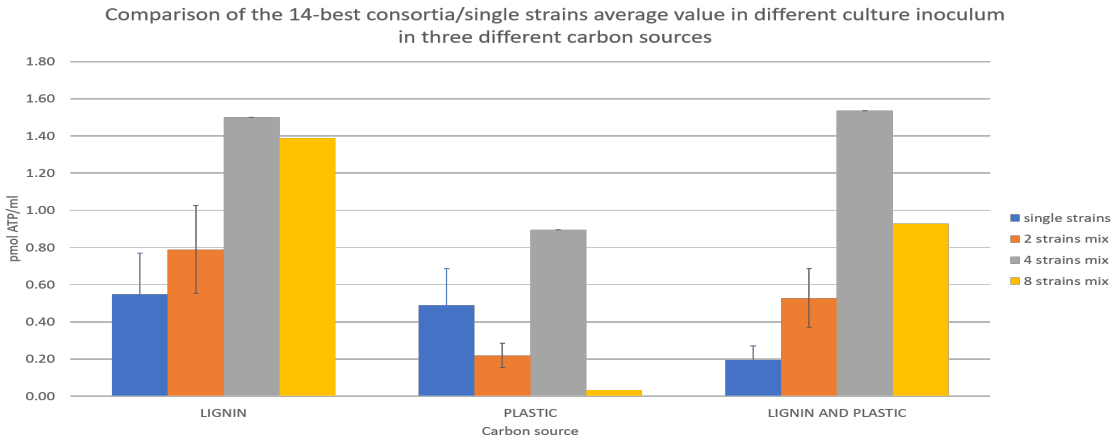


Figure 17: Average value of ATP content after 48h incubation in lignin, plastic and lignin combined with plastic for the single strains (blue bars) and the different artificially assembled bacterial mix culture; 2 strains mix (orange bars), 4 strains mix (grey bars) and 8 strains mix (yellow bars). Average of the standard deviation per each substrate are shown as error lines in the graph bars. Standard deviation for 4 strains mix is not shown due to only one of the duplicates was taken into consideration.

The main substrate studied in this project was plastic (LDPE). Consequently, a closer look was taken into the data of the 14 highest ATP content given by 4 strains mixtures and compared with their performance when growing on other carbon source.

In figure 18 it can be seen that within the 14 consortia assembled, 21% plastic is the substrate that gave them the highest ATP content, 36% was lignin and 43% was plastic and lignin.

The highest ATP content recorded was almost 2.5 pmol ATP/ml by *B. cereus, S. marcescens and P. putida* being around 0.7 pmol/ml higher than the best value on plastic and 0.5 pmol/ml higher than the best value on lignin.

The consortia assembled with *O. cicer, S. marcescens, Pseudoxanthomonas sp. and B. cereus* presents twice more activity for plastic than for lignin and almost non activity for the substrates combination.

Figure 18 confirms that the synergistic effect it was more productive, in terms of ATP content, when lignin and plastic are used together rather than alone.

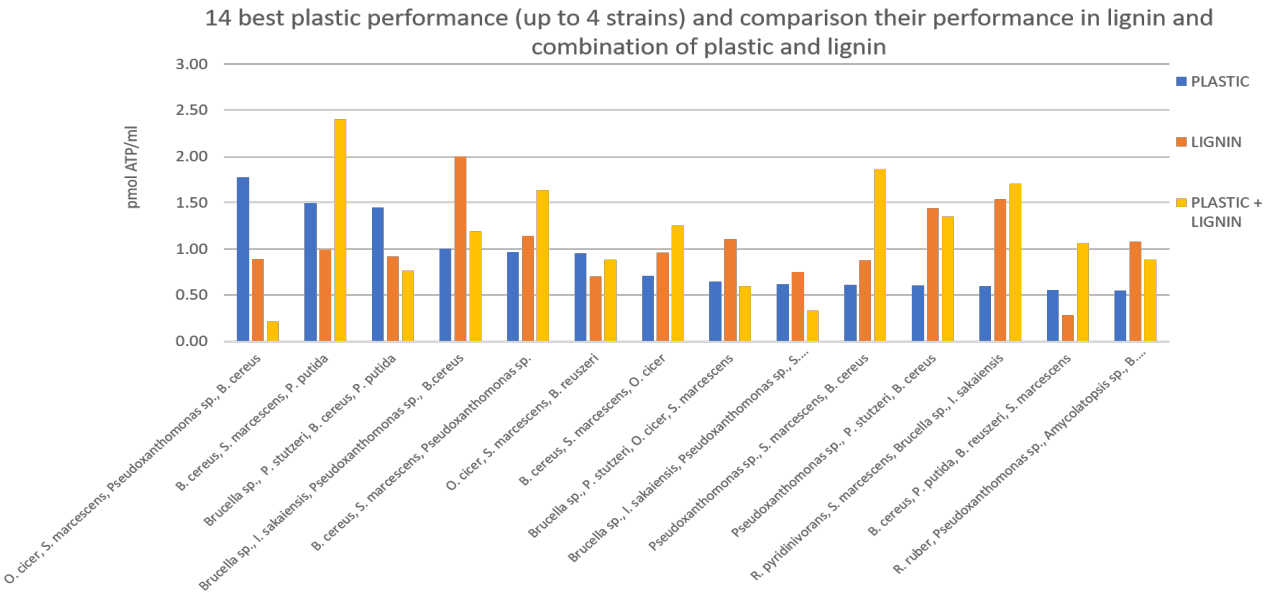


Figure 18: Best 14 values of ATP content after 48h incubation in plastic (4 strains mix, blue bars) and their respective values when cultured on lignin (orange bars) and lignin combined with plastic (yellow bars). The names of the species artificially assembled were added on the x-axis labels. Standard deviation was not shown due to only one of the duplicates was taken on consideration.

**Abundances of the best strains performing bacteria on plastic, lignin and plastic combined and lignin as the sole carbon source.**

Once the genomic sequencing of the isolates was done, it provided the opportunity to know exactly which bacteria appeared on the top 14-best ATP content in each of the substrates.

A table where all the different percentage abundance for each organism and substrate is shown in figure 19. More detailed pie charts for each of the substrates are shown in the Appendix section.

When using plastic as sole carbon source, it can be seen that *S. marcescens* occupied the first position, as the most abundance specie within the 14 consortia with the highest ATP content with an abundance of 22%, 5 points higher than the second more abundant, *B. cereus*, with a 17% .

On the other side, *S. marcescens* did not show as much presence when lignin was the carbon source (11%) being 11 points lower than the most abundant specie, *B. cereus* (22%). When lignin and plastic were combined, *S. marcescens* increased its abundance, sharing the first place with *B.cereus*, which can indicate that its relative presence was higher when plastic is part of the carbon source.

*B. cereus* resulted the most versatile specie, being highly active in all of the three substrates. Also, *Pseudoxanthomonas sp.* showed more presence on plastic and lignin, but was almost nonexistent when plastic and lignin were combined (4%).

*Brucella sp. 1, O. ciceri, Amycolatropsis sp. + B. cereus* showed almost the same abundance independently of the substrate.

It can be seen that organisms like *P. stutzeri and R. pyridinivorans,* were more present when lignin and plastic were combined as substrates than when they were cultured separately.

All the isolates were present at some extend in the highest positions, only isolate number 3 and isolate number 11 which species' names are: *Rhodococcus ruber 1* and *Brucella sp. 2*. Both are the only repeated species that were present within the 11 bacterial single strains screened from the enrichment. Therefore, they both appeared on the different consortia but under different numbers, 6 and 1 respectively.

As a result, all the strains played an important roll when combined and cultured on the different substrates, being some of them more protagonist than other but all of them appearing to be relevant in all the substrates.

It can be said that the most relevant species in terms of abundance and ATP content for all three substrates are: *B. cereus, S. marcescens, P. stutzeri, Brucella sp. 1* Moreover, each specie seems to react differently to the co-substrate culture.

| Table of the abundance of each organism within the 14-best performers in three different substrates | | | |
|---|---|---|---|
| **SPECIE** | **SUBSTRATE** | | |
| | Plastic | Lignin | Lignin and plastic |
| *1. Brucella sp. 1* | 11% | 10% | 8% |
| *2. Brevibacillus reuszeri* | 4% | 4% | 9% |
| *3. Rhodococcus ruber 1* | 0% | 0% | 0% |
| *4. Amycolatopsis sp + Bacillus cereus* | 2% | 2% | 4% |
| *5. Ochrobactrum ciceri* | 2% | 2% | 4% |
| *6. Rhodococcus ruber 2* | 2% | 4% | 2% |
| *7. Pseudoxanthomonas sp.* | 15% | 20% | 4% |
| *8. Bacillus cereus* | 17% | 22% | 15% |
| *9. Rhodococcus pyridinivorans* | 2% | 2% | 7% |
| *10. Pseudomonas stutzeri* | 9% | 11% | 13% |
| *11. Brucella sp. 2* | 0% | 0% | 0% |
| *12. Serratia marcescens* | 22% | 11% | 15% |
| *13. Pseudomonas putida* | 7% | 7% | 4% |
| *14. Ideonella sakaiensis* | 7% | 11% | 2% |

Figure 19: Table where all the different abundance percentage are compared on the different substrates. Best values are the numbers on the darker green, and as the color fades the numbers are decreasing accordingly.

**Analysing more in depth the best strains and their performance in the different mixtures and substrates**

Four strains (*S. marcescence, B. cereus, Pseudoxanthomonas sp. and P. putida*) were chosen according to their relevance on ATP production when cultured on plastic.

Firstly, the most abundant strain when it comes to ATP production on plastic, *S. marcescence* in

figure 20 showed as single strain the third best ATP content on plastic being 8 and 5 points lower than on the assembled bacterial mixture, while for lignin and plastic and lignin to be presented in a mixture represented a clear advantage than as a single strain, being its highest ATP content when plastic and lignin combined, almost 25 pmol/ml.

