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Fermentation of organic waste for bioplastic production

A potential sustainable alternative to fossil-fuel plastic

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Abstract

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Abstract:

The plastic pollution has become a massive problem in the Arctic, affecting aquatic, and terrestrial ecosystems, the cryosphere, and the atmosphere. One of the solutions proposed by the Arctic Council is to improve waste management by using renewable and sustainable materials. This is where bioplastics reveal their importance. They can be bio-produced by microorganisms from organic waste, they are biodegradable and can be reused. Their production relies on a circular economy system making it sustainable. Here lies the relevance of developing the bioplastic bioproduction and technology. The present research focused on the development of a specific production of polyhydroxyalkanoates (PHAs) from organic waste, in collaboration with the start-up Dionymer (Bordeaux, France). First, the purpose of the study was to up scale the process from the fermentation of chemical volatile fatty acids in flasks (400 mL culture medium) to 2 L bioreactors (BR) by characterizing the main differences in the two processes. Secondly, the research consisted in implementing and testing different set-up for the BR to enhance and improve bioplastic and biomass yields, including aeration and agitation. The characterization of the culture parameters differences between BR and flask pointed out; a higher viscosity of the medium at the end of the process, a darker PHA product and a lower final optical density (OD) (8 versus 12) respectively. Secondly, the focus was on the increase of the OD in BR and finding the origin of the stress, to do so, the following parameters were tested; - three aerations strategies; pO2<10%, <20% and >20%; - two agitations blades; marine and Rushton with baffles; - two aerations spargers; circular and micro. The results revealed that; the pO2 needs to be higher to 20% and it may be linked with the reduction of stress induced to the cells; the marine blades increased the OD and reduced the medium viscosity; the impact of the micro sparger seemed to improve aeration and tent to be very sensitive to antifoam agent that reduced the aeration of the medium. So far, the optimum BR set-up seemed to include the use of marine blades and a pO2 above 20%. More experiments of optimization still need to be performed to unsure a stable and higher production performance.

Fermentation of organic waste for a bioplastic production

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Lise Demeslay

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Statement

I state that this thesis is built upon my own work, is authored by myself and has not been used previously in part or in whole for another academic degree.

Lise Demeslay

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Abbreviation

BR	Bioreactor
CAFF	Conservation of Arctic Flora and Fauna
DCU	Digit Central Unit
EnCHiL	Environmental Changes at High Latitudes
EPS	Exopolysaccharides
EU	European Union
FID	Flame Ionization Detector
GC	Gas Chromatography
GC quality	ISO 9001, ISO 14001 and ISO 13485 certification
HB	Hydroxybutyrate
HPLC	High Pressure Liquid Chromatography
HV	Hydroxyvalerate
LDPE	Low Density Polypropylene
Mcl	Medium Chain Length
MP	Microplastic
OD	Optical Density at 600 nm
OECD	Organisation for Economic Co-operation and Development
PAME	Protection of the Arctic Marine Environment
PC	Polycarbonate
PE	Polyethylene
PLA	Polylactic Acid
PHA	Polyhydroxyalkanoate
PHB	Poly(3-Hydroxybutyrate)
PHBH	Poly(3-hydroxybutyrate-co-3-hexanoate)
PHV	Poly(3-Hydroxyvalerate)
PMMA	Polymethylmethacrylate
PP	Polypropylene
PS	Polystyrene
PU	Polyurethane
PVC	Polyvinyl Chloride
Scl	Short Chain Length
SDS	Sodium Dodecyl Sulphate
SDWG	Sustainable Development Working Group
STR	Stirred Tank Reactor
T_g	Glass transition Temperature
T_m	Melting Temperature
UTS	Ultimate Tensile Strength
VFA	Volatile Fatty Acid
WCB	Working Cell Bank
WHO	World Health Organisation

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Introduction

The plastic pollution has become a priority for the United Nation[1,2], the World Health Organization (WHO)[3] and the European Union (EU)[4]. Indeed, since 1950, the industrial plastic production has grown rapidly, reaching 368 million tonnes globally per year by 2019[5]. The major plastic market is packaging due to the shift from reusable to single-use containers. This induced an increase (by mass) from 1% in 1960 to 10% by 2005 of plastic in municipal solid waste in middle and high income countries[6]. The COVID-19 pandemic has exacerbated the use of plastic with the urgent demand for single-use plastic personal protective equipment (masks, gloves, face shields)[7]. Additionally, most plastics are derived from fossil hydrocarbons and non-biodegradable. As a result, most of them are accumulating in the natural environment or in landfills[8].

In the Arctic, the plastic pollution is becoming pervasive, even not highly populated areas or with no apparent human activity. A recent review from April 2022, published in Nature depicts the sources and impacts of microplastics and plastics debris that are affecting terrestrial, aquatics ecosystems, the cryosphere and the atmosphere[9]. Indeed, the poleward branch of the thermohaline circulation leads to a sink of plastic debris in the Arctic Ocean[10]. Furthermore, the plastic accumulation occurs also into the sea ice[11–13] and is expecting to be worst with global warming affecting Arctic fauna with plastic ingestion, especially in Artic marine organisms[14].

To face this growing issue, Iceland, as the chair of the Arctic Council between 2019 and 2021 decided to make Arctic pollution and marine litter a priority. To perform so, six working groups of the council are involved in the project[15,16]. The council provides a solution following three axis: managing, monitoring and preventing waste[16]. The action plan consists of improving the knowledge about how plastic pollution is affecting the wildlife, improving waste management sites, and using more sustainable materials, for instance. The production of bioplastic as a substitute to fossil-fuel based ones can be included in a solution of sustainable material.

In fact, biobased bioplastic refers to polymers that are biobased or biodegradable[7]. The purpose of these plastics in to turn the linear life cycle of the main fossil-fuel based ones in a circular life circle by producing plastics from renewable source, that are biodegradable and recyclable[7]. Then, biobased polymers can be obtained from various sources such as: sugar, corn, vegetable oil[17] or seaweeds[18]. Each source needs to be studied to be obtained sustainably. Then, bioplastics can be produced through chemical modifications or fermentation by microorganisms. Finally, they must be biodegradable by being decomposed by living microorganisms. Polyhydroxyalkanoates (PHAs) are a good example of such biobased bioplastic.

PHAs are a group of aliphatic polyester, natural biodegradable polymers synthesized by microorganisms[7]. Produced from organic materials such as cellulose, glucose, vinasse, food waste or rice[19,20], they can also be biobased. The material properties can be tuned by modifying the repeat unit chain length, the side chain functionalities and co-monomer compositions[21–23]. To perform so, the choice of the strain and its substrate are essentials. The productivity can be enhanced through genetic modifications of the strain and/or fermentation optimisation.

The start-up Dionymer, where I performed my traineeship, is producing poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBV), a type of PHA, from grape pomace with fermentation. It is part of the circular economy since the substrate is vinery waste to produce a biodegradable material. The aim of the research was to scale-up the fermentation from 400 mL flask culture to a 2 L bioreactor (BR) and solve the associated foaming problem. The goal was to find out, **which parameters in BR can be adjusted to increase scale, reduce foaming, improve and enhance the production of PHBV in comparison to the flask**. The hypotheses were that the scale-up can be carried out without cultivation-related problems, and the foam formation can be solved by using antifoam agents and controlling agitation or aeration. To answer these questions**, I first characterized and listed the differences in the culture parameters differences between flask and BR. Then, I tested three different parameters in the BR to correspond as closely as the ones in the flask.** The parameters were accepted and adopted in further experiences when the microorganism had a significant biomass production. I used the optical density (OD) as a proxy for biomass production.

This master thesis is divided in four sections. First, an overview of plastics in the Arctic and general information regarding this material are depicted. Then, the main properties of the polymer bioplastic, PHAs, are explained, including its fermentation production and my implication in the start-up Dionymer. Thirdly, the materials and methods of my work are presented before jumping the results and discussions of this work.

1. The Arctic and plastics

1.1 Different kinds of plastics

1.1.1 Plastics

Plastics are multipurpose polymers with high molecular weight, which can be degraded by various processes. They have various changeable properties: lightweight, cheap, durable and resistant[24]. Their use can suit a large range of purposes due to their properties. Plastics can be pressed, extruded into a variety of shapes for films, fibres, plates, tube, bottles, and other objects. Their production has grown from 1.7 million tons in 1950 to 335 million tons in 2016 and is expected to double in the next 20 years[25]. This is mainly due to their variety of applications. In 2019, the three main ones are packaging (31%), building and construction (17%) and transportation (12%) (Figure 1-1). In the main sector of application, plastics can be identified and sorted in seven categories, to indicate the type of polymer used to produce them and how they can be recyled[26] (Table 1-1). They have a number triangled framed with three arrows. Depending on the properties required: sun resistant, acid resistant, transparent, or light for example, different kinds of plastic can be used.

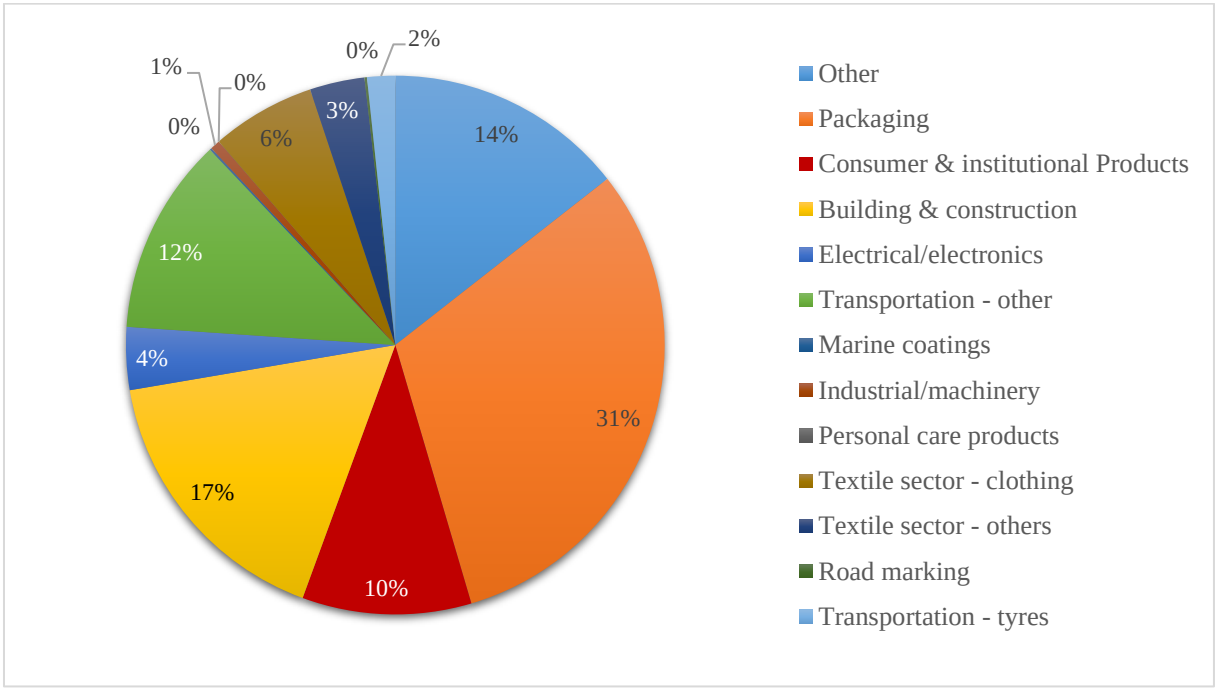


Figure 1-1. Global plastics application and repartition of 459.75 million tons of plastics in 2019[27].

Table 1-1. Plastics identification and recycling chart

1 PETE	**Number 1** • *PETE or PET (polyethylene terephthalate)* **IS USED IN** . . . . . microwavable food trays; salad dressing, soft drink, water, and beer bottles **STATUS** . . . . . hard to clean; absorbs bacteria and flavors; avoid reusing **IS RECYCLED TO MAKE** . . carpet, furniture, new containers, Polar fleece
2 HDPE	**Number 2** • *HDPE (high-density polyethylene)* **IS USED IN** . . . . . household cleaner and shampoo bottles, milk jugs, yogurt tubs **STATUS** . . . . . transmits no known chemicals into food **IS RECYCLED TO MAKE** . . detergent bottles, fencing, floor tiles, pens
3 V	**Number 3** • *V or PVC (vinyl)* **IS USED IN** . . . . . cooking oil bottles, clear food packaging, mouthwash bottles **STATUS** . . . . . is believed to contain phalates that interfere with hormonal development; avoid **IS RECYCLED TO MAKE** . . cables, mudflaps, paneling, roadway gutters
4 LDPE	**Number 4** • *LDPE (low-density polyethylene)* **IS USED IN** . . . . . bread and shopping bags, carpet, clothing, furniture **STATUS** . . . . . transmits no known chemicals into food **IS RECYCLED TO MAKE** . . envelopes, floor tiles, lumber, trash-can liners
5 PP	**Number 5** • *PP (polypropylene)* **IS USED IN** . . . . . ketchup bottles, medicine and syrup bottles, drinking straws **STATUS** . . . . . transmits no known chemicals into food **IS RECYCLED TO MAKE** . . battery cables, brooms, ice scrapers, rakes
6 PS	**Number 6** • *PS (polystyrene)* **IS USED IN** . . . . . disposable cups and plates, egg cartons, take-out containers **STATUS** . . . . . is believed to leach styrene, a possible human carcinogen, into food; avoid **IS RECYCLED TO MAKE** . . foam packaging, insulation, light switchplates, rulers
7 OTHER	**Number 7** • *Other (miscellaneous)* **IS USED IN** . . . . . 3- and 5-gallon water jugs, nylon, some food containers **STATUS** . . . . . contains bisphenol A, which has been linked to heart disease and obesity; avoid **IS RECYCLED TO MAKE** . . custom-made products

Since plastics are meant to last, their waste needs to be managed. After their use, these polymers can have different end-life[28]. They can be **recycled** by being collected, sorted regarding their polymer composition, and reused to form other valuable products. **Incineration** is also a performed. It consists of a complete combustion of the product, releasing water and carbon dioxide in the atmosphere[29]. Part of the waste is disposed and buried in **landfills** to protect the other areas to be contaminated. Additionally, **pyrolysis** refers to the process of converting gases and fatty oils to obtain hydrocarbons. Finally, **bioremediation** is a process where microorganisms decompose waste to decontaminate and detoxicate.

However, these waste management practices depend on the country and existing infrastructures. According to the organisation for economic co-operation and development (OECD), only 9% of the world plastic is recycled, 22% is mismanaged and uncollected, 49% is landfilled and 19% incinerated (Figure 1-3). Some noticeable management can be pointed out: OECD Asia mainly managed its plastics by incineration (70%) and OECD Europe seems to have the highest recycled management of plastic (15%) compared to other continents.

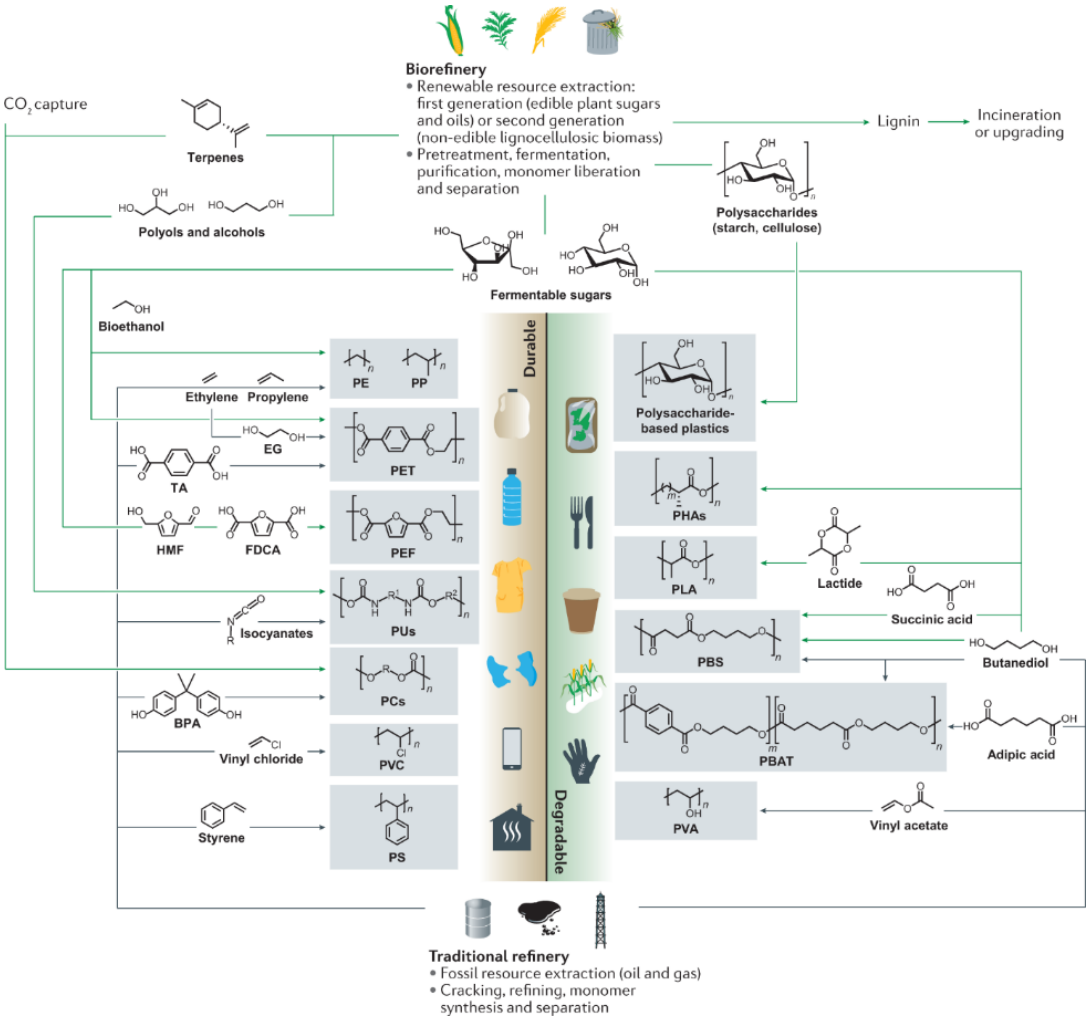


Figure 1-2. Structure and monomer origins for petrochemical/fossil-based and biobased polymers of plastic[7].

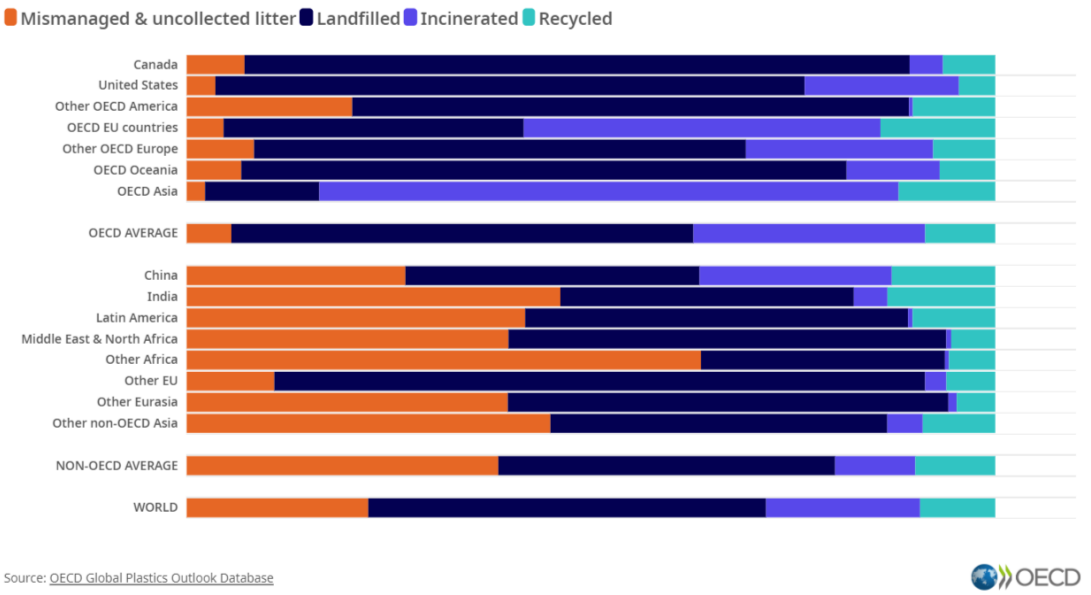


Figure 1-3. Share of plastics treated by waste management category in 2019[30]

1.1.2 Bioplastics

In 2019, the global production of bioplastic (2.33 millions of tons) represented 0.5% of the global plastic production (459.75 millions of tons)[27]. The global bioplastic production is expected to grow up to 6.72 millions of tons in 2024[4]. The prefix "bio" in bioplastic can be **biobased**, **biodegradable** or both (yellow frame in Figure 1-4). Indeed, it can refer to the renewable resources the monomers are from (**bio-based**) and then polymerized through chemical mechanisms (green squares). A **biodegradable** plastic correspond to a polymer which can be degraded through biological processes[7,24] (bottom squares). They are opposed to the fossil-based and non-biodegradable plastics (up left grey square).

In 2022, a review identified the five main challenges for bioplastics development in comparison to fossil-based ones[7]. Economically, they are **more expensive** to produce due to the low prices of crude oil. The production processes are **less energy efficient**, and the **recycling streams** have yet to be established and explained to the customers. Some bioplastics use **first-generation biomass** that can be directly used for food-production, which is controversial. For bioplastics using second-generation biomass, the processes need more development. Additionally, the **education** for customer needs to be developed, to reduce the confusion of labels and distribution associated.

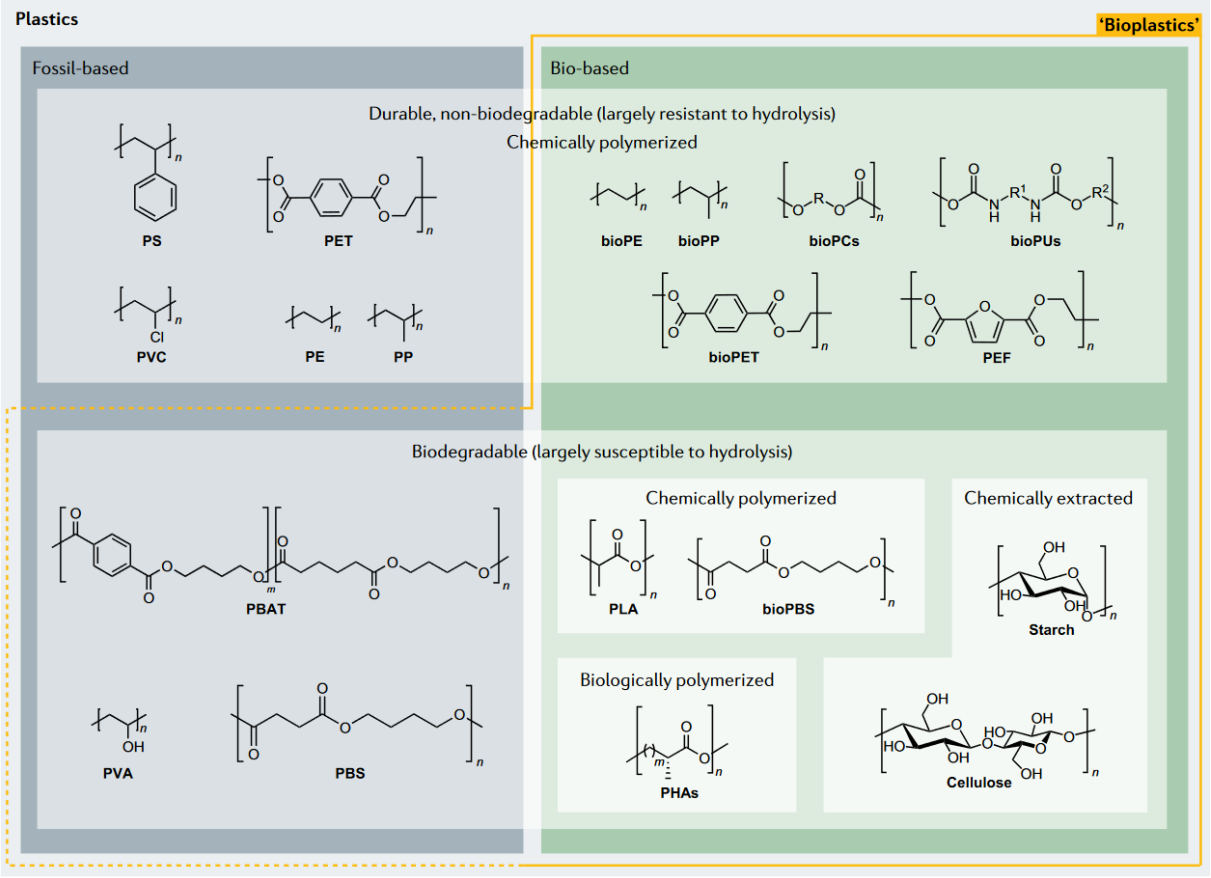


Figure 1-4. Scheme differentiating plastics in fossil-based and bio-based and non-biodegradable and biodegradable.[7]

1.2 The increase of plastic threat in the Arctic

1.2.1 The current state of plastics in the Arctic

Plastic accumulation in the environment is a substantial environmental problem. The residues of plastics in natural places are not fulfilling an intended function and stay in terrestrial and aquatic environment. The number of wildlife species impacted by plastics debris through entanglement, ingesting and laceration has doubled since 1997 to 2015[31]. Plastic debris can be categorized in four sections depending on their size[32]:

- Nanoplastics (1-1000 nm)
- Microplastics (1-1000 µm)
- Mesoplastics (1 – 10 mm)
- Macroplastics (1 cm and larger)

The macroplastics are becoming microplastics due to weathering: sunlight, mechanical abrasion, biotic interactions, wave action and temperature fluctuations[9]. Since they are widely used in fishing, aquaculture, shipping, and offshore operation, an important amount leaks into marine environments and get accumulated in some areas[33]. Their speed of transportation is different since microplastics are submerged and macroplastics exposed to environmental stress.

Since the Arctic region has few inhabitants compared to other areas in the word, the local plastic pollution would be expected to be very low. However, the pollution in the region exists and has different sources, impacting wildlife, Arctic biota and food webs[11]. Indeed, a model projections suggest the formation of a sixth accumulation area in the Nordic Sea and an increase in marine debris over time[34,35]. Figure 1-5 shows the localisation of different ecosystems where plastic pollution has been found in the Arctic Ocean in 2022. The accumulation circulates around the Beaufort Gyre and leaves in the Atlantic water through the Canadian Arctic and goes to west Greenland[9].

The origin of the plastic found in the Arctic can be from **local** sources, due to industrial activities: fishing, shipping, aquaculture and tourism[9]; and also from domestic sources: washing textile, personal care product that contains microplastics[8,10]. Additionally, **external** plastic pollution is from long-range transport from temperate regions to the Arctic through atmosphere and ocean currents[10,13,14]. Air plays a key role in the transport of microplastics since it helps the particles to fall down with precipitation and induce its presence in Arctic snow and ice[13].

The impact of plastics on Arctic biota are known to be harmful. Large plastics can have a mechanical: entanglement, ingestion, blockage of intestines or hindering limb movements; or toxicological: related to chemicals; impact on wildlife[33]. The food web is impacted from small plankton to top predators such as seas, whales and polar bears[11]. Plastic ingestion by animals have been reported: northern fulmars (*Fulmarus glacialis*) from the Svalbard Islands[36] or arctic foxes (*Vulpes lagopus*) in Iceland[37].

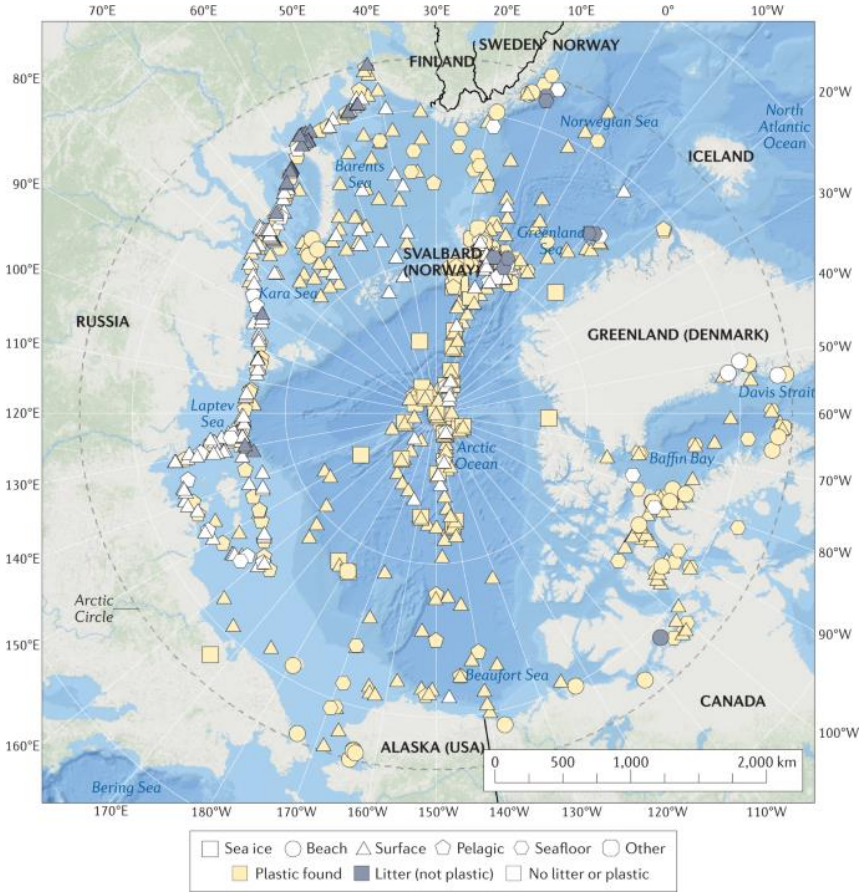


Figure 1-5. Plastic pollution in the Arctic Ocean[9]

1.2.2 The potential solutions

The Arctic situation regarding plastic pollution is not yet well understood. Transportation, food chain impact, plastic ingestion by wildlife, water contamination are subjects which require more research and understanding. However, scientists are facing assessment restrictions and the lack of infrastructure and harsh environmental conditions[9] preventing sampling. Working with local population on longer period could enable to fill the knowledge gap.

The Arctic Council gives three axes of development to solve the plastic problem in the North Pole[16] (Figure 1-6). First, **waste management** is taken care by the council's sustainable development working group (SDWG), which focuses on specific strategies, practices, behaviours, and infrastructures that protects the health of residents and the environment. Secondly, **waste monitoring** is performed by the protection of the arctic marine environment (PAME) and the conservation of arctic flora and fauna (CAFF). Their role is to study marine litter to develop a monitoring plan and technical guidelines, and to use sea birds to monitor plastic ingestion[15]. Finally, **waste prevention** is put in place thanks to the regional action plan on marine litter, an international collaboration between Arctic and non-Arctic states. It consists in finding sustainable materials, coastal clean-ups, stronger monitoring and research and outreach.

Figure 1-6. Logos of the working groups to solve the plastic issue in the Arctic. a. The arctic council. B. The protection of arctic marine environment. C. Conservation of arctic flora and fauna. D. Sustainable development working group.

1.3 Bioplastics for a circular economy

A circular economy "is a model of production and consumption, which involves sharing, leasing, reusing, repairing, refurbishing, and recycling existing materials and products if possible. In this way, the life cycle of products is extended"[38] (European Parliament 2022). The circular economy relies on reducing the amount of residual waste as much as possible (Figure 1-7). This model is the opposite of the traditional linear economic model, which relies on the large use of cheap, easily accessible materials and energy. The circular economy has several advantages. Since raw materials are limited on the planet, it is based on reusing waste as a resource, reducing in consequence the CO_2 emissions[38]. The case of the actual plastic industry is traditionally following a linear life cycle. Oil and gas are used to create polymers with extraction and polymerization, and formulation and moulding produces the plastic-based product. Finally, 79% of all plastics are dumped into landfill sites or into the environment, 12% are incinerated and 9% are recycled[39].

The case of polyhydroxyalkanoates (PHAs) bioplastic fits with the circular economy model (Figure 1-8). Indeed, the PHA can be biosynthesised in bioreactor though microbiological fermentation. The PHA granules are extracted, preferably with renewable solvents. The PHA items designed and produced are then composted. The CO_2 produced is then captured by plants, which are then reused for PHA production.[40] Additionally, food waste can be used as a substrate for microbial cells and instead of releasing the carbon in carbon dioxide form, they are stocked in PHA, as a carbon energy source for the microorganisms. The PHA production fits in a circular closed loop.

The use of renewable resources alone does not imply sustainability. It highly depends on how the material is made, where it is used and how it can be recycled[7]. There is a bioplastic substitute for almost all the fossil-based plastics. However, their quantity is very small and expensive, and they are not always holding substantial environmental benefits. Therefore, the production processes need to be improved to increase efficiency, reduce the energy demand and the use of toxic chemicals. The environmental sustainability has yet to be met with financial sustainability. The low oil prices, narrow profit margins and existing fossil-fuel subsidies reduce the cost-competitiveness of bio-based plastics producers. That is why, they often rely on products with high added value: specifically in the medical, nutritional, cosmetical and pharmaceutical sectors[7]. However, this is most likely to evolve in the following years due to the current increase and fluctuation of oil prices in the world. In fact, they are mainly connected to geopolitical crises, which create an unstable situation for industries, that may lead them to find a new source of energy with an impact on prices.

Figure 1-7. Circular economy scheme by the European Parliament 2022[38]

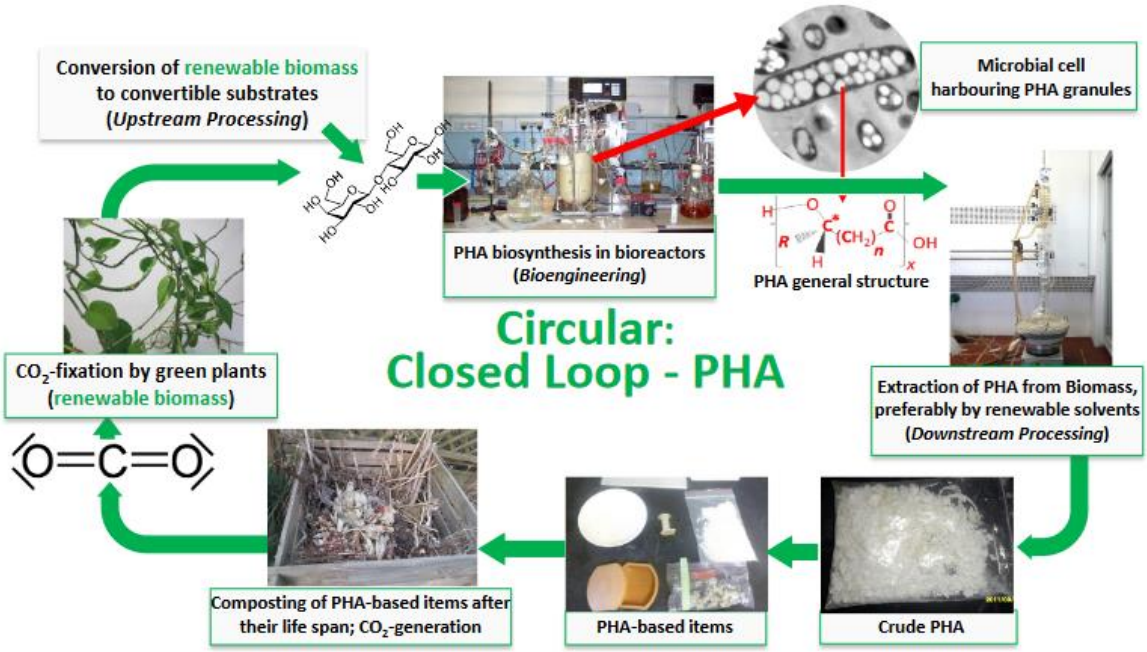


Figure 1-8. Circular economy model for PHA production[40]

1.4 Arctic species as bioplastic producers

The PHA production by microorganisms can be part of a circular economy, as explained above. This possibility can be enhanced by the choice of microorganism species. Indeed, some species can store carbon in the form of PHA in a harsh environment. In this condition, PHA can be an energy storage[41] or a resistance to a stress, in the case of extremophiles[42].