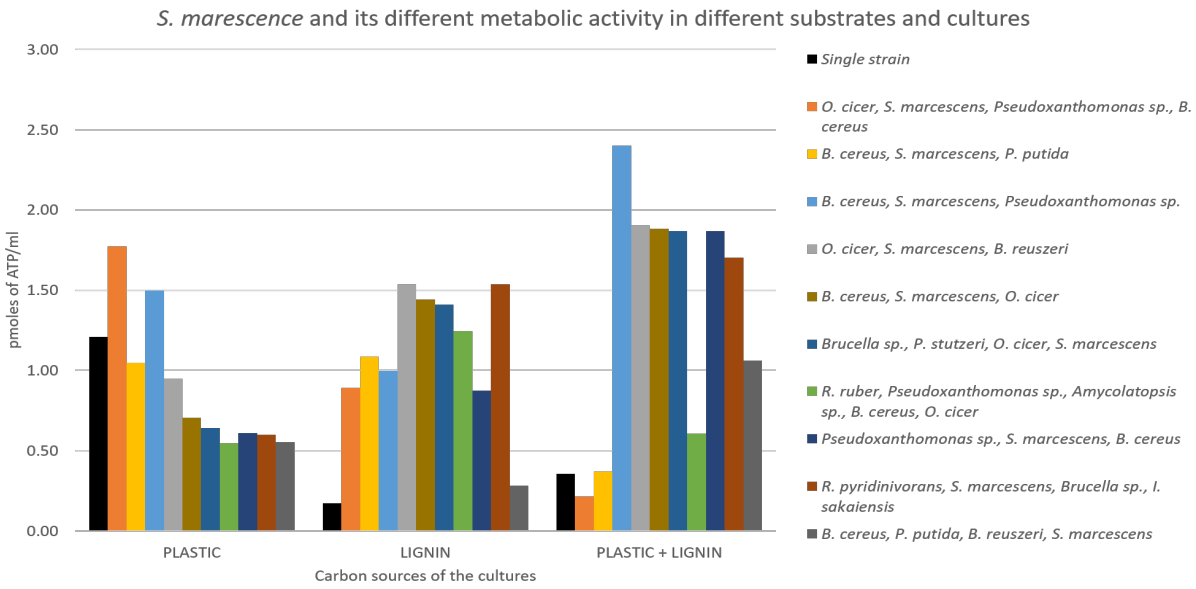


Figure 20: *S. marescence* and its different metabolic activity in different substrates and cultures, single strain cultures represented as black bars.

Next strain that plays a very important roll on the bacterial mixture was *B. cereus*. In figure 21, it can be seen that the organisms benefited in all the substrates from being in a mixture, as a single strain the maximum value obtained for plastic was almost 0.2 points lower than its higher value when is found on a mixture. The same phenomena happened when cultured on lignin. When plastic and lignin were cultured, almost no ATP production was detected and when it was found on mixture an ATP production of almost 2.5 pmol/ml was achieved.

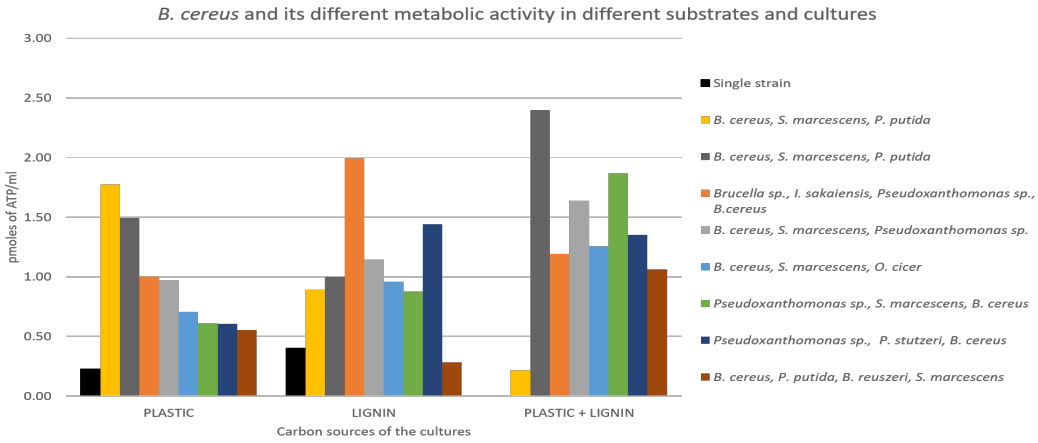


Figure 21: *B. cereus* and its different metabolic activity in different substrates and cultures.

*Pseudoxanthomonas sp.* ATP activity alone and combined are represented in figure 22. As single strain, obtained the second highest value with approximately 8 points less than the best value. Alternatively, when plastic is combined with lignin, as a single strain did not show any ATP signal, but when combined with other organisms, the mixture gave almost 2 pmol ATP/ml.

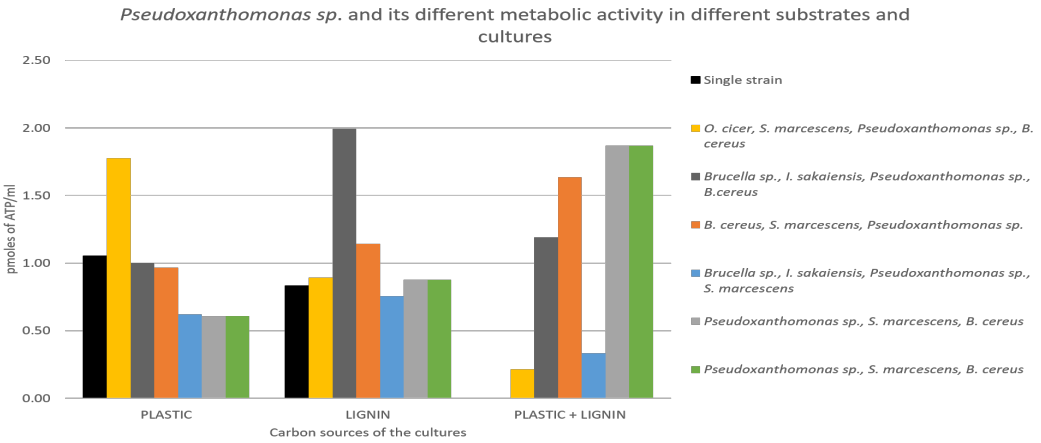


Figure 22: *Pseudoxanthomonas sp.* and its different metabolic activity in different substrates and cultures.

*P.putida* ATP activity alone and combined were plotted in figure 23. As single strain, obtained the second highest value with approximately 0.2 points less than the best value. This strain of *P. putida* has been enriched previously for 6 month, which can be justified its higher content of ATP when is cultured as single strain on plastic, but not on lignin or plastic combined with lignin.

In addition, when plastic was combined with lignin, as a single strain did show more signal than with lignin alone. For almost all the substrates *P. putida* reached higher ATP content values when combined with other species.

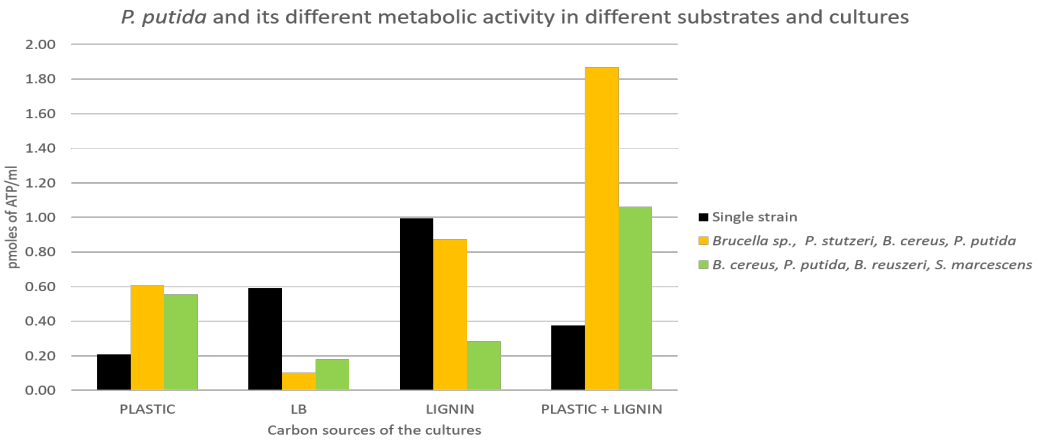


Figure 23: *P. putida* and its different metabolic activity in different substrates and cultures.

Overall, all the most abundant organisms within the best plastic ATP producers in a mix of up to 4 bacteria, increased their ATP content on an average of 0.75 pmol/ml of ATP when they were

combined with other bacterial species rather than when they were cultured alone.

Furthermore, their capacity to be metabolically active on plastic is rather high when they were found cultured on plastic as single strains. *B. cereus* was the only organism that did not perform well as single strain, not even achieving 0.5 pmol/ml of ATP content, and its success was clearly due to being on a mix culture.

## 4.6 Enriched isolates genomic sequencing

The whole genome of the 11 isolates was sequenced. The following species were found:

1. *Brucella sp. 1*
2. *Brevibacillus reuszeri*
3. *Rhodococcus ruber 1*
4. *Amycolatopsis sp + Bacillus cereus*
5. *Ochrobactrum ciceri*
6. *Rhodococcus ruber 2*
7. *Pseudoxanthomonas sp.*
8. *Bacillus cereus*
9. *Rhodococcus pyridinivorans*
10. *Pseudomonas stutzeri*
11. *Brucella sp. 2*

There were two species repeated within the isolates: *Brucella sp.* and *R. ruber*.

For strain 4, 7 (and 11) more than 1 contig resulted in relevant hits in the 16S database, indicating that these samples were mixes of more than one species.

The contigs for strain 4 were separated based on BLAST search analyses using genome sequences of *Amycolatopsis eurytherma* strain DSM 44348 and *Bacillus cereus* ATCC 14579 as subjects.

Contigs with no hit against any of these were included into the pile with the contigs having similar coverages.

The contigs for strain 7 were similarly split from BLAST analyses against *Pseudoxanthomonas suwonensis* 11-1 and *Bacillus toyonensis* BCT-7112. In this case the majority of the large contigs were against *Pseudoxanthomonas suwonensis* and the *Bacillus sp.* contigs formed an highly incomplete assembly, which was not analysed further.

## 4.7 FITR: detection of polymer structural changes due to consortium biodegradation

Seven different consortia were cultured on LDPE pretreated and co-substrate culture (LDPE and lignin) for 17 days.

A measurement after 4 days was taken, but any structural change on LDPE was found.

After 17 days, in the co-substrate culture, no structural changes on LDPE were found. In contrast, changes on LDPE structure were found when cultured on LDPE alone and in the samples without washing, once washed with distilled water it was found that there was a peak but with very few intensity.

It is a qualitative assay where the presence of a new peak after 17 days of culturing was found in the region of orbital sp2 hybridized carbons such as alkenes or aromatic compounds in the area 1650 $cm^{-1}$.

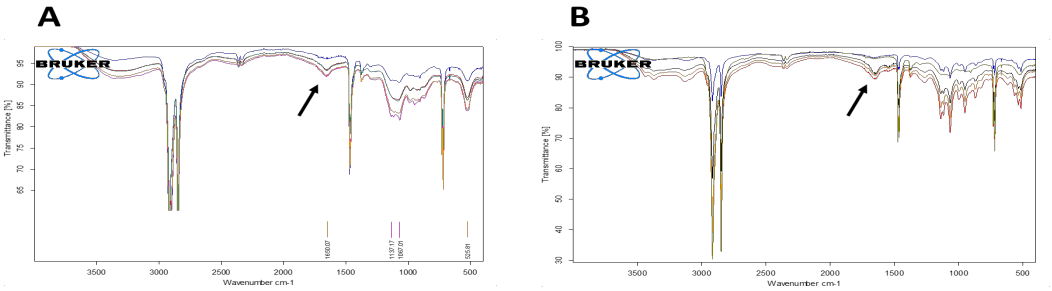


Figure 24: FTIR spectroscopy on LDPE after 17 days of being on a culture with consortia number 6 (**A**) and 7 (**B**), the arrow is pointing in the 1650 $cm^{-1}$. The blue line in **A** and **B** represents the control.

# 5 Discussion

## 5.1 Monitoring the enrichment culture: O.D. and extracellular protein content

### O.D. as reliable measurement for monitoring plastic biodegradation enrichment cultures

O.D. is not a measure of absorbance, but rather a measure of the light scattered by the cell suspension which manifest itself as a absorbance at 600 nm [84]. Consequently, in order to have a proportional ratio between the concentration of your cells and the intensity of scattered light, the cells need to be growing as a free-floating organisms homogeneously distributed.

It is well-known that most of the principal plastic degraders are growing on the plastic surface as a biofilm community rather than as a free-floating cells, due to it increases its effectiveness of degradation [85] [58] [86], but also because most of the organisms they rather live sessile attached to a surface because it confers them several advantages than as free-floating organisms [87]. In addition, the inoculum used in the short-time enrichment was soil which environment is usually solid.

Consequently, O.D. data is not a representative methodology to monitor the growth of a community that is expected to develop a biofilm-like ecology.

It can be seen in figure 7 that the maximum O.D. reached by the culture over a month of growth was at the second week, 0.18 units of absorbance at 600nm, the enrichment that the MDR strategy was used. If only the O.D. measurments are taken, it seems like no biomass is developing and the community is death because they are not able to uptake LDPE as carbon source. Contrarily to this, several bacteria isolated from this enrichment as *O. ciceri, B. cereus* and *R. pyridinivorans* were proven to be active in terms of ATP production growing on plastic as the sole carbon source.

To conclude, it can be stated that O.D. measurements when working with non-soluble substrates that leads to a non-homogeneous distribution of the population (biofilm formation) should not be a reference methodology to monitor growth.

**Monitoring the protein content during the enrichment: comparison of the enrichment strategies performance**

Extracellular protein concentration measurement it has been used by some authors as a supportive indirect growth data on biodegradation studies [88] [89] [77]. Moreover, plastic biodegradation is usually defined as an external digestion by the secreted enzymes, that happen to be proteins [7], consequently it could also be an indirect measurement of metabolic activity.

Baring in mind that the BCA assay is a fast, cheap and an easy method, it always can be added to any biodegradation study as supportive data.

As it is shown in figure 8, the MDR enrichment showed a more stable pattern of protein production than the classical approach. This phenomena could be explained because of the more stable conditions that MDR strategy suffered due to the lack of transfers, which also can be reflected in the lower standard deviation of the samples. While, in the classical approach where there has been a transferring step of the 10% of the volume to a 90% of fresh media, the standard deviation between triplicates was higher and the oscillations on extracellular protein content as well.

Stability of conditions it is not a desired characteristic for an enrichment, its finality is to provide adapted organisms that survived to the restrictive media. Therefore, even if it is harder to monitor the growth, it is expected that at some point, the population will reach to an equilibrium of the ultimate fittest organisms.

In this classical enrichment, the balanced situation on the community that would provoke a stabilization of the protein concentration detected was not seem to be reached within 3-month of culture.

It is important to remark that MDR enrichment were performed in 50 ml as w.v. while the classical enrichment was performer in 20 ml w.v. due to the lack of material avaiabile for such a long period. Thus, it cannot be compared more than in an overall view of both strategies.

However, the ultimate goal of both enrichments was achieved: isolate organisms adapted to degrade LDPE.

In figure 25 the origin of the samples and its performance as single strains did not show any pattern. Surprisingly, two of the strains that have not been adapted to plastic are in the 3rd and 4th best position of ATP producers (*Pseudoxanthomonas sp.* and *S. marcescens*).

Bacteria coming from the longest enrichment, contrarily that what it is expected, occupied lower positions on the plastic ATP activity. This could be explained because during all the 6 month that they have been enriched, they have not been under a selective pressure, so the population establish a more balanced ecosystem but not as well adapted as if there was a selective pressure.

| Single strains ATP content (pmol/ml) growing on plastic | |
|---|---|
| *11. Brucella sp. 2* | Classical enrichment |
| *5. Ochrobactrum ciceri* | MDR enrichment |
| *7. Pseudoxanthomonas sp.* | Malathion enrichment |
| *12. Serratia marcescens* | Not-enriched isolates |
| *10. Pseudomonas stutzeri* | Classical enrichment |
| *2. Brevibacillus reuszeri* | Long-enrichment |
| *1. Brucella sp. 1* | Classical enrichment |
| *6. Rhodococcus ruber 2* | Long-enrichment |
| *4. Amycolatopsis sp + Bacillus cereus* | Long-enrichment |
| *3. Rhodococcus ruber 1* | Long-enrichment |
| *9. Rhodococcus pyridinivorans* | MDR enrichment |
| *14. Ideonella sakaiensis* | Not-enriched isolates |
| *8. Bacillus cereus* | MDR enrichment |
| *13. Pseudomonas putida* | Enriched isolate |

Figure 25: Single strain ATP content and its enrichment type.

To conclude, even though MDR strategy reflects a much stable pattern, the adaptative pressure is weaker, in table 25 it can be seen that bacteria isolated from classical enrichment performed better than bacteria isolated from the MDR strategy. This could be because of the harsh pressure that was on them for 3 month.

Also, extracellular protein content analysis should be a supportive data, combined with another kind of analysis such as CFU counts.

## 5.2 Optimization of several factors on plastic degradation studies in two single strains and two enriched bacterial mixtures on microtiter culture.

### General considerations about microtiter culture on plastic biodegradation

High-throughput screening (HTS) represent a way of culturing organisms much faster and trying at once several variables. It is commonly used in biomedicin studies such as drug discovery, antibiotic resistance screening or drug development [90]. In the last decade, environmental biotechnology research started using HTS as a tool to test different enviornmental conditions [91] [92].

In this project, the microtiter plate format was taken inspired from the HTS methods, enabling to test several conditions in parallel with a limited amounts of microbial biomass. As far as it is concern, no papers were found that described any microtiter plate culture of plastic degraders. Consequently, the methodology was developed as described in materials and methods.

Baring in mind two crucial facts about plastic degraders: its potential biofilm ecology and using a non-soluble substrate.

Furthermore, the volume used in the microtiter was 200 $\mu$l. Cultures in *E. coli* demonstrated that the volume used for the culture it affects to the life-cycle of the bacteria, reaching the death phase faster as well as having different kinds of stressors as when culture in larger volumes [93]. Therefore, this consideration need to be taken when extrapolating results from the microtiter cultures.

This first attempt of testing plastic degraders culture on microtiter was made when optimizing the several factors recording O.D. data every hour.

A pre-cultured with rich media would have needed to be performed before transferring the culture from a 50 ml batch culture to a microtiter plate in order to avoid a longer lag period of the bacteria to get used to the new environment. When culturing for consortia assembling, always a pre-culture of the inoculum on a microtiter for 24 hours before starting the experiment has been done.

Firstly, too small air volume was left: only $1/3$, when usually the volume for air left is $9/10$. Thus, combined with the sticky membrane used that was not permeable to gases, created an non-homogeneous environment most probably lacking oxygen after a certain period of time. The culture was kept without aeration for 70 hours approximately. Afterwards, the sticky membrane was changed, provoking another atmospheric change and being the oxygen the limiting factor for aerobic metabolism.