- **Halophiles** live in high saline environment: *Natrinema pallidum*[43], *Halomonas* TD01[44] and *Haloferax Mediterranei*[45]
- **Thermophiles** grow best at high temperature (50-80°C): *Thermus thermophilus*[46], *Synechococcus* sp. MA19[47] or *Chelatococcus daeguensis*[48]
- **Cryophiles** grow best at a low temperature (15-20°C): *Pseudomonas extremaustralis*[49] (found in Antarctica), *Pseudomonas* sp. B14-6[50], *Pseudomonas* sp. PAMC 28620 (Arctic psychotropic bacterium)[51] (found in the arctic soil).

In the Arctic, the microorganisms must adapt and find tactics to survive the harsh conditions, especially to the low temperature and nutrition fluctuation. To do so, some of them store energy within the cells, as PHA granules[52,53], which also protect the internal cellular system[54]. They can produce short and medium-chain-length PHA depending on the carbon sources[55]. However, since the metabolisms of Arctic bacteria is quite slow, it weakens their potential as PHA producers[56]. There also exists some **cyanobacteria** that produces this bioplastic[57], under photoautotrophic conditions, using CO_2 and sunlight as carbon and energy sources. However, their productivity is not efficient enough to be used as a massive PHA producer[58].

2. Production of bioplastics: Polyhydroxyalkanoates through fermentation

2.1 What are polyhydroxyalkanoates?

2.1.1 Composition and structure

Polyhydroxyalkanoates are bioplastics polyesters, natural, biobased, and biodegradable that can have similar material properties as synthetic fossil oil-plastics based. PHAs have the advantage of being industrially produced by several microorganisms through fermentation of plant-oils or waste lipids from agricultural industry or pure and mixed carbohydrates[20,40,59–61]. PHAs are commonly found as storage compounds of carbon and energy in various microorganisms[62]. They can also act as part of protective mechanisms against extreme environments[54].They have similar properties to petroleum-based plastics[44], except that they are biobased and biologically degraded[63]. Polymers of PHA are a long chain-like molecules (macromolecules), composed of many repeating subunits (monomers) (Figure 2-1 A).

The polymers are defined by several properties, such as their **thermoplasticity**, **elastomer ability**, **melting point**, **glass transition temperature** or **molecular weight**. There properties are affected by the composition of monomers. It can be a single monomer repeated a certain amount of time (n) (Figure 2-2 (a)). It can also be a **co-polymer** consisting of a mixture of at least two different monomers. The structure can have different features (Figure 2-1 B):

- Random: "macromolecules in which the probability of finding a given monomeric unit at any given site in the chain is independent of the nature of the adjacent units.[64]
- Alternating: "macromolecules comprising two species of monomeric units in alternating sequence."[64]
- Block: "portion of a macromolecule, comprising many units, that has at least one feature which is not present in the adjacent portions."[64]
- Graft: "One or more blocks of homopolymer are grafted as branches onto a main chain"[65]
- Periodic: "macromolecules comprising more than two species of monomeric units in regular sequence."[64]

A polymer is defined as a PHA when its monomers follow the same structure (Figure 2-2 (a)). Depending on the number x and their alkyl radical R, these monomers can have variating length that describes three types of PHAs[60,66]. N corresponds to the number of carbons between carbonyl group (included) and the last carbon of the alkyl radical.

- $N \leq 5$, it is a short chain length-PHA (scl-PHA) (Figure 2-2 (b)).
- $6 \leq N \leq 13$, it is a medium chain length-PHA (mcl-PHA) (Figure 2-2 (b)).
- $N \geq 14$, it is a long chain length-PHA (lcl-PHA).

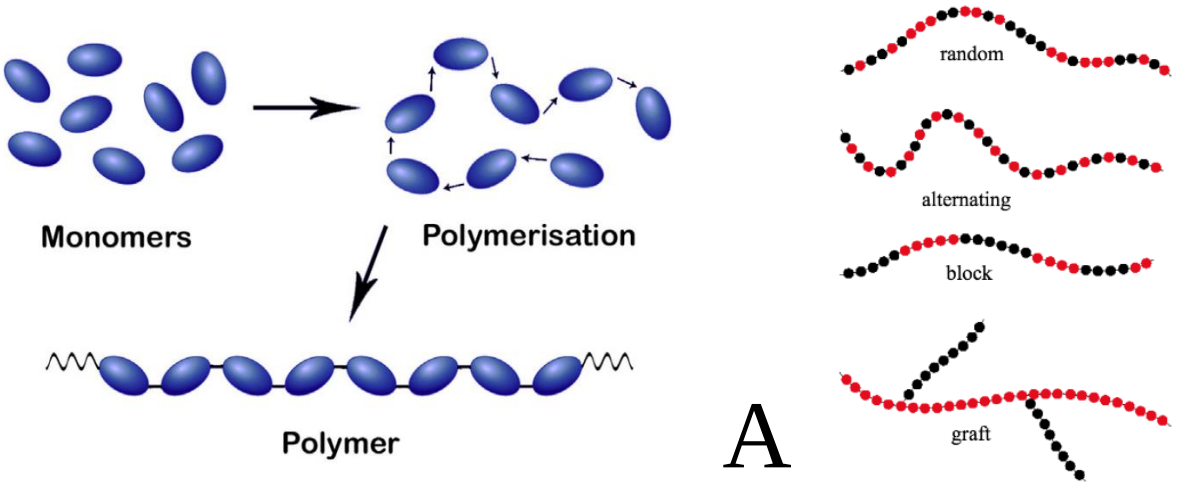


Figure 2-1. General molecular composition of PHA. A. Scheme of a polymer composed of many repeated units (monomers) after polymerisation[67]. B. Four basic copolymer structures[68,69].

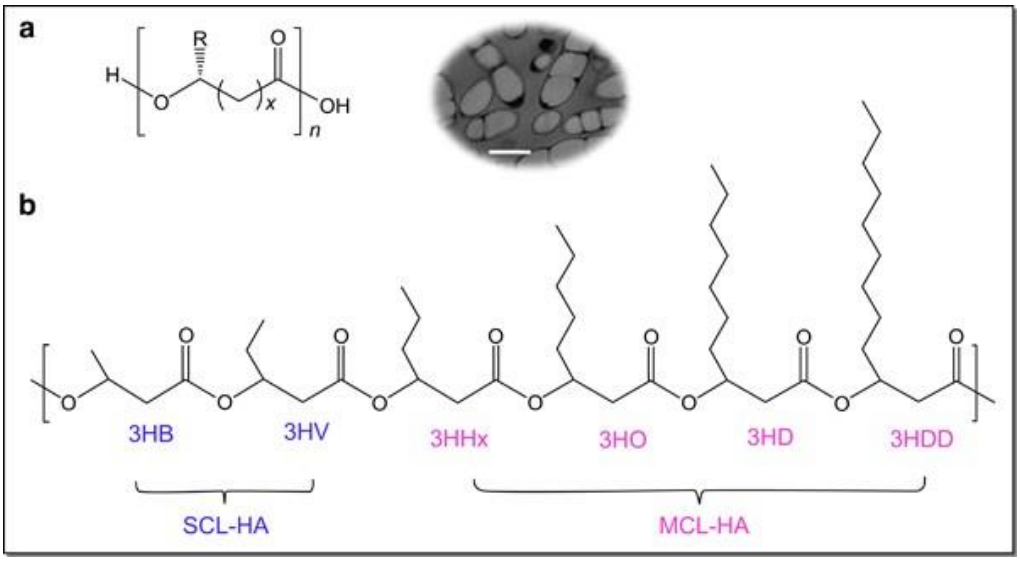


Figure 2-2. Molecular formula of PHA. a. General molecular formula of PHAs, typically, x=1-8, and n ranges from 100 to 1000. B. Some commonly synthesized short-chain-length PHA monomers (scl-PHA) and middle-chain-length PHA monomers (mcl-PHA). 3HB: 3-hydroxybutyrate, 3HV: 3-hydroxyvalerate, 3HH[70]

2.1.2 Material properties

First of all, a macromolecule becomes a polymer when the number of subunits is big enough to give stable properties to the molecule[67]. Its material properties, mechanical and physical, are defined by several factors:

- Crystallinity (%): refers to the degree as to which there are regions where the polymer chains are aligned with one another[67,71] (Figure 2-3). In terms of properties, the more crystalline the polymer is, the more brittle it becomes.[67]

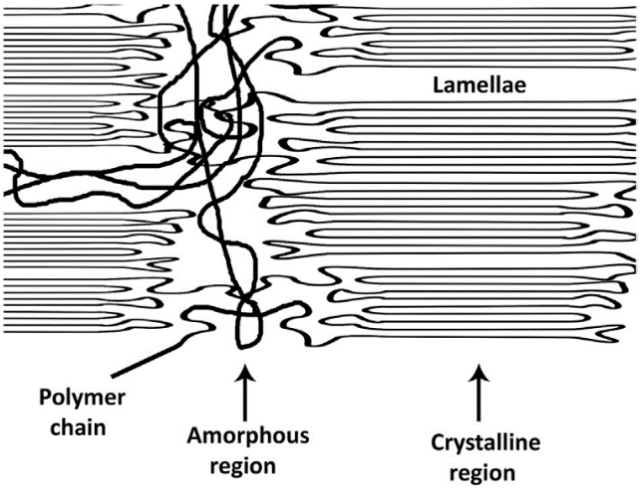


Figure 2-3. Scheme of a region of a polymer. Amorphous regions have disordered entangled chains while semi-crystalline polymer are interspersed with ordered crystalline regions where the chains are aligned.[67]

- Around 100% of crystallinity, which is theoretical and impossible, the polymer can be **crystalline,** where ideally, all chains would be aligned (see crystalline region in Figure 2-3). This induces the crystalline polymer to be harder, denser and has higher melting points (i.e., diamonds, table salt, sugar).
- From 0 to 100%, the polymer is **semi-crystalline,** meaning that the polymer contains both aligned and disordered regions. Depending on the amount of crystallinity, its material properties will be influenced (PE, PP, Teflon).[72]
- Around 0%, the polymer is **amorphous**, meaning that the arrangement of the chains is disordered. Inducing its material properties to be more brittle, clear, and stiff. They tend to have a lower softening points and higher impact resistance (PVC, PS, PMMA, PC).

The material properties affect their final use. Certain kinds of PHA will have different uses and potential applications. Since they have a non-toxic quality, they can be used for medical purposes. In general, mcl-PHAs are characterized by their large flexibility and limited crystallinity. This is leading their use to thermoplastic elastomers for packaging and some medical applications[73,74]. However, they degrade at a low temperature that makes their processing more difficult[75].

The crystallinity of a polymer depends on polymer arrangement in space. The more the chains are aligned, the more crystalline the polymer will be. The motifs in PHAs all have a chiral carbon (C* in Figure 2-4) linked with four different groups: Hydrogen, polymer backbone, ester, and another alkyl chain. This chiral carbon can have an R or S conformation. Depending on the conformation of the monomers that polymer is holding, it will have different names.

- *Isotactic:* when all the monomers have a conformation R or a conformation S. (R-R-R-…) or (S-S-S-…)
- *Syndiotactic:* the conformation will change regularly. (R-S-R-S-R-…)
- *Atactic:* the localisation of the motifs R and S is random. (R-R-S-R-R-R-S-…)

- T_g (°C): Glass transition temperature corresponds to the temperature at or above which the molecular structure exhibits macromolecular mobility, going from brittle to viscoelastic state (Figure 2-5). Regarding PHAs, the mobility concerns the amorphous parts of the macromolecule.[53]
- T_m (°C): Melting temperature corresponds to "the critical temperature above which the crystalline regions in a semicrystalline plastic are able to flow[72] (Figure 2-5).

T_g concerns only the amorphous parts of a polymer. Under that temperature, the polymers have a very low mobility. Between the T_g and the T_m the amorphous part becomes mobile, and above the T_m the polymer melts. In the case of a 100% crystalline polymer, T_g does not exist. Similarly, a 100% amorphous polymer does not have a T_m.

- Young's Modulus (MPa): Corresponds to the stiffness of a material, meaning how can it be bended or stretched.[76] It is a ratio of stress over elongation of the material.
- UTS (MPa): Ultimate Tensile Strength corresponds to the maximum stress that a material can withstand while being stretched or pulled before breaking.[77]
- Elongation break (%): It is the ratio between changed and initial length just before breakage of the tested material.[78] It corresponds to its elasticity.

The main proprieties of PHAs are biodegradability, **insolubility in water**, nontoxic, biocompatibility, piezoelectric, thermoplastic and/or elastomeric nature, which can have different use for industry[79]. They also have **thermoplasticity** defined by the ability to modify the proprieties when the material is heated, and the properties do not change when the material cools down[62,66].

Then, PHAs physical and mechanical properties are impacted by the structures and the chemical nature of their monomers. On one side, mcl-PHA are semi-crystalline elastomers with a low melting point, low UTS and high elongation to break.[80] On the other side, scl-PHAs are more crystalline (range between 60-80%), and have high T_m points and low T_g. However, in the case of PHBV, a scl-PHA, the more hydroxyvalerate (HV) motifs it contains in proportion to motifs hydroxybutyrate (HB) (Figure 2-2 b), the more crystalline the polymer will be (Table 2-1).

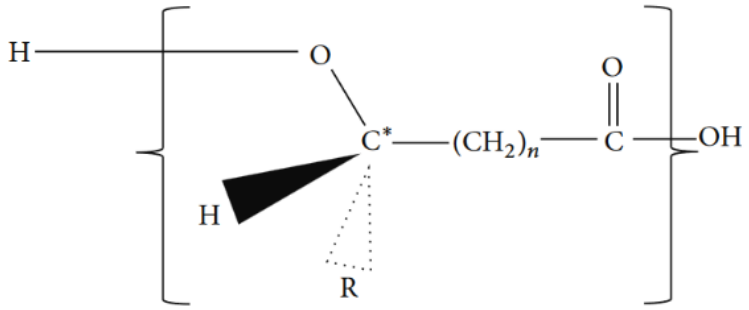


Figure 2-4. General Lewis structure of PHA monomer. R: Radical side chain monomer giving the name of the monomer. * Corresponds to the chiral carbon of most PHA building blocks.[81]

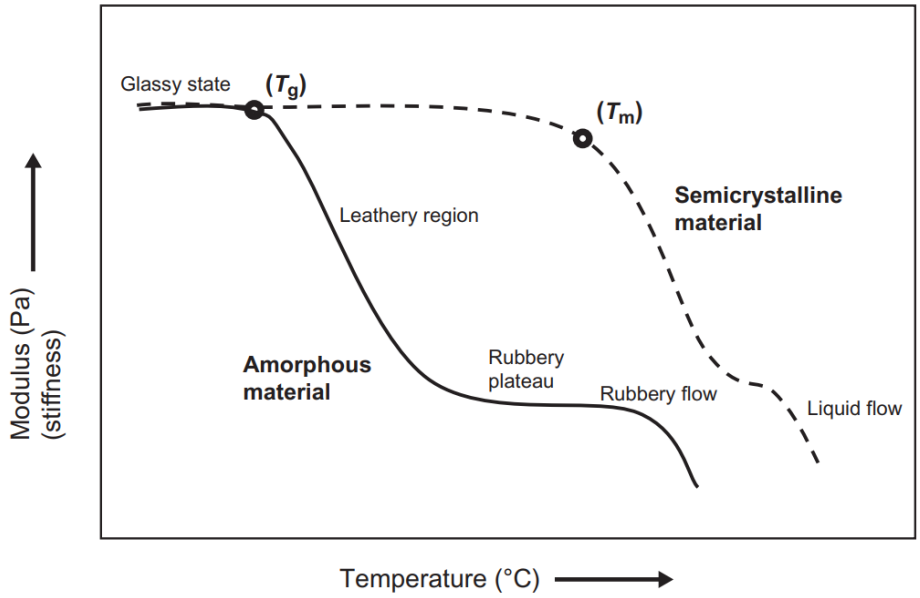


Figure 2-5. Thermal transitions between amorphous and semi-crystallin polymers[72]

Table 2-1. Physical and mechanical properties of some polyhydroxyalkanoates and fossil-fuel based plastics[80].

Polymer	Crystallinity (%)	T_m (°C)	T_g (°C)	Young's Modulus (MPa)	UTS (MPa)	Elongation break (%)
PHB	60	177	4	3500	43	5
PHV	-	106	-16	390	7-31	4
P(4HB)	25	54	-48	1000	104	1000
P(3HB-co-20%HV)	56	145	-1	1200	32	-
P(3HB-co-14%HV)	45	150	-	1500	35	-
P(3HB-co-9%HV)	34	162	-	1900	37	-
PLA	56	170	57	3500	41	4
PP	70	176	-10	1700	38	400
LDPE	20-50	130	-36	200	10	620

PP polypropylene, LDPE Low Density Polypropylene, T_m melting temperature, T_g glass transition temperature, UTS Ultimate Tensile Strength.

2.1.3 An energy storage for microorganisms

PHAs are biopolymers biosynthesized by a variety of microorganisms originated from a vast range of environments like marine microbial mats, estuarine sediments, rhizosphere, groundwater sediments or anthropogenic origin where there is a fluctuating nutrient content[82]. PHAs are mainly used by these microorganisms as an energy and carbon reserve[46]. It also fills purposes of maintaining redox potential and NADH oxidation[83,84], for maintenance of anoxic photosynthesis and sulphur cycle[85] or protection against exogenous stress factors[86]. For archaea and bacteria PHAs are produced under nutrient-limiting conditions with excess carbon source[87].

PHAs are synthesized as a form of storage material, and they accumulate in bacterial cell cytoplasm in the form of granules since the PHA molecules are insoluble in water (Figure 2-6). The average size is about 0.2-0.5µm [88,89]. To recover the PHA granules, the bacterial cells need to be broken and the protein layer over the PHA granules needs to be removed[83] (Figure 2-6 D).

The biosynthetic pathway to produce PHAs is highly dependent on the microorganism and the substrate available in the medium[66]. PHA production requires three steps. The first one is the entrance of the carbon source in the cell with a passive diffusion or with specific transporters. Then, the carbon source is metabolised into PHA precursor such as acetyl-CoA or propionyl-CoA and then transformed into (R)-3-hydroxybutyryl CoA or (R)-3-hydroxyvaleryl-CoA respectively. The last step consists of incorporating the monomer molecules into PHA polymer growing chain by a PHA synthase[90] (Figure 2-7). The production of PHBV or Poly(3HB-*co*-3HV) requires the precursors 3HB-CoA and 3HV-CoA. The latter is obtained from the condensation of acetyl-CoA and propionyl-CoA to 3-ketovaleryl-CoA that is then reduced to produce 3HV-CoA[41]. Propionyl-CoA can be produced thanks to different substrates (Figure 2-8).

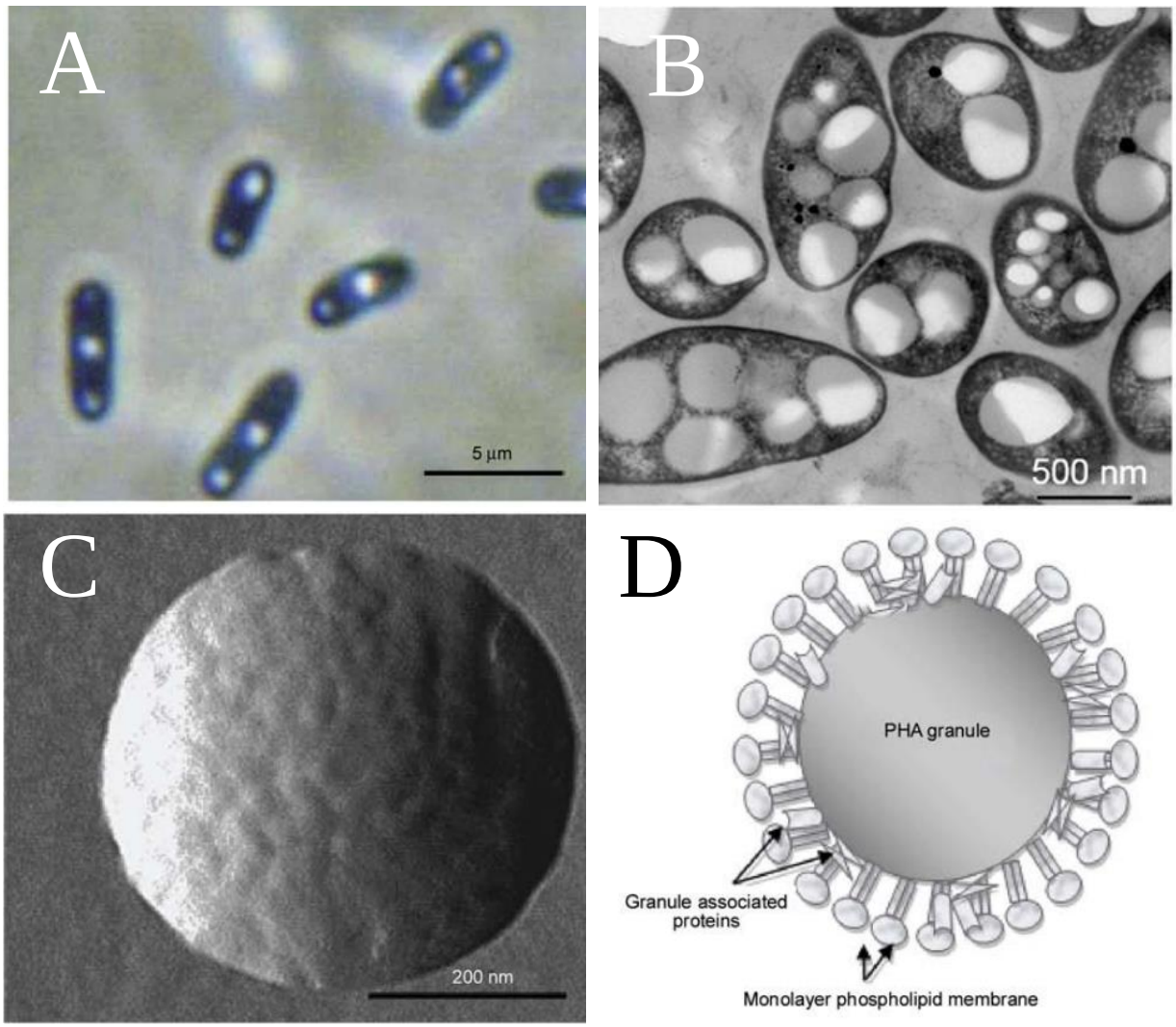


Figure 2-6. Photographs of PHA granules observation in the bacterial cells. They are observed under A. phase contrast, B. transmission electron microscope, and C. atomic force microscope. D. Model of PHA granule.[83,91]

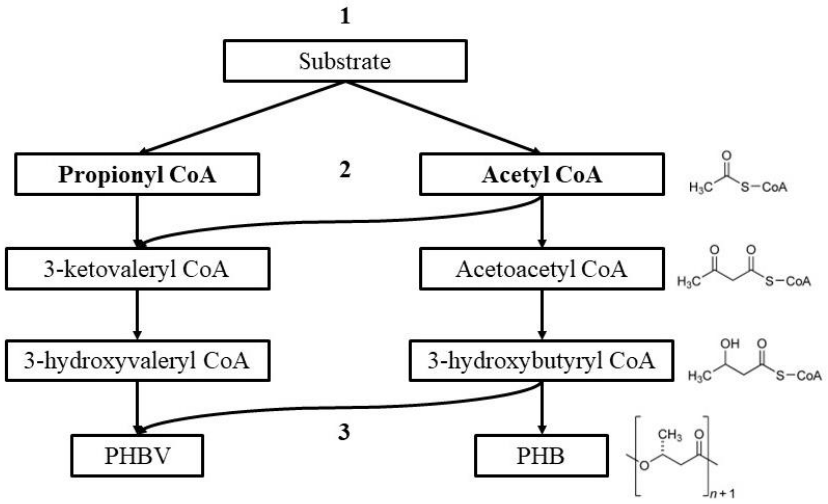


Figure 2-7. The metabolic pathway to produce PHBV. *In bold are the precursors required*[41,92].

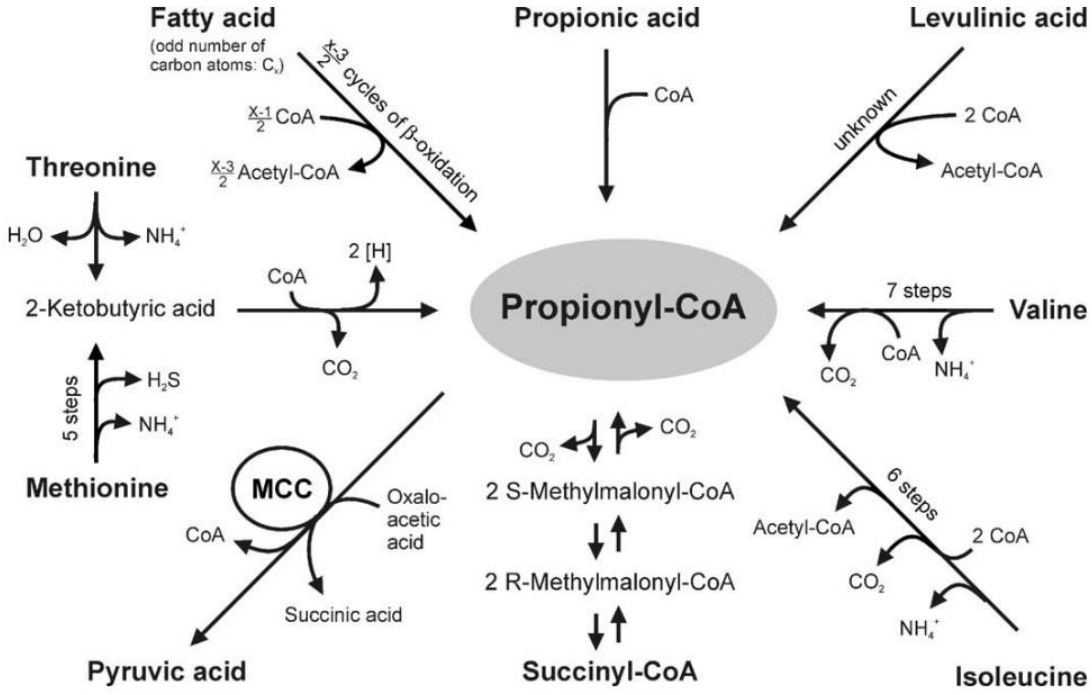


Figure 2-8. A source of propionyl-CoA for the biosynthesis of PHAs containing 3HV as a constituent[41]

The bacteria able to produce PHBV can be both gram-positive and gram-negative. There are different strains that have the potential for PHA production. The strain *Rastonia eutropha*, or *Cupriavidus necator* has the advantage of degrading a wide variety of substrates: sugars, alcohols and volatile fatty acids (VFA)[93] and it is resistant to potential toxic element as mercury[94]. The bacteria *Delftia acidovorans* and *Caldimnia taiwanesnsis* can also produce PHBV[95,96]. The extremophile, halobacterium *Haloferax mediterranei* or some *Pseudomonas* species present the advantage of operating under nonsterile conditions and have high PHBV production[97].

2.2 PHA industrial production methods

2.2.1 PHA producer strains

The application of the bioplastic is the main factor in choosing the type of PHAs to be produced. A strain is selected taken into consideration the substrate it can use to produce the desired type of PHA. Some strains can also be engineered to maximize bioplastic production by shutting off some metabolic pathways. Some examples of PHA producers are reported in Table 2-2. The most known strains are *Pseudomonas* and *Bacillus spp.*

2.2.2 Fermentation methods

The amount of PHAs produced depends on the strain, the substrate selection and especially the bioreactor design and process regime. To increase the production the necessity of upscaling the process is decisive[98,99]. The cylindrical stirred tank reactor (STR) is used in many studies. Figure 2-9 shows some common bioreactor (BR) design for fermentation production of PHA[98].

The batch cultivation

Batch cultivation can be performed with one-stage or two-stage cultivation depending on the strategy adopted (Figure 2-9 A and B). The one-stage one performs a biomass growth and PHA production simultaneously since the medium contains nutrients and others essential compounds. Whereas, the two-stages cultivation, consists in two step cultivation. The first step is focusing on biomass production (induced by the availability of nutrients) and the second on in PHA accumulation inside the cells due to the lack of essential nutrients[98].

Table 2-2. Bacterial strains used to produce PHAs, polymers produced, carbon sources and reference.

Strain	Type PHA	Substrate	Reference
Bacillus spp.	PHB, PHBV, copolymers	Nutrient broth, glucose, alkanoates, e-caprolactone, soy molasses	(Katircioglu et al. 2003; Tajima et al. 2003; Full et al. 2006)
Cupriavidus necator	PHB PHBV (from valerate)	Glucose, sucrose, fructose, octanoate, lactic acid, soybean oil	(Khanna & Srivastava, 2005)
Mixed microbial culture (MMC)	PHBV	Clarification of molasses; anaerobic fermentation of molasses sugar to volatile fatty acids (VFAs) as accessible carbon source.	(Albuquerque et al., 2007)
Pseudomonas aeruginosa	mcl-PHA	Glucose, technical oleic acid, waste free fatty acids, waste free frying oil	(Hoffmann and Rehm 2004; Fernandez et al. 2005)
*Pseudomonas Putida**	mcl-PHA organic aromatic alkanoic acids	Phenyl alkanoic acids Methyl phenyl alkanoic acids	(Mezzina et al., 2021)
Rhodospirillum rubrum	PHBV	Syngas (produced from discarded seed corn)	(Do et al., 2007)
Synechococcus sp. MA19 (Cyanobacterium)	PHB	CO_2, illumination	(Nishioka et al., 2001)

* Engineered modified species

The feed-batch cultivation

Feed-batch cultivation consists in performing a culture under minimum nutrient concentration and the regular addition of substrate or nutrient during the cultivation when the substrate concentration reaches a minimum value[48] (Figure 2-9 D). The fed-batch can be also implemented with a cell-recycle unit to maximize the accumulation of cells in the tank[100] (Figure 2-9 C). There is also a strategy, pH-stat control, where the pH is controlled by addition of substrate[56]. These strategies require the addition of concentrated substates in pulses to avoid significant expansion of the working volume.

The chemostat cultivation

Chemostat cultivation consists in a continuous culture, where the volume is fixed since the fresh medium is continuously added and the used medium extracted the same way. It can be a one-stage, with one tank or a multistage with several tanks (Figure 2-9 E and F). It works in a way that a dilution rate (D), which corresponds to the ratio of flow rate to working volume, is fixed to correspond to the maximum growth rate (μ) to increase biomass concentration[101]. The dilution rate is the key to avoid the wash-out of the strain during the culture.

2.2.3 Extraction method

The PHA extraction method consists of the biomass recovery and the removal of PHA granules out of the cells, and their purification, which is highly dependent on the strain[60]. It starts by the separation of the cells from the medium. It can be performed with centrifugation or filtration. Then the cells will have a pre-treatment to break the cell wall envelope (Figure 2-10). The intracellular PHA can be recovered using **solvent extraction**. The organic solvents could be acetone[102], chloroform, methylene chloride, dichloromethane or dimethyl carbonate (a green solvent). It is based on the fact that PHAs are water insoluble but soluble in a limited number of solvents[60]. However, the large amount of solvent required makes the procedure economically and environmentally unattractive, even though it results in a high purity PHAs[103]. That is why it also exists a **chemical disruption**, corresponding to surfactants as sodium hypochlorite[104] or dodecyl sulphate (SDS)[105] that can disrupt the microbial cells and release their content. The other methods can be **enzymatic cell disruption** with the use of proteases, nucleases, and lysozymes, for example[106]. S**upercritical fluids** are also used by modifying the pressure and temperature. Also, extraction can be performed by taking advantage of the native **cell fragility** or by engineering the productive strain, the extraction can be optimized and become more convenient. The purification method is about how the PHA needs to be purified depending on its final application. The treatment can use hydrogen peroxide, but requires high temperatures[107]. It exists also an ozone treatment[108] or the use of chloroform[109].