The bacterial inoculum were: 2 single strains, one of them enriched on plastic degradation batch culture of 50 ml w.v. and 2 mixed culture enriched on plastic cultured in 50 ml w.v.

*P. putida* is an organism that can tolerate microaerophilic conditions when growing on a biofilm [94], but when using plastic as carbon source, oxygen is the electron donor needed for one of the key enzymes involved in the process: Alkane monoxygenase [95]. As a result, bacteria could persist in the environment but without reaching the exponential phase due to having oxygen as limiting factor, as it is shown in graphs 28-33 which are found in the appendix section.

When the mix culture is present instead, oxygen is a limiting factor which will provoke that the ecology would shift selecting rather autotophs organisms [96] or anaerobic facultative organisms capable of using LDPE as carbon source, like for instance *Staphylococcal sp.* [97]. As a result, the community would have been shifting its compositions according to the oxygen availability, making it difficult to achieve an equilibrium that allowed to developed a consistent plastic degrading ecology.

Furthermore, the microtiter cultured suffered evaporation losses that up-concentrated the cells presents in the microtiter, they can be seen with the increase of O.D. in late period of time in the control samples.

Also, O.D. does not distinguish between viable cells and death cells, consequently, if there was a construction of biomass while oxygen was present but followed by a massive extinguish of organisms it would not could have been appreciated in the O.D. data.

## 5.3 Biosurfactant as enhancer for LDPE degradation with an enriched mixed culture

MEL is a biosurfactant produced by a non-conventional yeast *Candida antarctica* when growing on high-concentrations of vegetable oil or n-alkanes [98]. Therefore, its biological function when is produced by the yeast is to facilitate oil or n-alkanes degradation, which both are highly hydrophobic substrates. Polyethylene' chemical structure is a numerous repetition of n-alkanes and extremely hydrophobic. Thus, could explain its high activity showed on figure 11 due to the hydrophobic nature of rapeseed oil.

No reports were found regarding MEL as surfactant agent on polyethylene or any plastic biodegradation, but there are several reports acknowledging its properties for crude oil or petroleum biodegradation [99] [100] [82] [101].

Moreover, MEL was described to have antimicrobial activity in *Pseudomonas sp.* and *Bacillus sp.* which are two of the isolates found in the enrichments, its microbial inhibitory concentration (MIC) is approximately ten times higher than its CMC for *Pseudomonas sp.* and twice higher for *Bacillus sp.* [102].

However in the enrichment only its CMC concentration was used and *Pseudomonas stutzeri* was isolated from one of the flasks that contained MEL, which confirms this data.

On the other side, in this study five times its theoretical CMC was used, which could have been harmful for *Bacillus sp.* organisms and even though an average of 4 millions of UFC/ml and 500 $\mu$g/ml extracellular protein content was recorded in two weeks. In other biodegradation studies, where the substrate were PE blends with 6% starch, values close to 700 $\mu$g/ml of extracellular proteint content after two weeks were recorded in a single strain cultures of bacteria and fungi [103]. Also, in a PHA-rubber blend degradation by a *Pseudomonas sp.* study, its maximum protein content was around 300 $\mu$g/ml after 5 days, being 100 points less after 15 days.

If compared with tween 80 performance, tween 80's CMC is ten times higher than MEL's theoretical CMC. Each CMC has a different CMC depending on the carbon source and the strain that makes the different types of MEL be present in different distributions. Therefore, could be that the CMC of this specific MEL used was higher than what is thought initially.

However, using 5 times less amount of MEL than tween 80, MEL exhibits higher activity translated on higher yield of protein and biomass production.

Using a biosurfactant like MEL, is a more sustainable choice due to its biological synthesis instead of a chemical synthesis, and the optimal sustainable production would be to produce it from a waste substrate [104].

## 5.4 Microtiter culture of artificial assembled consortia with the enriched isolates on plastic and lignin biodegradation studies

Another attempt of microtiter culture was done, this time knowing all the crucial steps that went wrong in the previous microtiter culture.

Therefore an incubator with 85% of humidity was used in order to prevent evaporation losses, also ATP was measured instead of O.D. and a higher air-ratio was used to avoid being the oxygen the limiting compound.

### Effect of co-substrate cultivation on the degraders

A new substrate was tested in this part of the project in order to be eligible for the BRISK2 funding, which required a research that has lignocellulosic material involved.

As stated in section 2.4.2, lignin and plastic biodegradation have common enzymatic pathways, such as MnP, DyP, LiP and laccases [105]. Another common thing that they have is a high interest from the scientific community to disclose a cost-effective and sustainable depolymerisation strategy.

Consequently, using the same organisms for both substrates it is likely to encounter activity even though they have never been in contact with lignin.

In this paper it has been found that ATP production when bacteria grew on a co-substrate cultivation varies highly depending on if they are cultured as single strains or in a mix culture.

Co-substrate culturing in single strains resulted in a lower ATP production than when cultured with lignin or LDPE alone, none of the cultures showed any benefit from the co-substrate culture.

Contrarily, when different bacterial mixes were in a co-substrate culture, they showed better ATP production than when cultured on plastic alone but worst performance when cultured on lignin alone as it can be seen in figure 17. Thus, indicates a clear synergistic effect that allowed them to degrade both substrates more efficiently than when they are on their own.

### Artificially assembled consortia performance on plastic degradation

During all the process when bacteria was cultured in a microtiter plate, 0.05% of glucose was used as metabolic enhancer instead of PHB due to the previous microtiter cultured performed, where glucose and PHB were combined, the results were not reliable.

As shown in graph 38 (Appendix section), most of the single strains did not process very quickly glucose, it could be as a result of the enrichment, that their adaptation was towards a complex polymer degradation rather than a soluble substrate like glucose. However, the pre-activation step for being accustomed to the microtiter environment would have been better to be performed on LB media which can be up-taken efficiently by the organisms, thus it was their isolation media. It is

acknowledged that the pre-activation step could not have been very efficient for most of the strains, and further experiments will have to be carried in order of knowing the impact of this step.

The process of selection of the best-performers was done in terms of selecting the most active consortia and expect an accumulative effect of them when co-existing. Other principles could have been applied to it, but the lack of literature and short time-period leaded to select the 14 best ATP producers on plastic degradation as the criteria.

In addition, the amounts of biomass were only normalized when manipulating single strains, otherwise the inoculum consisted on the consortia developed at 48 hours whose proportion and distribution is unknown, but its effectiveness was the important data.

Also, another critical point on the study would have been to use another indirect measurement of metabolic activity or cell growth. Due to its tremendously amount of work that required the microtiter culture set up, together with the difficulty of working with non-soluble substrates, analysing a total of 273 combinations of bacterial mixtures, it was not possible to find another reliable assay that could be supplementary to the ATP content.

Nevertheless, if comparing with other plastic degradation literature where the ATP content was measured, the results obtained in this study are consistent.

A study where biodegradation of two biofilm-forming non-enriched bacterial species: *Rhodococcus rhodochrous* and *Nocardia asteroides* cultured on HDPE and LDPE pre-treated in a photo-aging chamber followed by 300 h of exposure in an aerated oven at 60 °C altogether resulted an equivalent of being 2-3 years of thermo-oxidation at room temperature in a dark, the film already fragmented was passed through a metallic screen with hole diameter of about 1 mm. After 3-4 days of culture, the ATP recorded was 100 pmol/ml for the HDPE and 10 pmol/ml for the LDPE [76]. In this study, the highest ATP production achieved after 48 hours of mix culture on LDPE pretreated under UV light for a month almost approximately 10 points less of ATP content were found, 1.8 pmol/ml, while under co-substrate culture, 2.6 pmol/ml.

Taking into consideration the pretreatment that they performed and the pretreatment that it has been performed in this study, the results are congruent. Accessing to the polymer it is a limiting step when colonizing the surface, therefore having the LDPE pretreated in such an exhaustive way have a significant impact on the metabolic activity of the cells. As a result, baring in mind the differences within the pretreatment of the LDPE, ten times lower ATP activity it is a positive result. Nevertheless, in order to have a fair comparison, scaling-up the project to the same working volumes as Koutny et al. will be required to see if the microtiter culture has any effect on the detection of ATP.However, this results indicate a novel approach of assessing short-term plastic biodegradation using a microtiter culture.

**Abundances of the best strains performing bacteria on plastic as the sole carbon source**

The abundance of one specie in an artificial assembled consortia, could be defined by different criteria such as its ecological interactions (symbiont, antagonist or neutralist) or its metabolic interactions. Therefore, the abundance of each specie does not necessary indicates its direct

activity with the substrate. Also, metagenomic studies should be carried on in order to disclose the proportion of each bacteria respect the other within each sample.

Nonetheless, a non-homogeneous distribution of each bacterial specie is shown, which points to a relative abundance selected by its metabolic activity. As a result, even not having the metagenomic data, some trends can be hypothysised from the results show in table 42.

Four of the best plastic-degrading strains were analysed more in depth in graphs 20, 22, 21, 23. In all of them their performance when cultured on a co-substrate it is enormously improved by the consortia. For example, if taking the best value of co-substrate culture in figure 20, which is around 2.4 pmol/ml and the bacterial mixed responsible for it is formed by *B. cereus, S. marcescens and Pseudoxanthomonas sp.*, only *S. marcescens* showed ATP activity when co-substrate cultured as single strain, which was 0.3 pmol/ml. Thus, the 2 points of improvement on ATP production were due to the synergistic effect when bacteria are combined.

As a result, they acquired the ability of producing more ATP on LDPE as carbon source, but its ability to use lignin and plastic it is highly dependent on the interactions with the other bacteria.

## 5.5 FTIR: detection of polymer structural changes due to consortium biodegradation

When the polyethylene recovered from a 17 days culture of 7 different bacterial culture was measured without a pre-washing step, in all the cultures but in the control one, there was a clear peak in the area 1650 cm $^{-1}$. After a washing step with deionized water, the peak that at 1650 cm $^{-1}$ was lowered considerably due to a possible washing of the compounds.

The area where the new peak was formed, indicated a formation of a double bound C=C of alkenes or aromatic compounds, which could be due to enzymatic oxidation. This finding would match with other biodegradation studies [71] [106] [107].

In all of studies cited, peaks were more pronounced, but in the articles they have a more compelling pretreatment of the LDPE and the incubation time was longer.

This peak could also be due to biomass attached on the surface, but the absence of other characteristic biomass noise peaks indicates that most probably could be as a result of enzymatic oxidation.

In conclusion, a peak on the area 1650 cm$^{-1}$ was found, most probably indicating that the enzymes secreted by the consortia oxidised the C-C bonds to C=C bonds to obtain energy, no reports were found where FTIR measurements were taken for such a short period of time, therefore it cannot be compared with literature. Although, if a change can be detected by 17 days, the biotic effect looks promising.

## 5.6 Sequencing of the enrichment isolates

### Ecology of the isolates and its relation to plastic-lignin degradation

*Serratia marcescens*:

Naturally, *S. marcescence* it is a gram-negative rod-shaped anaerobic facultative bacteria, it is an ubiquitous bacteria that can be found in several ecological niches: water, soil, air, plants and animals. It has a wide range of substrates, being one of the most efficient organism for chitin biodegradation. Also, it has a high affinity for hydrophobic surfaces[108].

*S. marcescence* has been already described as lignin [109] [110] and polyethylene degrader [107].

Its degrading mechanism for lignin is the use of lacasses, MnP and LiP [109] [110], a similar mechanism is though to be the responsible for LDPE biodegradation [**?**].

The genomic material of this organism was not sequenced in this study, so only a theoretical role of its enzymatic metabolism can be proposed.

*Pseudoxanthomonas suwonensis 11-1*:

This sample was taken from a malathion enrichment.

*Pseudoxanthomonas suwonensis* was isolated a decade ago for the first time from a cotton waste composts in Korea [111]. It is an gram-negative aerobic rod-shaped bacteria. It showed negative activity for hydrolysing chitin or starch, but positive in gelatin hydrolysation. It has also been isolated from leaf- and wood enriched soil, using carboxymethylcellulose as carbon source with a high-activity cellulase [112].

A close-related specie was found in an oil-contaminated soils in China [113].

In its genome, the following interesting enzymes were found:

**Dye-decolorizing peroxidase (DyP):** it is a lignin peroxidase that it is found in several lignin degraders like *P. putida and Rhodoccocus sp*. Belongs to the family of heme-peroxidase which LiP and MnP are members too. They are known for fungal plastic biodegradation. A potential role of this enzyme to plastic degradation is proposed.

**Catalase-peroxidase (KatG):** aromatic compounds degradation [114].

**Multicopper polyphenol oxidase (laccase):** enzyme that is found in processes of lignin and plastic oxidation [114] [21].

**Phospholipase/carboxylesterase family:** hydrolitical function, pesticide degradation [115].

*Bacillus cereus*

It is a well-known gram-positive facultative anaerobic bacteria, commonly found on soil and food. It has chitinolytic activity [116]. Different species of Bacillus have been tested successfully for lignin degradation [114] using laccases, but no reports citating specifically B. cereus on lignin degradation have been found.

Its activity to degrade LDPE films with a pro-oxidant agent within a consortia has been reported [80] [117].

In its genome, the following interesting enzymes were found:

**Multicopper polyphenol oxidase (laccase):** enzyme that is found in processes of lignin and plastic oxidation [114] [21].

*Brucella sp.*

It is a gram-negative, facultative anaerobes, coco-bacilli shaped and most of them are intracellular pathogens transmitted via zoonosis and inhalation. It has been reported *Brucella sp.* the ability to degrade phenolic compounds [118].

In its genome, the following interesting enzymes were found:

**Multicopper polyphenol oxidase (laccase):** enzyme that is found in processes of lignin and plastic oxidation [114] [21].

**Protocatechuate 3,4-dioxygenase:** play an important role in the use of aromatic compounds [114].

Consequently, in the bacteria whose genomic data was searched for degrading enzymes, a presence of at least one potential oxydising enzyme was found, together with data that corroborates its function in either lignin or LDPE or both.

Yet, the possible health risk for humans of two of the strains: Brucella sp. and *S. marcescens* needs to be consider for considering its culture and its use. It rather would be safer to use the target enzymes or find a strain whose category is GRAS (Generally Recognize as Safe).

Lastly, *Pseudoxanthomonas sp.* and *Brucella sp.* have never been reported to degrade LDPE. In this study, it can be seen that they showed ATP production after 48 hours of being in contact with LDPE as the sole carbon source (1 pmol/ml and 0.6 pmol/ml), and their contribution to a consortia is also benefitial and competent.

### Potential LDPE/lignin degrading enzymes found on the genomic sequence of the 11 isolates

In table 5 the presence of the different potential LDPE/lignin degrading enzymes is listed.

Hydroxylase (Cyt P450) are heme-containing proteins that catalyze the insertion of oxygen into a wide variety of substrates [119], alkane hydroxylases are within this family and its role within LDPE biodegradation have been described already.

It is remerkable the ubiquitous presence of laccase amongst all the isolates, which is expected taking into consideration the board substrate spectrum that they catalyse. Even though they already have been described as crucial in *Rhodococcus sp.* further studies need to be carried to understand exactly the importance of this enzymes within all this different range of organisms.

Also DyP is present 5 out 11 strains, no report has been found about its activity on LDPE, but it would be interesting to alayse in deep its potential role within PE metabolism.

| Organism | Laccase | Peroxidase | Hydroxylase (Cyt P450) |
|---|---|---|---|
| Brucella sp. 1 | Yes | No | No |
| *B. reuszeri* | Yes | KatG, DyP | Yes |
| *R. ruber* 1 | Yes | KatG, DyP | Yes |
| *Amycolatropsis sp.* | Yes | KatG | No |
| *B. cereus* | Yes | No | No |
| *O. cicer* | Yes | No | Yes |
| *R. ruber* 2 | Yes | KatG, DyP | Yes |
| *Pseudoxanthomonas sp.* | Yes | KatG, DyP | No |
| *B. cereus* | Yes | No | No |
| *R. pyridinivorans* | Yes | KatG, DyP | Yes |
| *P. stutzeri* | Yes | KatG | Yes |
| *Brucella sp.* 2 | Yes | No | No |

Table 5: Comparasion table of the different potential degrader enzymes amongst the 11 isolates' genome

# 6 Conclusion

Two different enrichment were made; 3 and 6 month-long. The enrichment that was monitored (3-month long) resulted on 11 isolates that were successfully cultured on LDPE as sole carbon source and showed high ATP activity. In addition, no evidence was found indicating that bacteria isolated from the long enrichment was more efficient than bacteria isolated from short enrichment. Which can indicate that the pressure (transfer) is more important than the length of the enrichment. Nonetheless, OD measurments were not reliable for following-up the enrichment development and instead, UFC counts are proposed as substitute strategy for monitoring the enrichment, together with the total protein content (BCA assay).

Two enrichment (3-month long) methodology were tested: MDR and classical enrichment. Classical enrichment inferred a more effective pressure because of a tougher selection, providing isolates with higher ATP activity when cultured on LDPE as sole carbon source.

MEL was proven to have a highly positive action towards plastic biodegradation but when 5 times of its CMC was used. More studies assessing the influence on biodegradation would need to be carried, but it is proposed as a novel biosurfactant that can be applied for LDPE biodegradation studies with mix cultures.

The first microtiter experiment, where multiple conditions were tested, did not show any reliable result due to different setting up errors. Consequently, PHB still is an interesting source of enhancing metabolic activity that need to be studied.

Microtiter culture was proven to be effective for biodegradation studies, yet very time-consuming, within a short time-frame and once learnt the correct set-up. It would be interesting to study its consequences in terms of ATP production when scaling up in small batch flasks. However, the consortia that scored the highest ATP content with LDPE as sole carbon source showed approximately ten times less ATP activity for the same period of time as *R. rhodochrous* and *N. asteroides* cultured in a LDPE that had been pretreated the equivalent of 3 years of weathering. Also, in this study, co-substrate culturing of the same consortia doubled the ATP activity in comparison of when LDPE was the sole carbon source.

Bacterial consortia of 4 strains performed better than 2, 8 or single strains cultures. Due to an methodological error during the experiment and the lack of considerable duplicates, the experiment should be repeated to confirm this assumption, otherwise it can be confirmed the benefitial synergistic effect of he co-culture in a consortia for LDPE biodegradation. Also, studies about persistence and stability of the consortia should be carried out in order to ensure the optimal performance along the generations.

The genomic content indicated that laccases are present in all the 11 isolates and DyP, KatG and possible Alkane hydroxylase are broadly present on them. Thus, suggesting that the paper of laccase on LDPE biodegradation should be further analysed as a general mechanisms rather than an isolate characteristic. Finally, DyP role on LDPE biodegradation should be deeply studied to see if it has any role within LDPE bacterial biodegradation as their close-related heme peroxidases have it in fungal biodegradation.

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# 8 Appendix

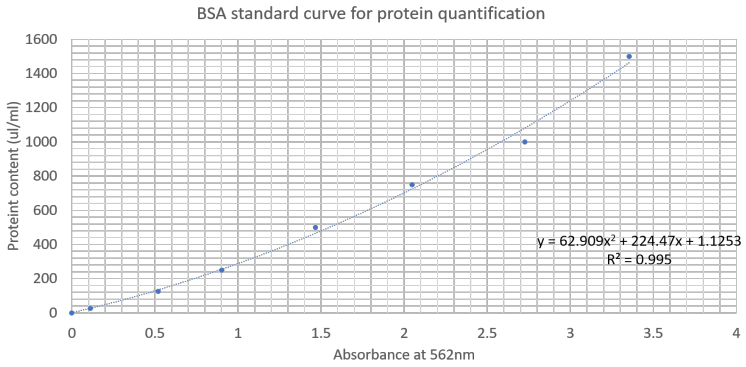


Figure 26: BSA standard curve used for protein quantification.