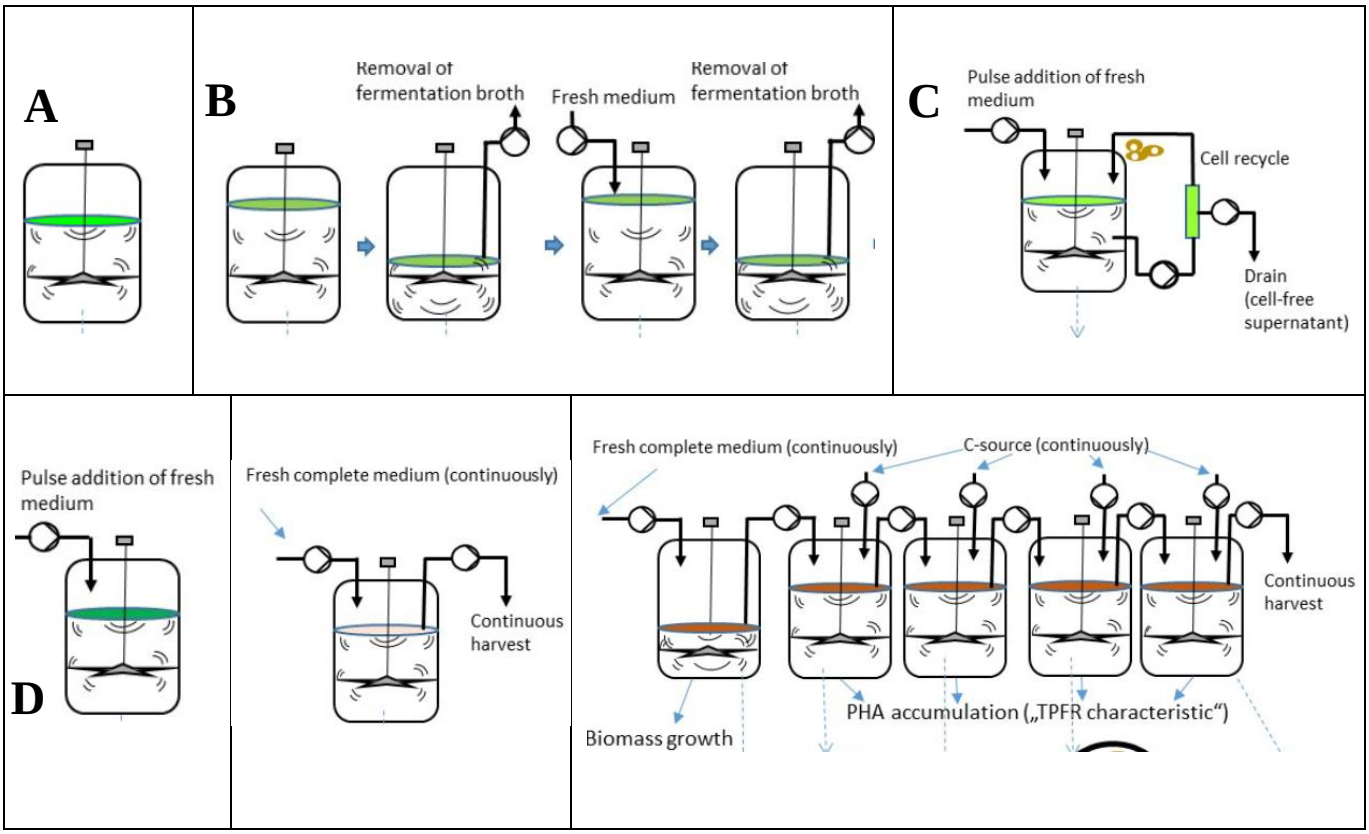


Figure 2-9 Schematics fermentation production methods for PHA production. A Batch culture. **B** Repeated Batch. **C** fed-batch + cell recycle. **D** Fed-Batch. **E** One stage Chemostat. **F** Multi-stage Chemostat[98].

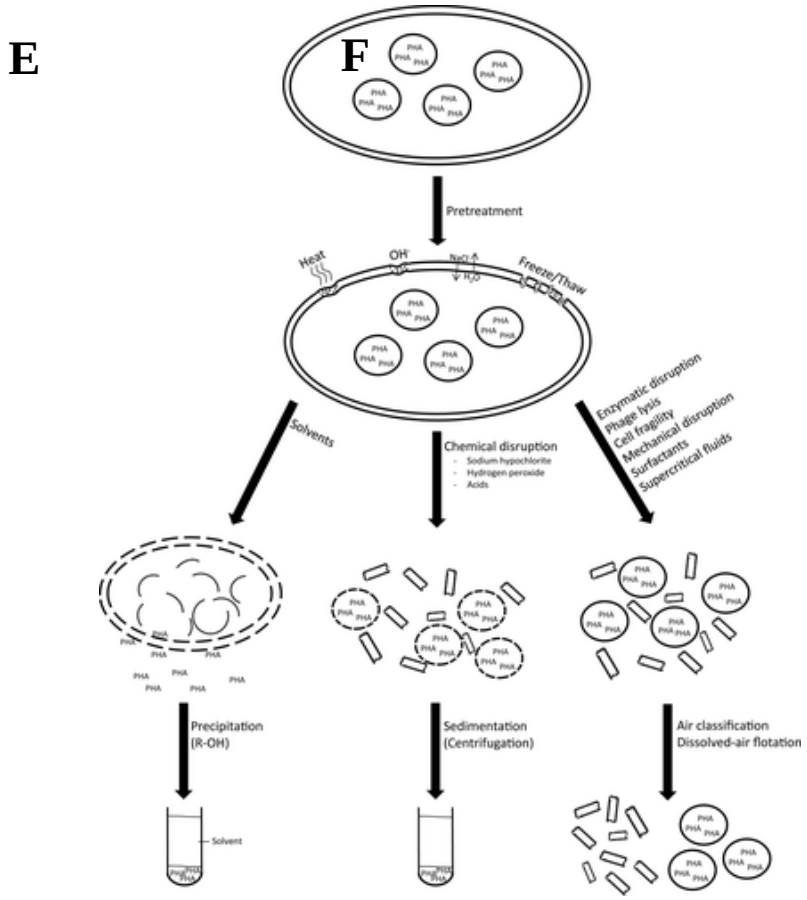


Figure 2-10 Principal methods for PHA recovery form bacteria cells[60]

2.3 The PHA production in the master thesis project

The bioplastics reveal to be a huge opportunity to produce sustainable plastics. Their bioproduction is more and more studied to enable companies and start-ups to develop the technology and its production. This is where my master thesis project stands, in collaboration with the start-up Dionymer, in Bordeaux, France. I worked for four months to assist the chief technology officer to establish and improve the fermentation strategy. The company is producing PHBV from organic waste. They first imply a pre-fermentation treatment to the initial substrate and secondly use it for PHA production.

My research focuses on setting up the scale-up process from 400 mL flask cultivation to 2 L bioreactor and solve the associated foaming problems. I first characterized the culture parameters differences between flask and BR. Then, I tested three parameters in BR to correspond as closely as the ones in flask, to optimize and increase PHA ad biomass production: three aeration strategies, two agitation blades and two aeration spargers. Additionally, I implemented the monitoring fermentation. I used as microorganisms an uncharacterized bacterial sp. My substrate was pure VFA from petrochemical based to create a simple and controlled medium. The equipment used belongs to Bordeaux INP, ENSTBB. My work was part of a development of a new technology for future uses.

3. Materials and Methods

3.1 Working cell bank

The strain was obtained from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures. The lyophilised cells were reactivated following the guidelines of the manufacturer. The working cell bank (WCB) of 75 cryotubes (13/07/2022) was stored at -80°C. To prepare new WCB cryotubes, 1 mL of the cell culture was first thawed, then injected to a preculture medium of 400 mL, and incubated in a shaking incubator at 37°C, 140 rpm for 66 h (Infors Multitron HT, Bottmingen, Switzerland). The liquid culture medium consisted in 1000 mL of distilled water: yeast extract, 1.0 g; glucose, 10.0 g or two volatile fatty acids, 9.58 g and 1.06 g respectively for substrate; Na_2HPO_4, 1.31 g; NaH_2PO_4, 0,71 g; $ZnSO_4 \times 7\ H_2O$, 0.10 g; $MnCl_2 \times 4\ H_2O$, 0.03 g; H_3BO_3, 0.30 g; $CoCl_2 \times 6\ H_2O$, 0.20 g; $CuCl_2 \times 2\ H_2O$, 0.01 g; $NiCl_2 \times 6\ H_2O$, 0.02 g; $Na_2MoO_4 \times 2\ H_2O$, 0.03 g; pH to 7.0 - 7.2. In the late exponential growth phase, monitored by an optical density OD_{600} (Safas Uvmc2, Safas, Monaco, Monaco*)* value of 8, 400 mL of an aqueous glycerol solution (40 % w/v) was added to the culture. Then, 75 cryotubes with 1 mL of 20 % glycerol and an OD_{600} of 4 were stored at -80°C.

3.2 Precultures

Precultures kinetics

To enhance the procedure and to establish the preculture kinetics, the experiments were performed at 37 and 42°C and with two different inoculation ratios. An amount of 4 flasks of 500 mL with a working volume of 100 mL were inoculated with an inoculation ratio of 1:100 each, corresponding to 1 mL of cryotube from the WCB. Then, 2 flasks are incubated at 37°C, 140 rpm and the 2 other flasks at 42°C, 140 rpm in a shaking incubator. To test the inoculation ratio of 1:400, corresponding of 0.25 mL of cryotube from the WCB for 100 mL of culture, 2 flasks were inoculated and incubated at 37°C, 140 rpm. The flasks were sampled twice a day for OD measurement (3.5.1). The average OD data were calculated for each temperature. The growth rate for each temperature was calculated with the method reported in 3.5.1. The two precultures at 37°C with ratio of 1:100 was used for inoculation after 80h of culture in a BR experiment.

Precultures to inoculate the bioreactor

Precultures were obtained by adding 1 mL cryotube from the WCB to 400 mL of the previously described culture medium with glucose. It corresponds to an inoculation ratio of 1:400; 1 stands for the cryotube volume and 400 for the medium volume (Fig. 3-1). The culture was incubated for 66 h, over a weekend, at 37 °C, 140 rpm in a shaking incubator. The $OD_{600\ (0)}$ was then measured to reach approximately OD_{600} = 8. At that time the microorganism was at the end of its exponential growth and was used to inoculate the cultures. The volume needed (V_0) for the preculture was calculated to reach $OD_{600\ (1)}$ = 1.6 in the bioreactor of volume V_1. I used the following formula: $V_0 = OD_{600(1)} * V_1 / OD_{600\ (0)}$. The volume V_0 was then centrifuged at 15 000 × g for 10 min at 20 °C (Centrifuge 5804R, Rotor FA-45-6-30 TL026, Eppendorf, Hamburg, Germany). The supernatant was discarded, the pellet was washed with the same volume of culture medium and inoculated in the 2 L culture.

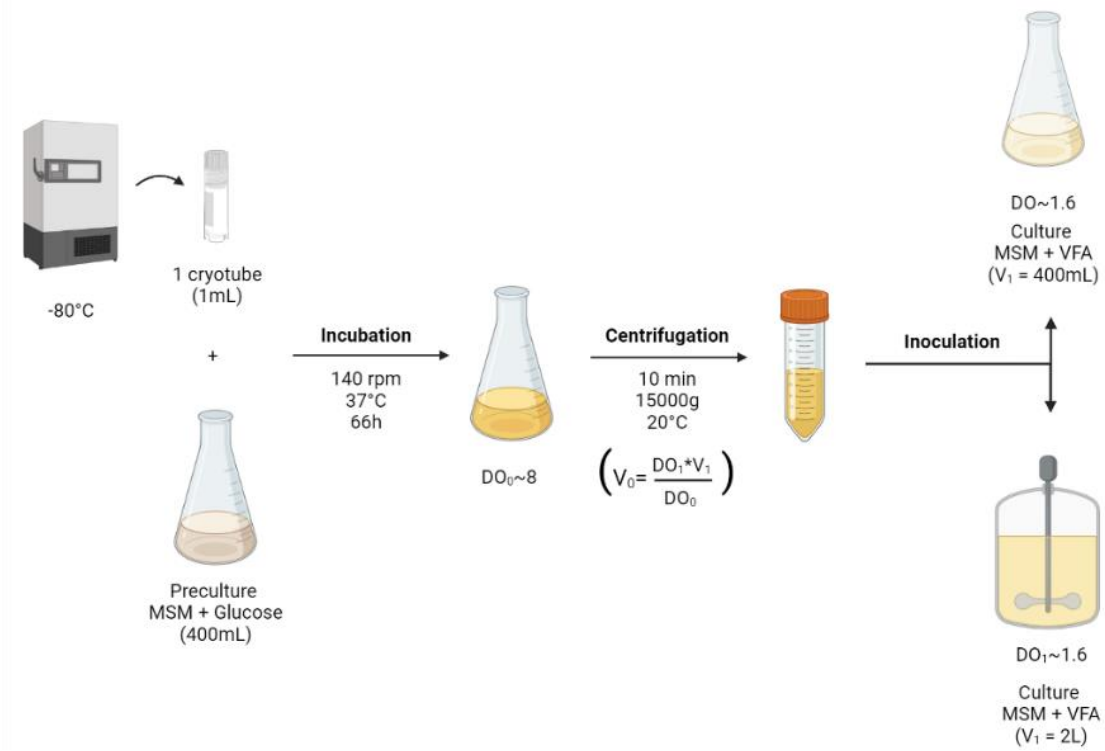


Figure 3-1 Schematic representation for the procedure to inoculate the production flask or bioreactor from the working cell bank.

3.3 Culture production in flask and kinetics

The preculture pellet required for inoculation, calculated as described in chapter 3.2 to achieve an OD_{600} of 1.6, was separated by centrifugation, washed with an equal volume of culture medium, and inoculated into 400 mL of culture medium (Figure 3-1). A number of 4 cultures of 400 mL were performed in 2 L flask with baffles. The culture medium was the same as previously described, with VFA. The cultures were incubated at 140 rpm at 37 °C for 90 h. The pH was adjusted manually twice a day (at 9 am and 6 pm) from Monday until Friday by adding a drop of medium on pH paper (from 5.5 to 8.0) and drops of 37 % HCl solution were added to the medium to reach a pH of 6.8. All samples from the 4 flasks were collected at the same culture time and before pH adjustment and their average OD was then calculated. The growth rate of the average data was obtained with the method described in 3.5.1.

3.4 Culture production in bioreactor

3.4.1 Bioreactor culture setup and equipment

The preculture was inoculated into the bioreactor to reach an OD_{600} of 1.6 using the same procedure as shown in chapter 3.3 for flasks, i.e. the separated preculture pellet was washed and then added in 2 L of culture medium. Its composition was the same as that used in flask culture production without Na_2HPO_4 and NaH_2PO_4 buffer. The cultivation was carried out in a 2 L bioreactor with a double-jacketed tun with a digit central unit (DCU), benchtop (bioreactor Biostat® B, Sartorius, Göttingen, Germany) (Figure 3-2). The schema on Figure 3-2 shows the probes and the setup of the BR.

The pH fixed at 7.25 was measured with a pH electrode (InPro3250i, Mettler Toledo, Colombus, USA) and adjusted with hydrochlorid acid (HCl 2M) and sodium hydroxide (NaOH 2M) solutions, directly monitored with the DCU. Temperature was fixed at 37°C and measured with a probe (Onigrad TR88, Endress & Hauser, Reinach, Switzerland) and adjusted and maintained with the double jacket. Turbidity was measured with the probe BioPat Fundalux 12mm 2L OPL 10mm, PN BPF2L10, SN 82921, Sartorius, Göttingen, Germany. Antifoam used was Antifoam A (Sigma-Aldrich, St. Louis, MS, USA) and then J673 (Struktol®, Böblingen, Germany). A volume of 200µL was manually added every 10 hours, from 4 h of culture as drops when the foam started to be visually important.

The pO_2 measured with a pO_2 probe (Visiferm DO Arc 120, Hamilton, Bonaduz, Switzerland) was automatically adjusted automatically from the DCU by air flow rate (vvm) or agitation (rpm). The probe was first calibrated at 0% of oxygen with an electrolyte gel (zeroing oxygen gel, Mettler Toledo, Colombus, USA) and the 100% aeration was performed with emerging the probe in distilled water with an aeration pipe in the water. Two strategies of medium aeration were used: agitation then aeration cascade or aeration then agitation cascade. To regulate the pO_2, the first parameter increased and when it reached its upper bound, the second parameter increased. Stirring was performed by Rushton and baffle or only marine blades (Figure 3-3 and Figure 3-4). Aeration was performed with an air flow into a circular sparger and a micro sparger in one of the experiments (Figure 3-4). Once the pH, temperature, agitation, and aeration were adjusted, the bioreactor was inoculated. One BR was performed every week. Samples were collected twice a day and OD was measured. The VFA composition was done at the start and end of the culture. The cultures were stopped after 90h.

3.4.2 Foaming bioreactor culture

To perform a first scale-up of the flask culture, the bioreactor was inoculated with preculture described in 3.2 in the culture medium described in 3.4.1. The aeration of the medium was setup that the pO_2 had to stay over 20%. The cascade strategy was first agitation then aeration by the DCU. The Rushton blades were used for carrying the agitation and the speed was between 200 and 350 rpm. The aeration was made with 1 vvm through a circular sparger. The BR was performed for 70h. The equipment used did not have an antifoam probe preventing its automatic addition but was performed manually. In this first experiment antifoam A from Sigma-Aldrich was used. A second experiment with the same parameters was performed and the antifoam was exchanged with J673 from Struktol.

3.4.3 Flask and bioreactor comparisons, pO_2, agitation and aeration test setups

Once the fermentation and production in BR were not inhibited by the foam production and overflow, the culture monitoring revealed several differences between flask and BR cultures of 400 mL and 2 L respectively. In fact, the final quantity of PHA produced in BR was lower and the biomass extraction was more challenging than in flask. Then, the objectives were in two steps: to characterize the biological properties between the BR and flask and to modify the physical parameters to improve the biological performances in the BR. Then all the biological characteristics of each are first listed after the end of the culture: final OD, time to reach OD_{max}, final VFA concentration, cells extraction, PHA visuals, final PHA concentration, purity of PHA and weight % HB - weight % HV. Then, an overview of the physical parameters in flask and BR was established containing: pO_2 strategy, agitation

blade, aeration sparger, k_La, Antifoam, medium composition, and pH adjustment. Three parameters out of them were tested: pO_2, agitation blade and aeration sparger (Table 3-1). For each experiment, a comparison between BR and flask cultures was performed, the OD curve was first used to compare cases. In the context of productivity, if the OD was too small at the end of the culture, further analysis was not performed.