**Variation coef. (%) of the triplicates at the beginning and at the end of the culture**

| *Condition* | *Time (hours)* | PP1 | PP0 | SW1 | L2 | C |
|---|---|---|---|---|---|---|
| PE pret | 1 | 41% | 75% | 44% | 72% | 5% |
| | 130 | 63% | 11% | 21% | 37% | 57% |
| PE non-pret | 1 | 90% | 36% | 25% | 1% | 42% |
| | 130 | 15% | 60% | 8% | 28% | 60% |
| PE pret + T80 | 1 | 5% | 40% | 1% | - | 9% |
| | 130 | 3% | 62% | 10% | - | 26% |
| PE pret + MEL | 1 | 4% | 5% | 11% | 0% | 1% |
| | 130 | 18% | 5% | 13% | 43% | 13% |
| PE pret + Glu | 1 | 19% | 85% | 7% | 2% | 19% |
| | 130 | 3% | 10% | 14% | 80% | 31% |
| PE pret + PHB | 1 | 51% | 6% | 6% | 36% | 8% |
| | 130 | 34% | 4% | 19% | 2% | 26% |
| PE pret + MEL + PHB | 1 | 5% | 2% | 6% | - | 5% |
| | 130 | 35% | 37% | 33% | - | 62% |

Figure 27: percentage of the variation coefficient of the triplicates in all the samples (SD/average)*100 at the beginning and at the end of the experiment.

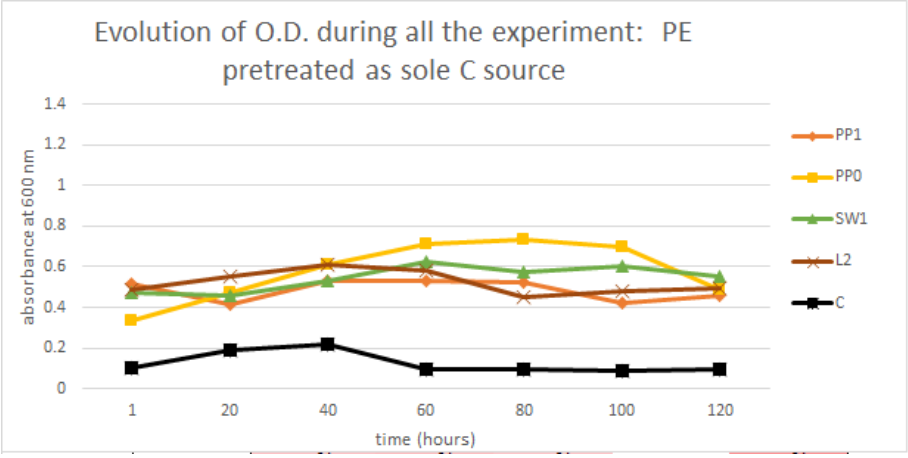


Figure 28: Evolution of OD during all the microtiter experiment: PE pretreated as sole carbon source

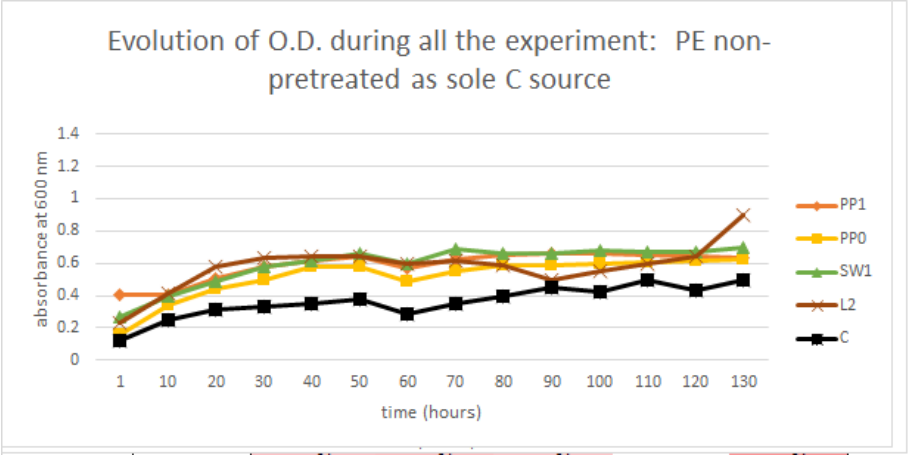


Figure 29: Evolution of OD during all the microtiter experiment: PE non-pretreated as sole carbon source

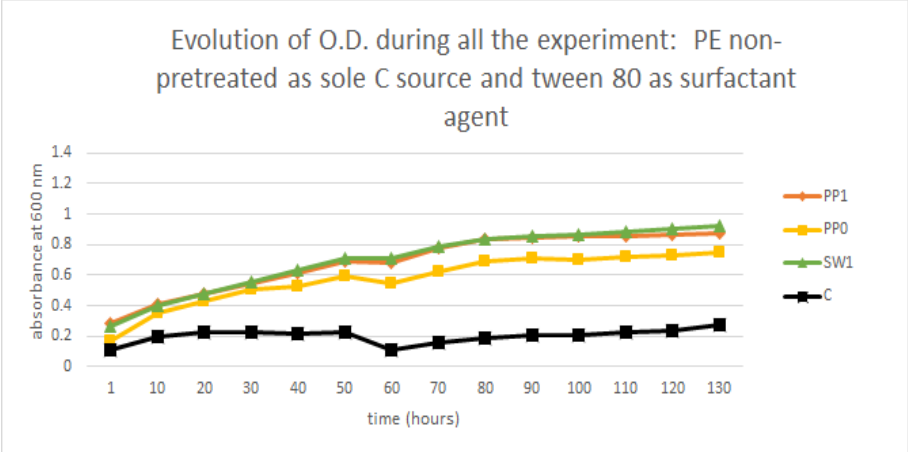


Figure 30: Evolution of OD during all the microtiter experiment: PE pretreated as sole carbon source and tween 80 as surfactant agent.

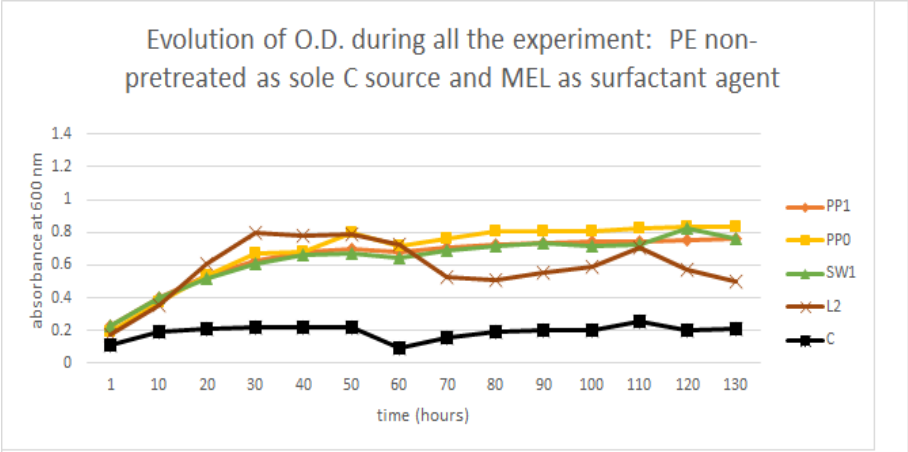


Figure 31: Evolution of OD during all the microtiter experiment: PE pretreated as sole carbon source and MEL as surfactant agent

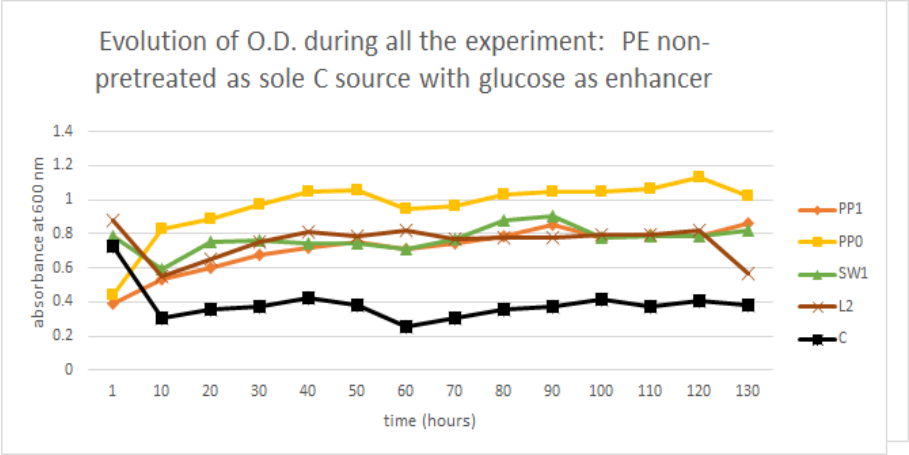


Figure 32: Evolution of OD during all the microtiter experiment: PE pretreated as sole carbon source and glucose as metabolic enhancer

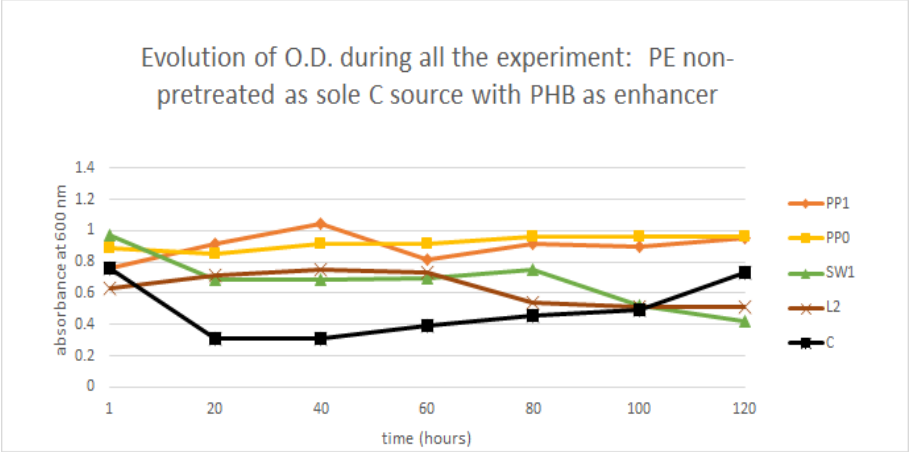


Figure 33: Evolution of OD during all the microtiter experiment: PE pretreated as sole carbon source and PHB as metabolic enhancer.

COUNTS OF COLONY-FORMING UNITS (CFU/ml)

| | PE + NO MEL | PE + NO MEL | PE + 1/2 CMC MEL | PE + 1/2 CMC MEL | PE + 1 CMC MEL | PE + 1 CMC MEL | PE + 5 CMC MEL | PE + 5 CMC MEL | 1 CMC CMC + NO PE | 1 CMC MEL + NO PE | PE + 1 CMC MEL | PE + 1/10 CMC T80 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Time 0 | 1527 | 1487 | 2840 | 2433 | 2160 | 1613 | 2667 | 1600 | 3867 | 5533 | 5067 | 2400 |
| Week 1 | 111 | 18 | 1453 | 2440 | 2153 | 3173 | 111333 | 169333 | 9800 | 15067 | 14400 | 10067 |
| Week 2 | 1207 | 1860 | 1840 | 14600 | 17400 | 11400 | 6000000 | 2120000 | 20333 | 13333 | 268667 | 345333 |

Figure 34: CFU counts in biosurfactant optimization experiment

EXTRACELLULAR PROTEIN CONTENT AVERAGE OF THE REPLICATES (- blank value of the medium)

| | PE + No BS | PE + No BS | PE + 1/2 CMC BS | PE + 1/2 CMC PE | PE + 1 CMC PE | PE + 1 CMC BS | PE + 5 CMC PE | PE + 5 CMC BS | 1 CMC BS | 1 CMC BS | PE + T80 (10 CMC BS) | PE + T80 (10 CMC BS) |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Time 0 | 5.11 | 1.50 | 17.46 | 17.30 | 21.52 | 17.85 | 63.05 | 53.55 | 20.97 | 21.60 | 72.56 | 75.88 |
| Week 1 | 3.53 | 3.30 | 36.70 | 25.55 | 82.49 | 87.04 | 543.78 | 305.34 | 79.75 | 21.84 | 99.97 | 152.08 |
| Week 2 | 6.85 | 9.67 | 130.24 | 67.27 | 159.86 | 162.36 | 763.84 | 137.47 | 36.54 | 31.05 | 80.28 | 83.38 |
| | BS01 | BS02 | BS11 | BS12 | BS21 | BS22 | BS31 | BS32 | BS41 | BS42 | BS51 | BS52 |

Figure 35: Proteint content in biosurfactant optimization experiment

CV (%) UFC BIOSURFACTANT EXPERIMENT

| Sample | T0 | T1 | T2 |
|---|---|---|---|
| **BS01** | 3% | 16% | 18% |
| **BS02** | 7% | 10% | 23% |
| **BS11** | 10% | 10% | 5% |
| **BS12** | 3% | 27% | 22% |
| **BS21** | 9% | 23% | 77% |
| **BS22** | 75% | 9% | 24% |
| **BS31** | 28% | 22% | 0% |
| **BS32** | 70% | 17% | 28% |
| **BS41** | 88% | 55% | 20% |
| **BS42** | 6% | 23% | 5% |
| **BS51** | 20% | 15% | 19% |
| **BS52** | 17% | 31% | 2% |

Figure 36: percentage of the variation coefficient of the duplicates in all the samples (SD/average)*100 at the beginning and at the end of the experiment.

Coefficient of variation of the replicates for extracellular protein content in the biosurfactant experiment

| | BS01 | BS02 | BS11 | BS12 | BS21 | BS22 |
|---|---|---|---|---|---|---|
| T0 | 146% | 262% | 22% | 23% | 13% | 21% |
| T1 | 84% | 63% | 28% | 81% | 90% | 33% |
| T2 | 84% | 81% | 15% | 53% | 7% | 5% |
| | | | | | | |
| | | | | | | |
| | | | | | | |
| | BS31 | BS32 | BS41 | BS42 | BS51 | BS52 |
| T0 | 20% | 2% | 9% | 3% | 8% | 6% |
| T1 | 1% | 9% | 55% | 27% | 15% | 2% |
| T2 | 9% | 28% | 7% | 20% | 4% | 16% |

Figure 37: percentage of the variation coefficient of the duplicates in all the samples (SD/average)*100 at the beginning and at the end of the experiment.

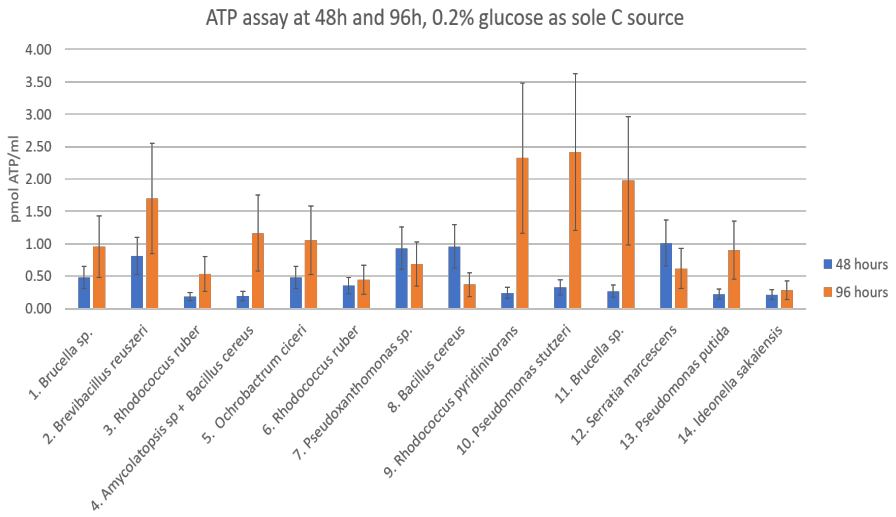


Figure 38: ATP content after 48 hours (blue bars) and 96 hours (red bars) of each of the isolates growing on 0.2% of glucose as sole carbon source. Average standard deviation is represented as error bars.

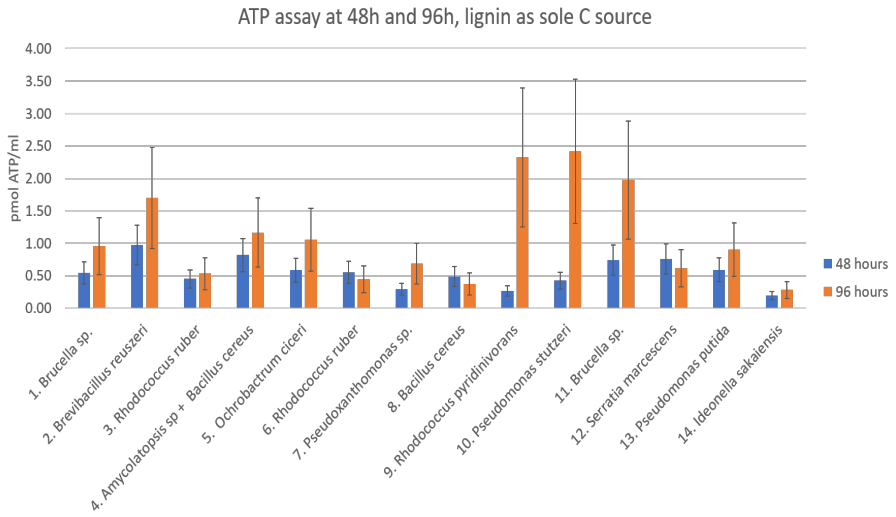


Figure 39: ATP content after 48 hours (blue bars) and 96 hours (red bars) of each of the isolates growing on lignin as sole carbon source. Average standard deviation is represented as error bars.

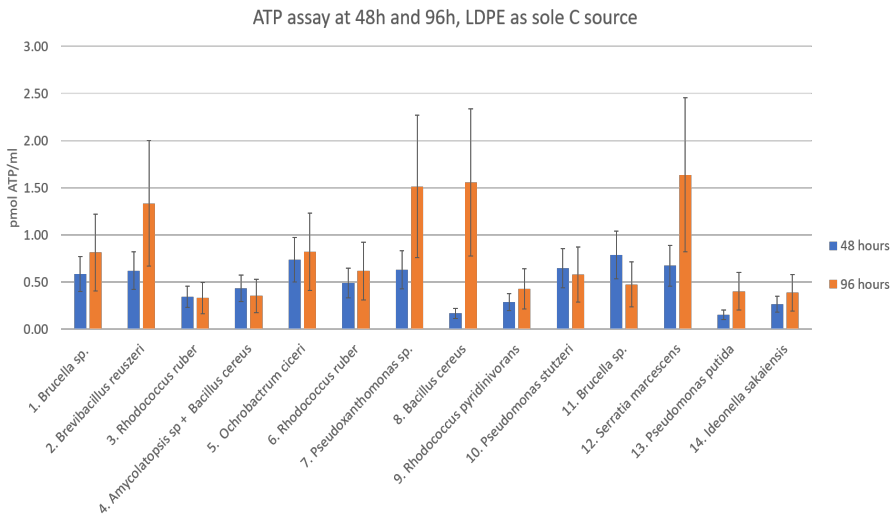


Figure 40: ATP content after 48 hours (blue bars) and 96 hours (red bars) of each of the isolates growing on LDPE pretreated as sole carbon source. Average standard deviation is represented as error bars.