To determine the differences between pO_2, agitation and aeration strategies, two BR were performed and used as **reference** (Table 3-1). The pO_2 parameter, pO_2<20%, consisted in forcing it to reach 20% at the start of the culture by stopping the aeration and maintaining it under 20%. The aeration was performed with circular sparger and pulses from 0 to 1.5 vvm. The agitation was set between 200 and 500 rpm and performed with Rushton blades. For the **pO_2 strategies** comparison, the same agitation and aeration parameters as described above are used; pO_2>20%, <20% and <10%. And the condition pO_2>20%, consisted in its natural decrease at the beginning of the culture, due to microbial respiration) and maintaining it above 20%. And the condition pO_2<10% was the same as pO_2<20% but with 10%. For the **agitation blade** comparison, a BR with the same parameters as the reference was carried out except that the Rushton blade was exchanged with a marine blade without baffles (Table 3-1). Concerning the aeration comparison, a BR with the same parameters as the reference was performed and the circular sparger was exchanged with a micro sparger (Table 3-1).

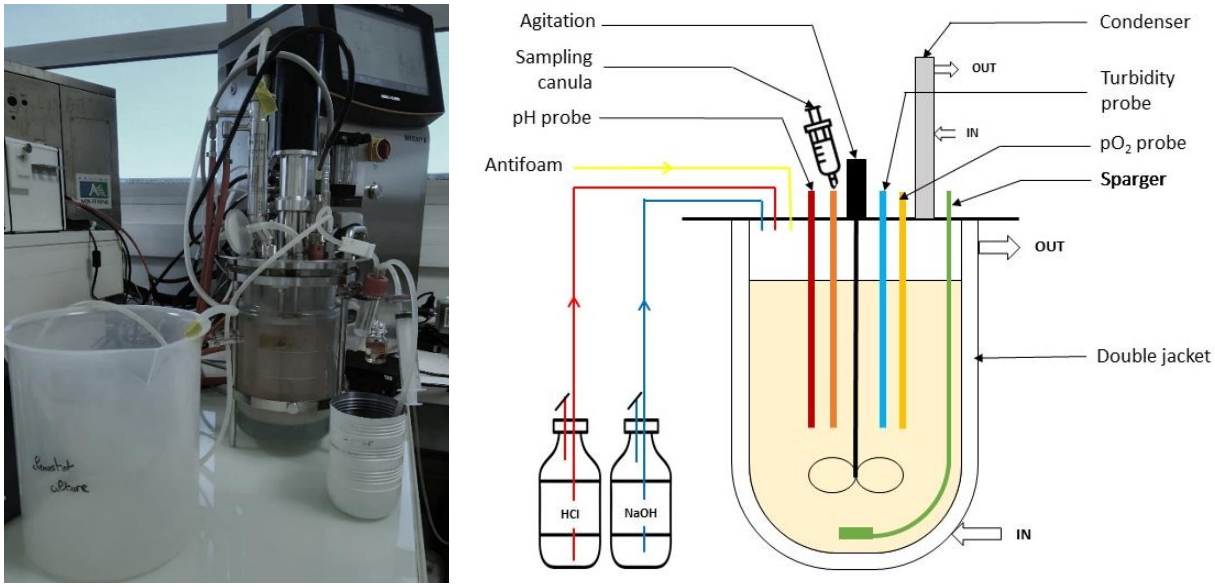


Figure 3-2. Bioreactor setup to produce PHA from VFA in the experiment

Table 3-1. Aeration, agitation, and aeration strategies tested for bioreactor experiments.

Bioreactor's experience name	pO_2	Aeration strategy	Stirring blades	Aeration sparger	Antifoam
Antifoam	pO_2>20%	Agitation>Aeration	Rushton	Circular	**Antifoam A**
REFERENCE	**pO_2<20%**	Aeration>agitation	**Rushton**	**Circular**	*Struktol*® J673
pO2>20%	**pO_2>20%**	Aeration>agitation	Rushton	Circular	*Struktol*® J673
pO2<10%	**pO_2<10%**	Aeration>agitation	Rushton	Circular	*Struktol*® J673
Marine blades	pO_2<20%	Aeration>agitation	**Marine**	Circular	*Struktol*® J673
Micro sparger	pO_2<20%	Aeration>agitation	Rushton	**Micro**	*Struktol*® J673
Flask	-	-	-	-	-

In bold are the compared parameters.

Figure 3-3. Photography of blades used in the experiments in bioreactor. Rushton blades with baffles (up) and marine blades (down)

Figure 3-4. Photography of aeration sparger used in the experiments. Micro sparger (left) and circular sparger (right)

3.5 Analytical techniques

3.5.1 Optical density, growth rate, and substrate concentration

The optical density was measured with a UV-visible spectrophotometer (Safas Uvmc2, Safas, Monaco, Monaco) at 600 nm. An uninoculated solution of the medium was used for the blank. The growth rate (μ in h^{-1}) was calculated from the OD results. First, the ln (OD) was computed and plotted against culture time (h). Then a linear regression was calculated from ln (OD) between the time corresponding to growth, the given slope corresponds to the growth rate when $R^2>0.97$. The sample of 1 mL was first centrifuged for 25 min at 14 800 × g. The supernatant was then filtered with a 0.2μm polytetrafluoroethylene (PTFE) or nylon filter. The sample was then analysed on a high-performance liquid chromatography (HPLC) column Agilent, Hiplex H (Agilent technologies, Santa Clara, USA). The analysis was done at 60 °C at a flow rate of 0.2 mL of H_2O containing 0.005 M of H_2SO_4. The calibration curve was performed by Dionymer (Pessac, France).

3.5.2 PHA extraction and analysis

The extraction started by separating the solid biomass from the liquid with three successive centrifugations of 10 min at 20 °C and 15 000 × g (Sorvall Lynx 6000 centrifuge; Rotor: F9-6x1000 LEX PN: 096-061075, ThermoScientific, Waltham, USA) (Figure 3-5). After cell lysis, the pellet was resuspended in 0.1 % SDS at a ratio of 1:2 (SDS: volume initially extracted) and stirred for an hour, followed by the same centrifugation. The pellet contained water insoluble PHA, which was resuspended in distilled water at a ratio of 1:2 and stirred for 30 min. It was then separated by the same centrifugation method and washed with pure ethanol in a ratio of 1:2 and stirred for 30 min at 900 rpm (Atlantic Labo, Lbx instruments OS20series, Bruges, France). After centrifugation, the pellet was dried in an oven at 68°C over the weekend.

To quantify the purity and composition of PHBV in motives HB and HV, an acidic methanolysis was performed (Figure 3-6). Approximately 8 mg of the PHBV sample was added to 1 mL of chloroform ($CHCl_3$ of GC quality), 1 mL of standard solution (methyl benzoate at 2.3 g/L in chloroform) and 1 mL of acidic methanol (H_2SO_4 at 3% in methanol) in a tightly sealed tube. For the internal standard, the PHBV used was CAS 3976-69-0 (SigmaAldrich, Melbourne, Victoria, Australia). The tubes were heated for 4 h at 100°C in a thermostat-equipped oil bath under chemical hood. Then, the samples were cooled in an ice bath for 30 min, and 500 µL of NaCl 1 M aqueous solution was added in each tube and softly hand agitated for 30 seconds. After mixing, 30 minutes of settling time was allowed to achieve phase separation. Approximately 1 mL of the organic phase (chloroform phase at the bottom) was extracted with a syringe and placed in a vial for gas chromatography (GC). Pure chloroform was used as a blank control. The chloroform phase was analysed with ThermoScientific Trace 1300 Gas Chromatograph. The chromatograph was operated with a SGE BPX70 column (30 m length x 0.025 mm I.D. x 0.25 m film) and H_2 as the carrier gas (1.5 mL/min). A flame ionisation detection (FID) unit was operated at 300°C with an injection port temperature of 250°C. The oven temperature was set to 80°C for 1 min, increased at 10°C/min to 120°C, and then o 270°C at 45°C/min and held for 3 min[110].

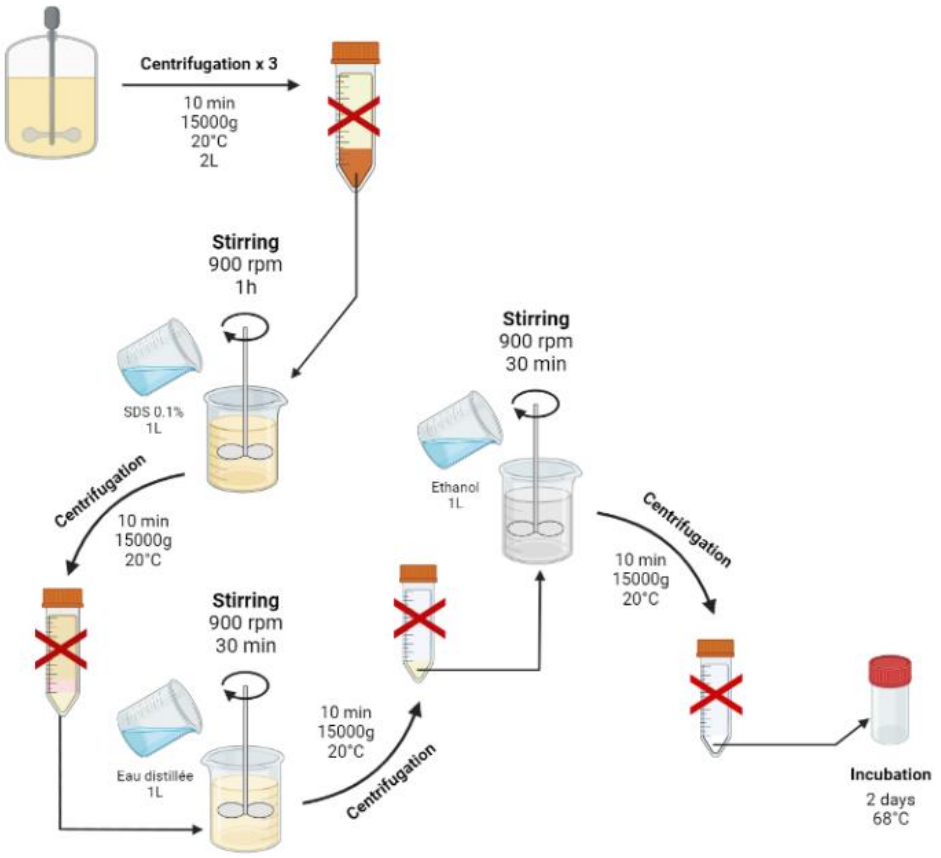


Figure 3-5. Scheme of the PHA extraction method at Dionymer. The red crosses represent the supernatant that is discarded.

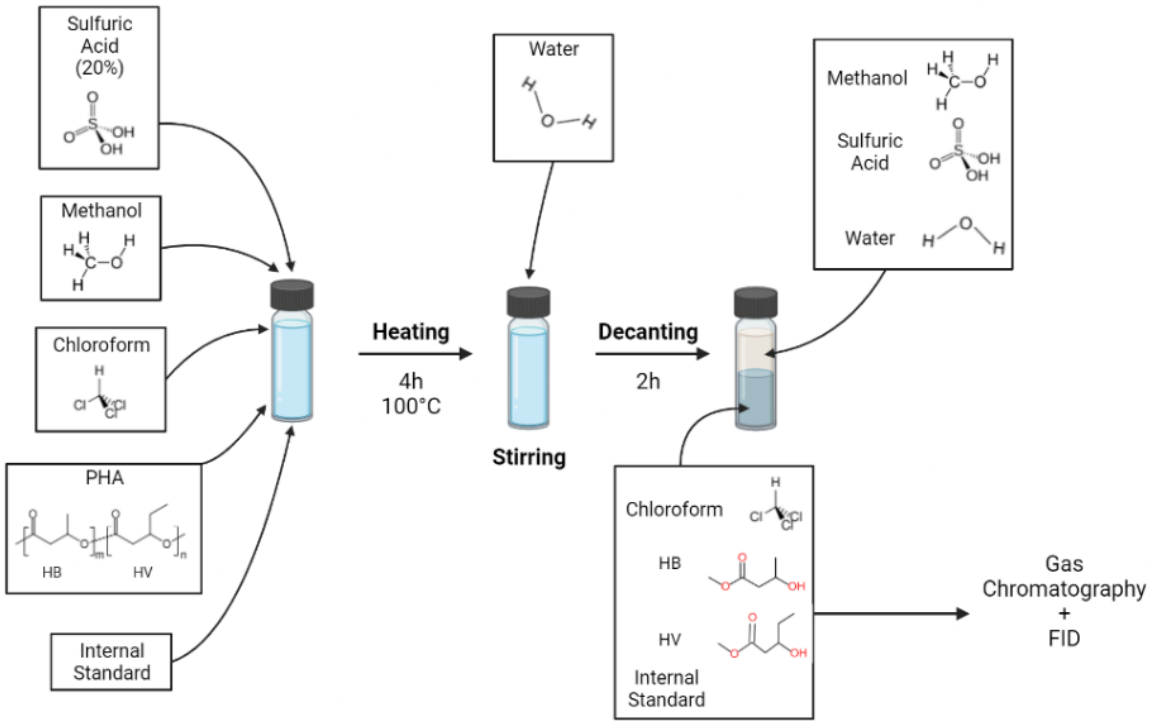


Figure 3-6. Scheme of the methanolysis to estimate the purity and the composition of PHBV.

3.5.3 K_La measurement

Probe acquisition time determination

The acquisition of the pO_2 probe was started in a deoxygenized medium (performed by nitrogen gas addition), where the dissolved oxygen was first calculated to be zero ($pO_{2\ min}$ at t_{min}). Then, the probe was directly and quickly immersed in a 100 % oxygenized medium and its dissolved oxygen was calculated ($pO_{2\ max}$) from the equation $pO_{2\ 63\%} = 0.63*(pO_{2\ max} - pO_{2\ min})$ to find the corresponding time ($t_{pO2\ 63\%}$). Finally, t (63%) = $t_{pO2\ 63\%} - t_{min}$) was determined.

K_La measurement

The oxygen transfer coefficient K_La measurement for the flask was performed to emulate the incubator 's conditions. A 2 L flask containing 400 mL of the culture medium described above was placed in the incubator at 37°C for 5 h. The flask was then placed on a platform shaker with a temperature and a pO_2 probes and a pure nitrogen gas pipe in the medium. The platform was shaken at 140 rpm, and pure nitrogen was injected into the medium to reach 0 % pO_2. Then, the pure nitrogen gas pipe was removed from the medium. Once the bioreactor was set-up for the culture; probes and culture medium in, the K_La was determined. Pure nitrogen was first injected into the medium with vigorous stirring. When the medium reached 0 % of dissolved oxygen, the stirring speed and air aeration rate were set (Table 4-4). Then, the nitrogen pipe was changed with the air pipe. Rushton blades and circular sparger were used for this experiment. The acquisition was started in both cases when the dissolved oxygen in the medium was about 0 % and the nitrogen pipe was removed from the medium and exchanged with the air pipe. When the pO_2 measurement reached 100 %, the acquisition was stopped and the C* and C_0 were obtained (Figure 4-8 A and B). The function $\ln\left(\frac{C^*-C}{C^*-C_0}\right) = f(t)$ was plotted (Figure 4-8 C). Then a linear regression was performed on aligned data. Since, $f(t) = -K_L at + b$, the K_La was determined (Figure 4-8 D). Then, the K_La value was adjusted to 20°C: $Kla_{20°C} = \frac{Kla_{T°\ exp}}{1.024^{(T-20)}}$

3.5.4 Correlation method

Correlation tests were performed to check if the turbidity and acid addition can be used as a proxy for the optical density. The turbidity data were collected with the previously presented probe. The acid addition corresponded to the volume quantity in mL of hydrochloride acid (2 M) to adjust the pH to 7.25. The ODs were collected through the method describe above.

The linear regressions were performed with the Microsoft® Excel®, Microsoft 365 MSO (Version 2208 Build 16.0.15601.20526) 64 bits, Microsoft, Redmond, USA under the regression data analysis.

3.5.5 Statistics

One-way ANOVA was performed using Anova: single factor from Microsoft® Excel® (Microsoft 365 MSO Version 2208 Build 16.0.15601.20526 64 bits, Microsoft, Redmond, USA). The data presented for the flask culture corresponds to the average of four experiment in the case of growth curves and growth rate; and biological duplicate were performed for the reference BR and each preculture (37°C, 42°C and 1:400 ratio). Statistical significance was determined by *p*-value at $p < 0.05$.

4. Results and Discussion

4.1 Precultures kinetics

The preculture kinetics revealed that for an inoculation ratio of 1:100, the growth rate at 42°C was faster than at 37°C; 0.09 h^{-1} and 0.07 h^{-1} respectively. Additionally, the incubation time to reach an OD of 8, was reduced at 42°C in comparison to 37°C; 43 h and 65 h, respectively (Figure 4-1 A and B). At 37°C, changing the inoculation ratio from 1:100 to 1:400 increased the incubation time to obtain OD 8 from 65 h to 80 h. This allowed to perform precultures on weekend, save cryotubes from WCB and to monitor BR and flask fermentation cultures on weekdays.

4.2 Optimization of batch bioreactor

4.2.1 Foaming issue production

The first BRs fermentation revealed a massive foam production during cultivation, which inhibited future development of the technology. The first antifoam agent used was Antifoam A from Sigma-Aldrich under specific conditions listed in Table 3-1. The foaming seemed to be linked to the overflow that was coming from the massive addition of HCl and NaOH used for pH regulation (light blue and purple curves in Figure 4-2). In fact, when the foam was forming, the pH probe was misreading the pH-induced additions of the two substances to control it, that can be seen with lots of ups and downs on the dark green curve. Then, the foam formation started around **45 h** of culture. Additionally, it seemed to be linked to the increase of the stirr (brown curve) and the end of the exponential growth (black curve). The use of the antifoam agent Struktol J673A appeared to inhibit the foam formation and was then used for the rest of the experiments in BR. The antifoam program finally adopted was the sequential addition of 122 mg of pure Struktol J673A successively at 45, 55, 65 and 75 h of culture.