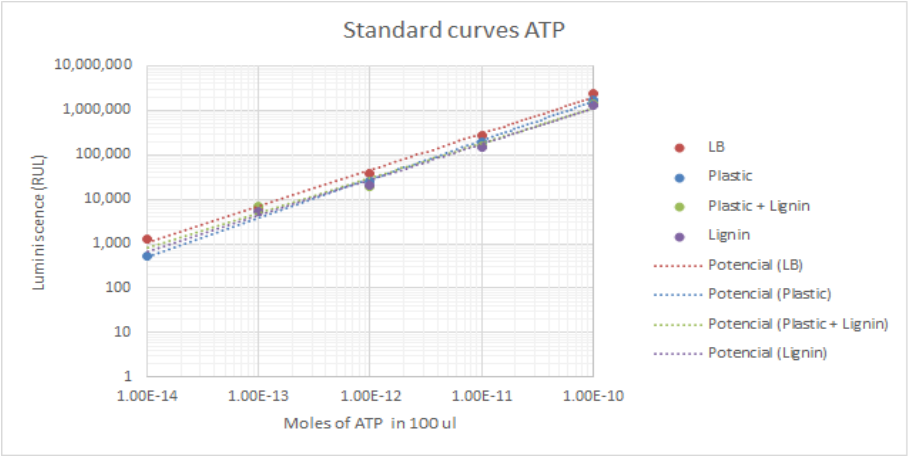


Figure 41: Standard lines for ATP quantification in the different media and substrates used.

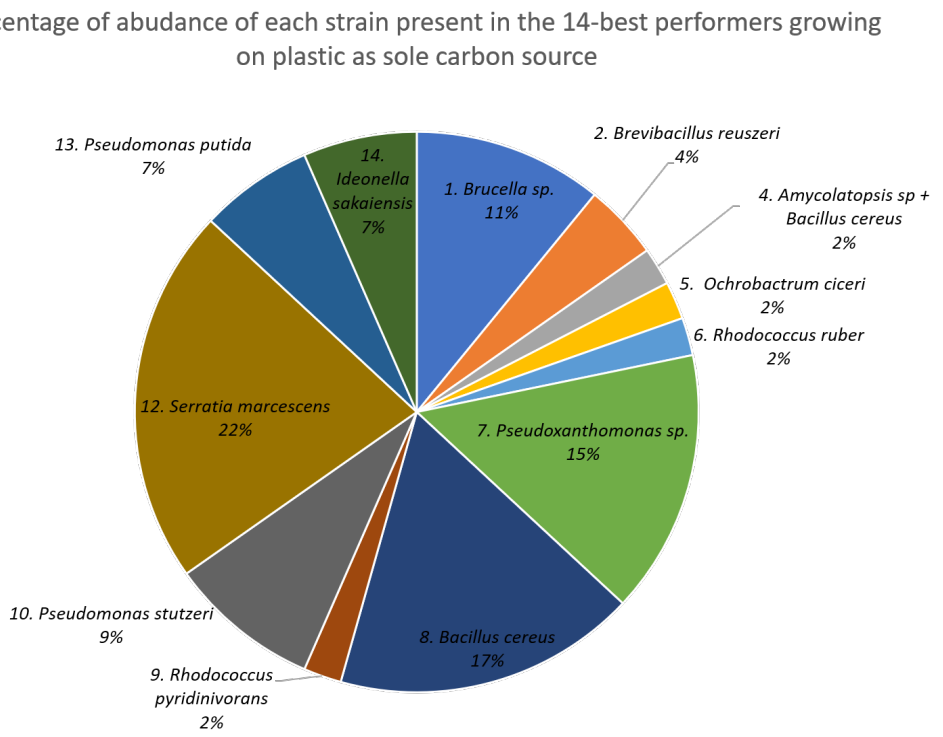


Figure 42: Abundance of each isolate in the 14-best positions of ATP content using plastic as carbon source

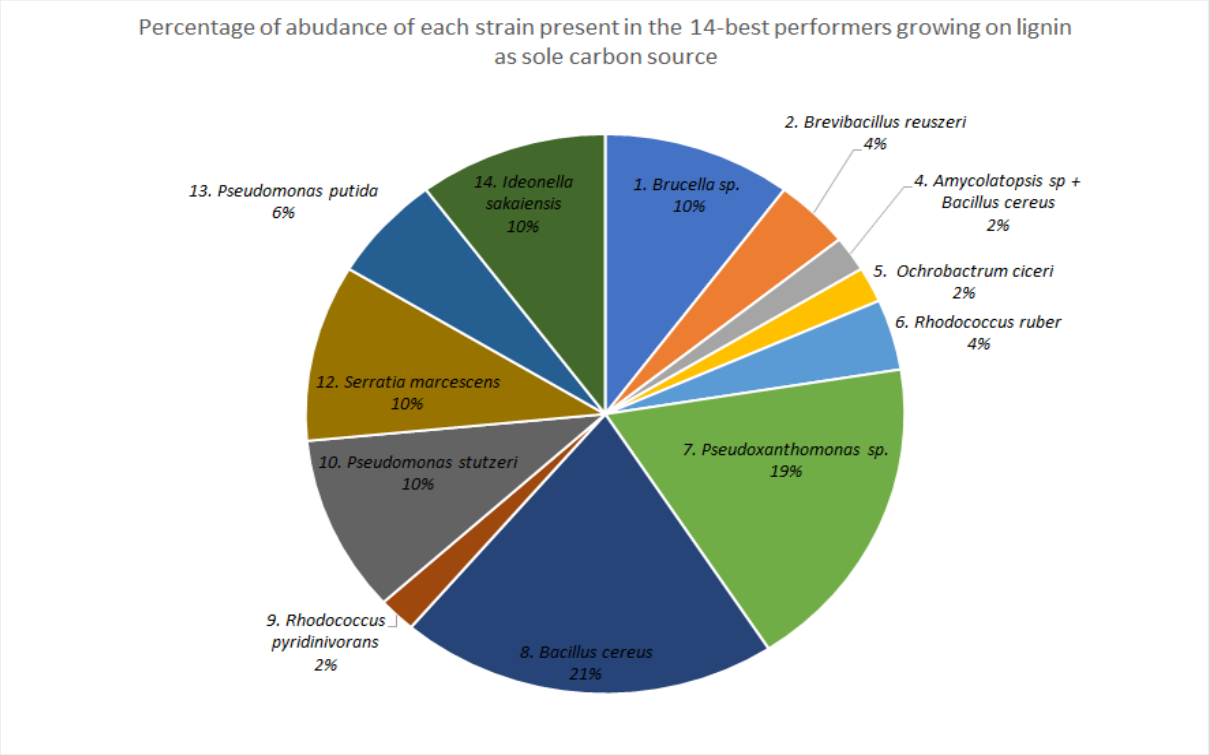


Figure 43: O.D. evolution can bee seen in the first 4 transfers (1 transfer per week).

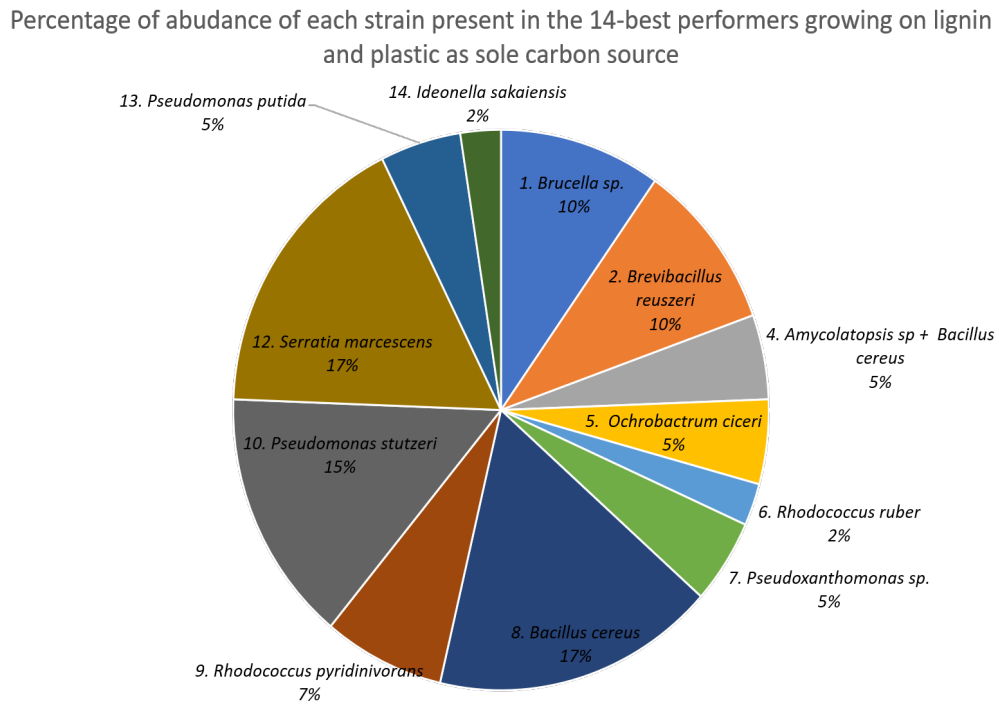


Figure 44: O.D. evolution can bee seen in the first 4 transfers (1 transfer per week).