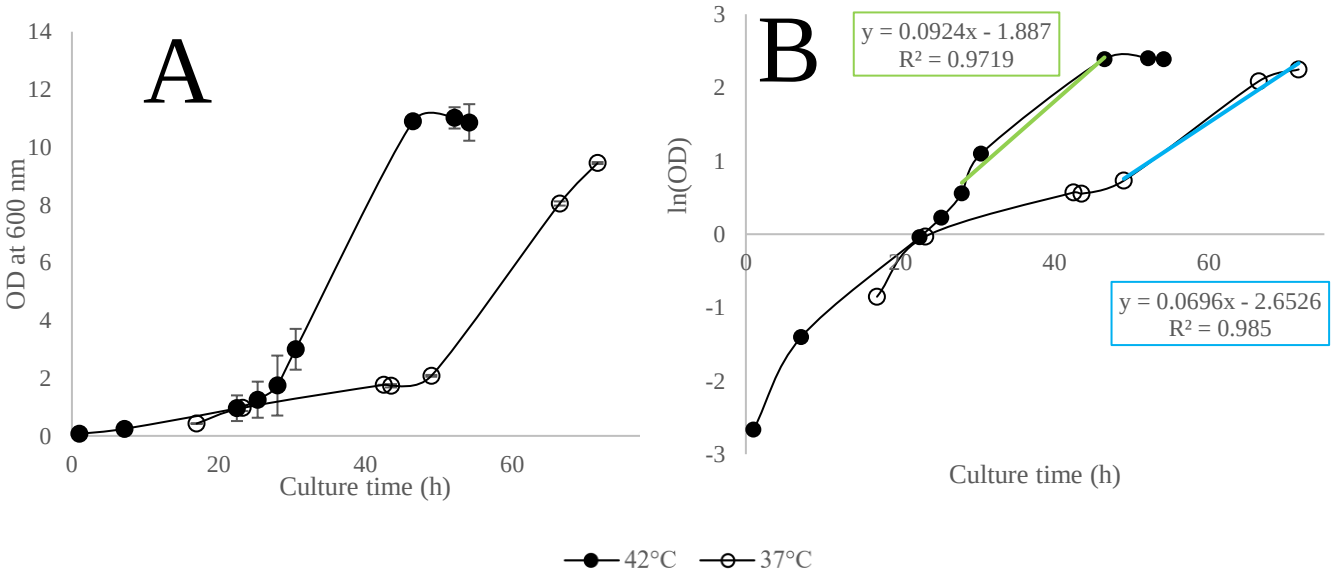


Figure 4-1. Determination of growth rate in preculture flask at 42°C and 37°C. A. Average optical density plotted against culture time for 37°C and 42°C experiments. B. Determination of growth rate for each experiment. The standard deviation of the two 42°C and 37°C experiments are plotted on A. $p < 0.05$.

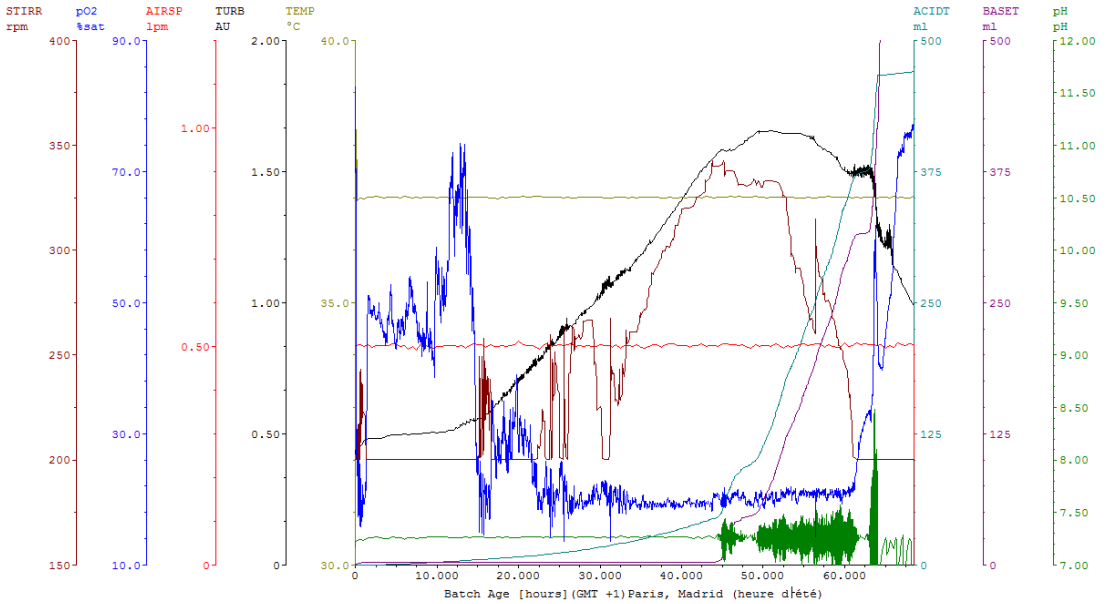


Figure 4-2. Culture monitoring for the experiment that had a massive foam production inducing an overflow.

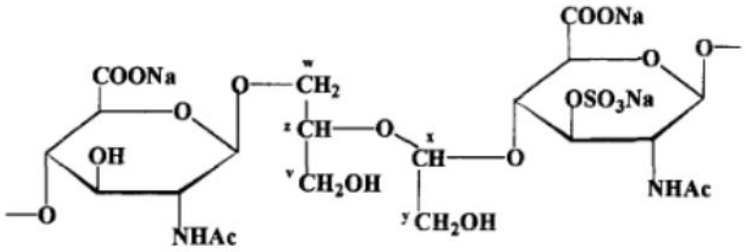


Figure 4-3. Molecular structure of the EPS produced by the strain

The foam formation could be induced by a stress, the bacterum strain was producing exopolysaccharides (EPS) instead of PHA, inducing a medium viscosity increase, which lowered the oxygen transfer. To compensate it the stirring was then increasing to keep the pO_2 at 20%. However, the cells were too stressed by the culture conditions due to agitation or aeration, and the cells lysed and released DNA. The bacterium strain could be producing EPS, since the conditions were already harsher in BR than in flask. According to the literature, the EPSs produced by the strain are responsible for the foam and the strain produces it under stress. The structure of the EPS is presented in Figure 4-3. This molecule has a pseudoplastic behaviour and strong viscosity[111–113]. The presence of -OSO_3Na groups can be related to the important presence of sulphate in the culture medium that enhances the production pathway to EPS.

4.3 Characterisation of the differences between flask and bioreactor cultures

4.3.1 Biological characteristics in bioreactor and flask

The flask cultures revealed a reproducibility with a maximum OD that reached 12 in about 75h (Figure 4-4 A) and a growth rate about $0.0348h^{-1}$ for flask (Figure 4-4 B). However, concerning the bioreactor of reference (Table 3-1), the OD reaches on average a maximum of 8 and a growth rate of $0.0266h^{-1}$. The growth rate between flask and BR is still very small but it seems that the culture growth is slower in BR than in flask. The growth rate

from each experience can be found in Table 4-1. The Table 4-2 summarizes the biological characteristics for BR and flask cultures. In BR, the growth rate was lower than in the flask, the substrate was not completely consumed at the end of the culture, and the final product seemed to contain two products: the white PHA and a residue. In addition, the final PHA concentration in BR was lower than in the flask. However, the PHA purity and the culture time were similar in both cases.

4.3.2 Testing of the physical parameters to enhance the biological characteristics

The main differences between flask and BR cultures lied in the control of the dissolved oxygen in the medium, or pO_2, related to the agitation and aeration techniques (Table 4-3). In the flask, the agitation was performed in an incubator with baffled flask on a stirring plate, whereas in BR, the pO_2 was controlled through air injection and agitation blades. Additionally, no antifoaming agent was added, the medium contained a buffer, and the pH was manually adjusted twice a day in the flask. Three parameters: pO_2, agitation blade and aeration sparger; were tested and a summary of their results is presented in Table 4-1.

Table 4-1. Summary of the experiments and data obtained at the end of the experiment.

Final data	Culture time (h)	OD	Growth rate μ (h^{-1})	PHA (g/L)	Purity (%)	%HB	%HV	VFA A (g/L)	VFA B (g/L)
REFERENCE (pO2<20%-Rushton-circular psarger)	95	8.0	0.0266	0.607	**>95**	**91**	**9**	0.18	0
pO2>20%	77	6.7	0.0326	-	-	-	-	3.93	0
pO2<10%	91	9.3	0.0224	-	-	-	-	3.1	0
Marine blades	93	9.0	0.0324	1.044	*>95*	*90*	*10*	0.3	0
Micro sparger	93	10.7	0.0316	-	-	-	-	0.1	0
Flask	90	11	0.0348	1.65	>95	92	8	0.0	0.1

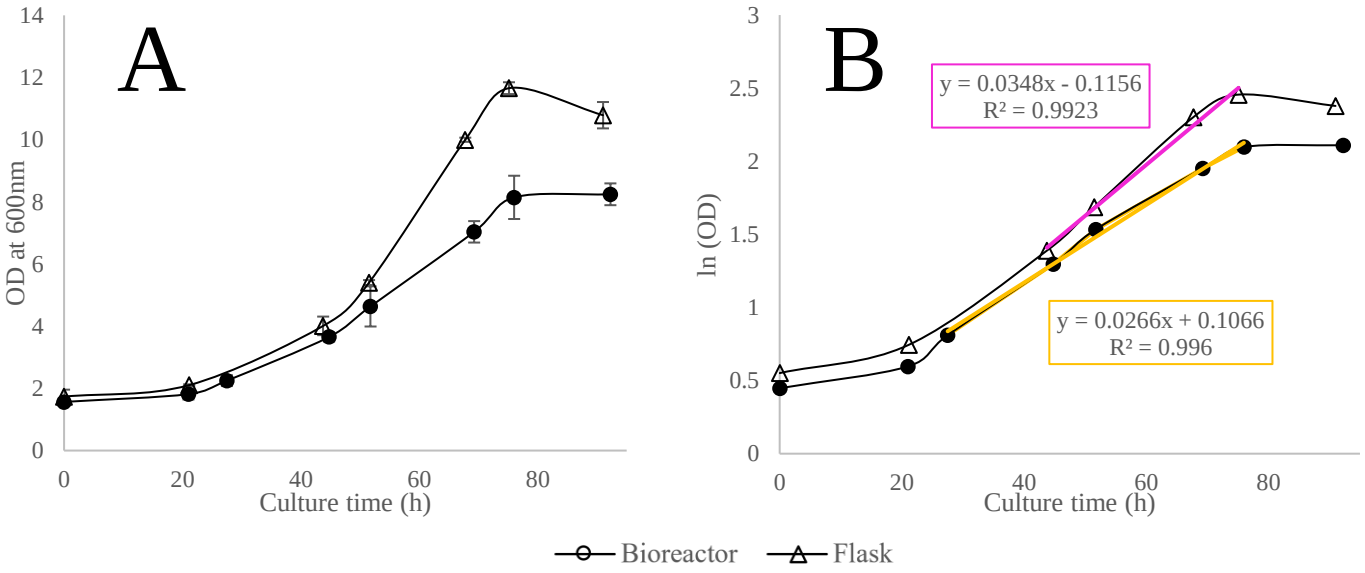


Figure 4-4. Determination of the growth rate in a flask and in bioreactor. A. Optical density over time of the reference flask and reference bioreactor. The flask culture corresponds to the average data of the four experiments, with a statistical significance $p<0.05$. The reference bioreactor culture corresponds to the average data of two experiments, with a statistical significance $p<0.05$. B. Growth rate of reference flask culture and reference bioreactor culture. The growth rate is determined with average data. The flaks experiment was compared to the reference BR to evaluate statistical significance $p<0.1$

Table 4-2. Summary of average values of the biological characteristics in BR and flask cultures.

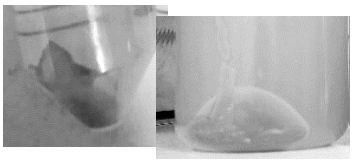
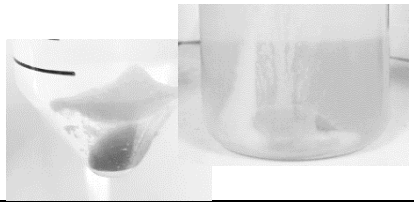
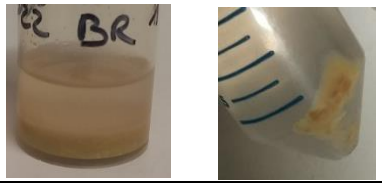
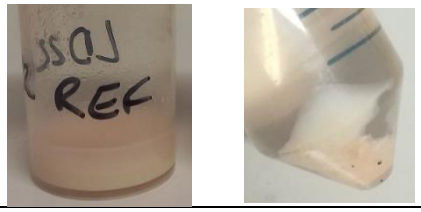
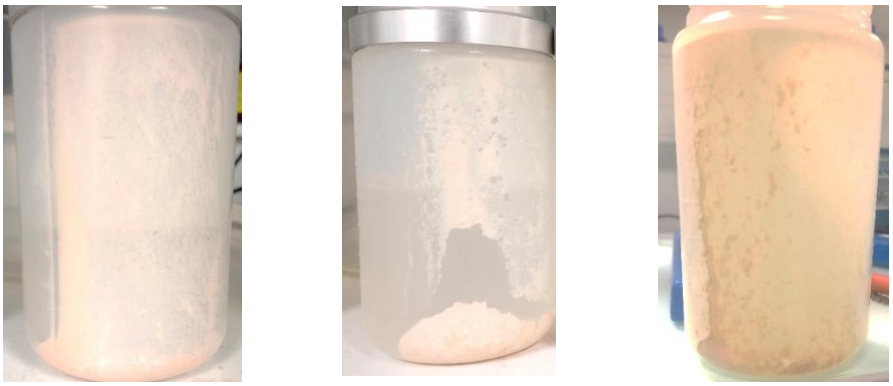
Parameters	Bioreactor	Flask
Final OD	~8	~12
Time (h) to reach OD_{max}	75	75
Growth rate (h^{-1})	0.0266	0.0348
Final VFA concentration (g/L)	2-4	~0
Cells extraction from supernatant	Two populations; cells and a yellowish deposit. The pellet is harder to obtain. 	One population, the cell pellet 
PHA	Two populations, one white and a yellow residuals 	One population, white PHA 
Final PHA concentration (g/L)	0.8	1.7
PHA purity after extraction	>95%	>95%
%wt HB - %wt HV	91% - 9%	92% - 8%

Table 4-3. Summary of the physical parameters in both BR and flask

Parameters	BR	Flask
pO_2 (Stirring-aeration pair)	Controlled by: Cascade: • Agitation>Aeration • Aeration>Agitation	Not controlled • Stirring of 140 rpm on a stirring platform • Ambient aeration
Agitation	Rushton blades and baffle	Baffled flask on a stirring plate at 140 rpm
Aeration	Flux through circular sparger	None
k_La	Dependant of the stirring-aeration pair	Fixed K_La (20°C) = 9.98h^{-1}
Antifoam	5 additions of 122mg each	No addition
Medium composition	(**Erreur ! Source du renvoi introuvable.**)	Same as BR plus addition of buffer
pH adjustment	Automatic by addition of HCl 2M and NaOH 2M	Manually adjusted to 6.8-7.1, twice a day 2 times a day with HCl 37%



Flask Marine blades Rushton blades

Figure 4-5. PHA visuals after extraction for comparing the impact of stirring blades.

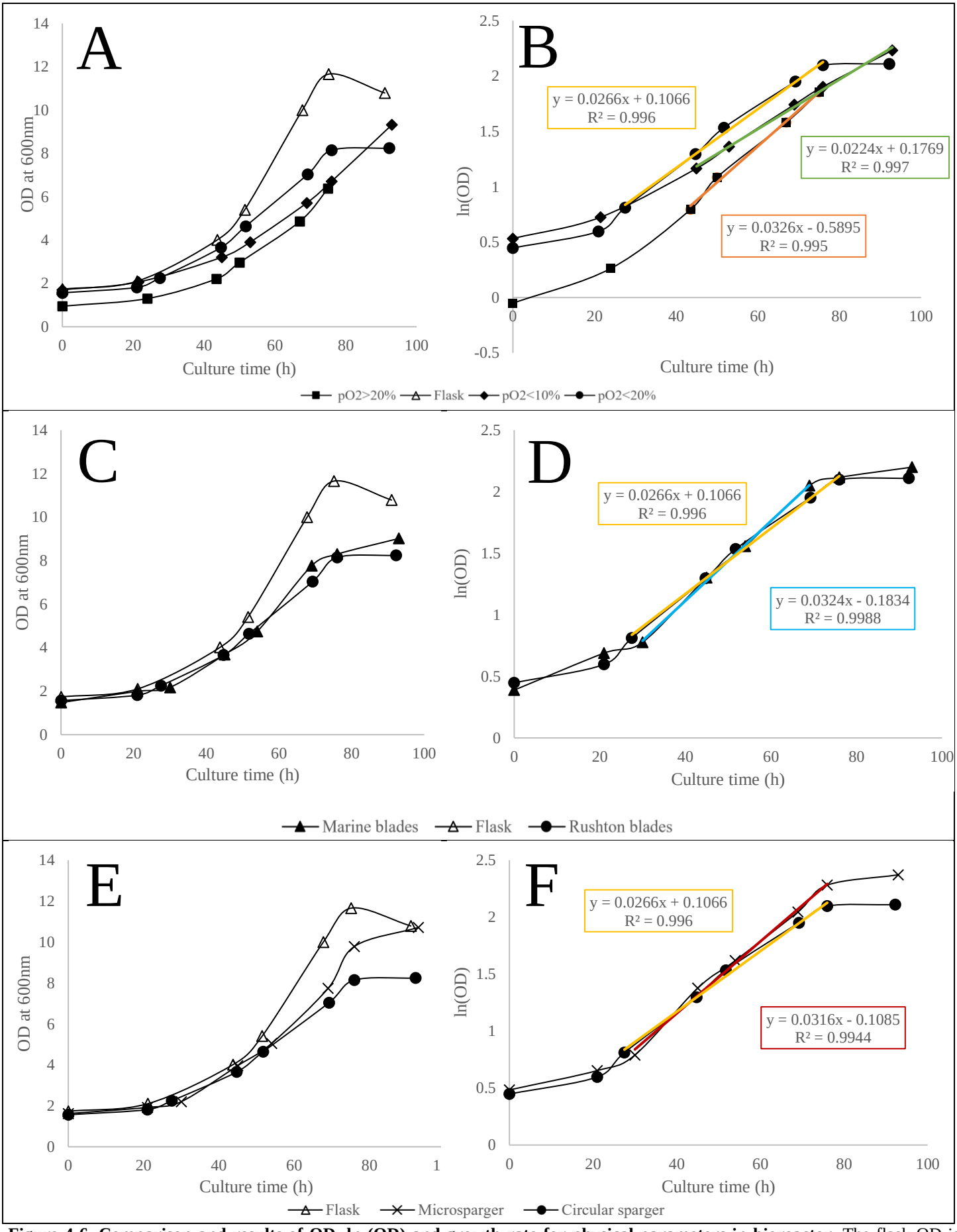


Figure 4-6. Comparison and results of OD, ln (OD) and growth rate for physical parameters in bioreactor. The flask OD is added as a standard. A & B. Results of three dissolved oxygen (pO_2) strategies. C & D. Results of agitation blades strategies. E & F. Results of aeration sparger equipment. The bioreactor (yellow) is used as a reference. The parameters of each experiment are listed in Table 3-1 and the results in Table 4-1. Each experimental sample was compared to the control (reference BR) to evaluate statistical significance $p < 0.15$.

pO_2 and stirring-aeration pair

The experiment was first performed to verify if too high oxygen level was responsible for stressing the cells. The parameters used are listed in Table 3-1. The experiment seems to reveal that unforced pO_2 (>20%) had a growth rate higher ($\mu=0.0326h^{-1}$) than forced pO_2 (<20% and <10%, $\mu=0.0224h^{-1}$ and $\mu=0.0266h^{-1}$ respectively), suggesting that the aeration pulses were inducing stress to the strain (Figure 4-6 B). However, the pO_2>20% experiment starts with an OD two times lower than the other experiments. The pO_2>20% provided a constant aeration to the culture medium and seems to be an important parameter to keep reducing the stressed on cells and for further BR productions.

Agitation blades

The stirring experiment consisted in comparing the marine blades with Rushton blades and baffles (Figure 3-3. Photography of blades used in the experiments in bioreactor. Rushton blades with baffles (up) and marine blades (down) and Table 3-1). The comparison of OD between the use of marine blade to Rushton blades (Figure 4-6 C and D) did not reveal a major change since they both reach an OD of 8.5 after 80h of culture. Even if the growth rate was higher for the marine blades ($\mu=0.0324h^{-1}$) than for the Rushton blades ($\mu=0.0266h^{-1}$), their behaviour seemed to be similar. This difference was too small to conclude on a significative faster growth. However, after the extraction, there was a significant change (Figure 4-5). The PHA obtained with marine blades after extraction was whiter and without a yellowish pigmentation compared to the one obtained with Rushton blades or reference BR. It seemed that the marine blades were inducing less shear stress to the cells and can be a parameter to keep for further productions.

Aeration spargers

The aeration experiment consisted of comparing the use of a circular and micro sparger with Rushton blades (Figure 3-4; Figure 4-6 E & F respectively and Table 3-1). The growth rate was faster with the micro sparger with $\mu=0.0316\ h^{-1}$ than with the circular one, $\mu=0.0266\ h^{-1}$. It appeared that the maximum OD reached 7.7 after 69 h of culture for the micro sparger and only to 7 for the circular one. In two experiments, pO_2 was maintained <20 % with a cascade regulating first with aeration and then with stirring. In Figure 4-7, the regulation required a higher air flux for the circular sparger, going to 1 vvm after 40 hours of culture. The micro sparger was very sensitive to the antifoam addition. Indeed, the sudden rise in aeration (from 0.1 to 1.5 vvm) and stirring (from 200 to 500 rpm) was linked to the antifoam addition for the micro sparger. There were no visible changes for the other experiment. Before the addition, the micro sparger did not need to modify the agitation or aeration to regulate the pO_2 suggesting that the dissolved oxygen was sufficient. Then, the micro sparger seemed to improve the aeration of the medium but was very impacted by antifoam additions.

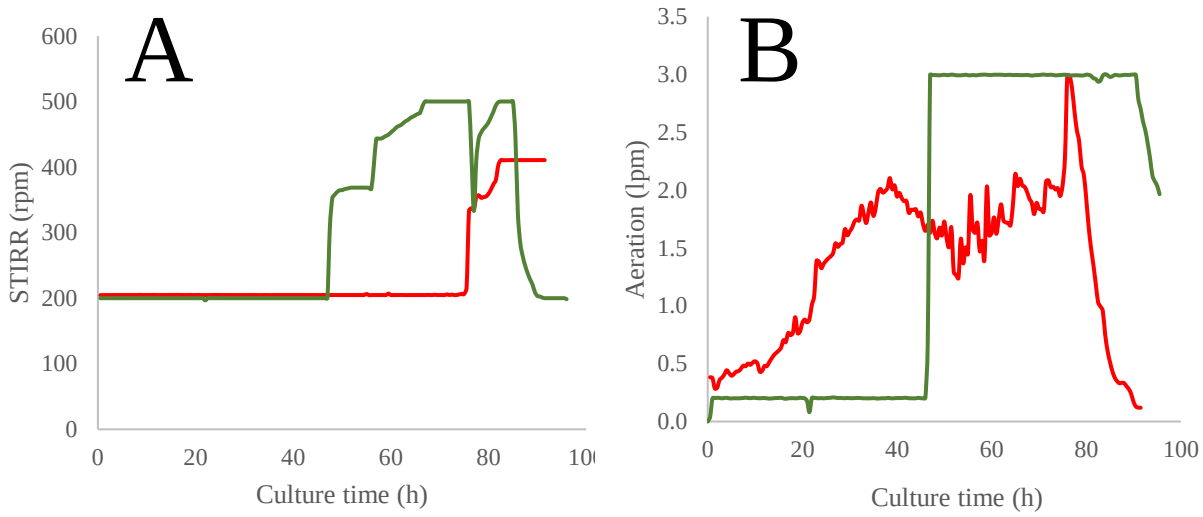


Figure 4-7. Stirring and Aeration behaviour during the experiment of aeration impact. A. Stirring behaviour. B. Aeration behaviour. Micro sparger in green and circular sparger in red.

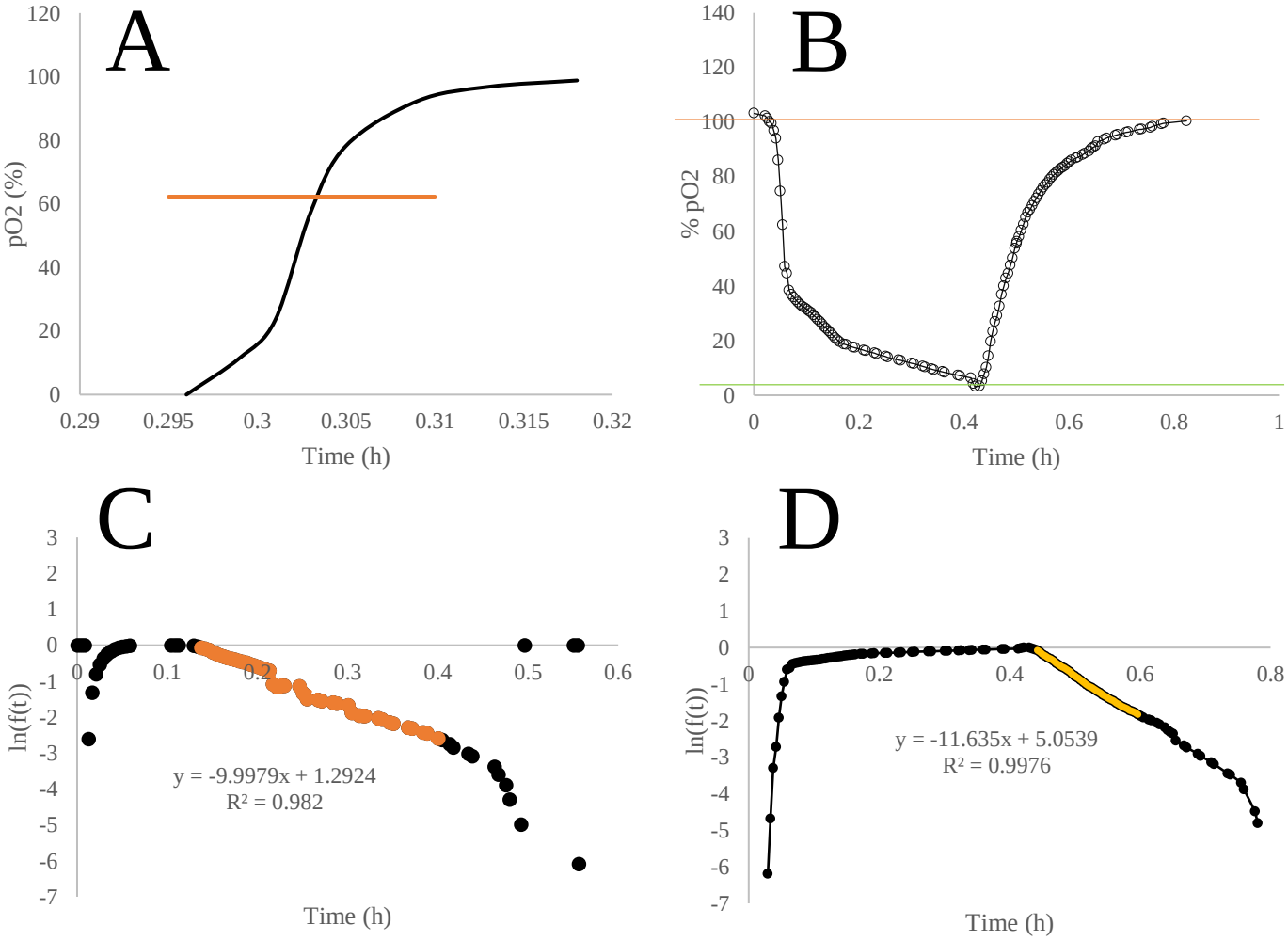


Figure 4-8. Results of the k_La measurement. A. Determination of the probe acquisition time. pO_2 as a function of time (h). The orange line corresponds to pO_2 63%. B. pO_2 measurement for k_La determination in flask. The orange line corresponds to the pO_2 max (C*) and the green line to the pO_2 min (C_0). C. K_La determination for flask culture. D. K_La determination for BR culture with 100rpm and 1lpm.

Oxygen transfer coefficient (k_La)

First, the acquisition time of the probe was calculated to estimate if the probe was fast enough. The time to reach 63 % of the maximum pO_2 corresponded to t(63 %) = 11.52s, which is 1/t (63 %) = 125 h^{-1} (Figure 4-8 A). All calculated k_La values must be below this value, otherwise the experiments were related to the acquisition time of the probe and not to the oxygen transfer. The oxygen transfer coefficient corresponds to the ability of a medium to transfer oxygen from the air bubbles to the microorganism. The higher the value, the more oxygen is available for the strain. The k_La measured in the flask was about 11.64 h^{-1} at 26.5°C which gave a k_La of 9.98 h^{-1} at 20°C (Figure 4-8 D). After several experiments in BR with Rushton blades and baffles, with the same culture medium (containing phosphate buffer), the closest k_La value was found with the following stirring and aeration parameters: 100 rpm and 1vvm with a k_La of 6.68 h^{-1} at 20°C (Figure 4-8 C and Table 4-4).

4.4 Correlation results

The linear regression between turbidity and OD revealed a positive correlation (Figure 4-9 A), with R^2=0.9504, suggesting that using the medium turbidity as a proxy for OD is possible. The linear regression between the acid addition and OD also showed a positive correlation (Figure 4-9 B), with a R^2=0.8327. It seemed that the acid addition can also be used as a proxy for OD measurement. As the strain was consuming the VFAs, the pH of the medium rose and required an acidic adjustment to maintain the pH at 7.25. Additionally, it was assumed that the OD can be used as a proxy for the growth, since in 2017, Mahler et al. (2017) have found the following formula linking the biomass (g/L) to the OD at 600 nm:

$$\text{biomass (g/L)} = 0.7863 \cdot OD_{600} + 0.1562$$

Table 4-4. Results of the kla experiments in flask and bioreactors

	Agitation (rpm)	Aeration (vvm)	K_La at 37°C (h^{-1})	K_La at 20°C (h^{-1})
Flask	**140**	**-**	**11.64***	**9.98**
BR 1	100	0.25	6.4387	4.3
BR 2	**100**	**1**	**9.9979**	**6.68**
BR 3	100	1.5	22.914	15.31
BR 4	200	0.25	11.218	7.5
BR 5	200	0.5	18.3	12.23
BR 6	200	1	41.8	27.9
BR 7	500	1	94.312	63.02

*The value was measured at 26.5°C due to the cool down of the flask. BR stands for bioreactor.

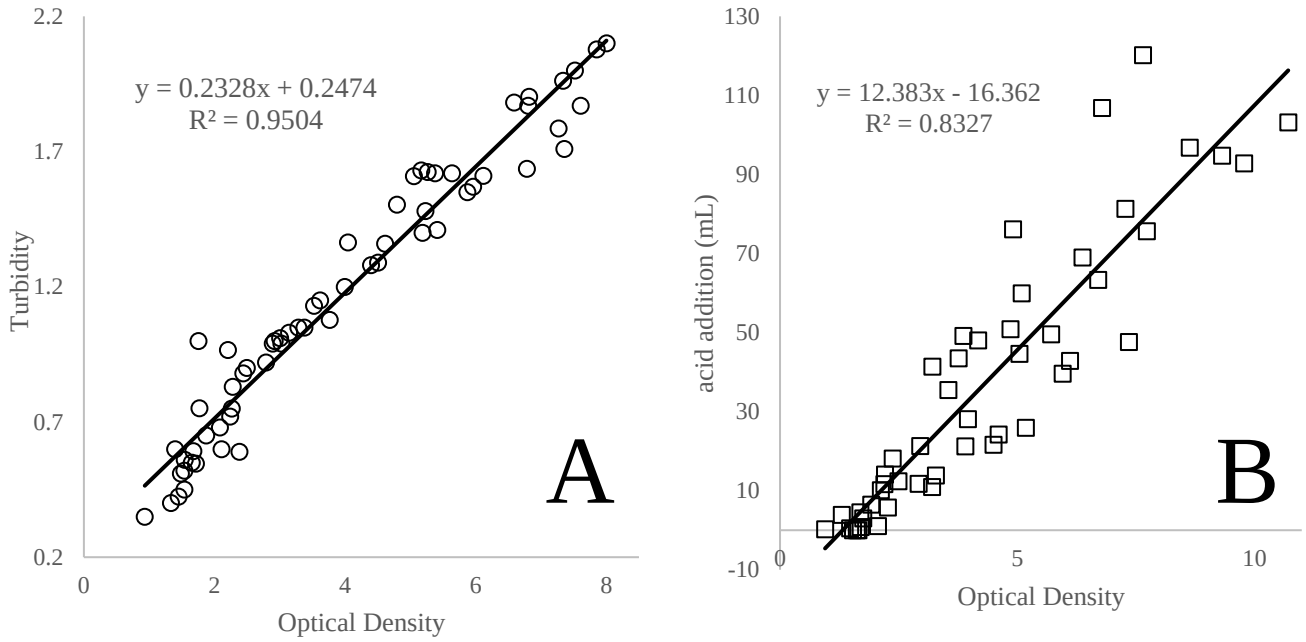


Figure 4-9. Correlation results for turbidity and acid addition with the optical density at 600 nm in bioreactor. A. Correlation of turbidity and optical density. B. Correlation between acid addition and optical density.

Conclusion

The PHBV production by fermentation was finally scaled up from a 400 mL flask culture to a 2 L bioreactor production. Several parameters still need to be optimized to stabilize and enhance PHBV and biomass productivity and production yield. From the experiments, very interesting and promising results were obtained. The precultures could be performed over the weekend at 37°C to be able to reach the end of their exponential phase. In bioreactor, the use of a new antifoam agent Strucktol appeared to be efficient in reducing foaming formation. This helped to conduct new experiment in bioreactor. This foam seemed to be induced by too much shear stress on the microorganisms and was directly linked to aeration, agitation, and aeration strategies. The biological parameters differences between flask and BR cultures were a higher OD and growth rate for flask culture. The first physical parameters tested was the aeration of the medium strategy, revealed the pO_2 needs to be higher to 20% and it may be linked with the reduction of stress induced to the cells. The use of marine blades increased the OD and reduced the medium viscosity in bioreactor. the impact of the micro sparger is not yet determined even though it tends to be very sensitive to antifoam agent that reduces the aeration of the medium. The k_La method was implemented during this work to better understand the aeration and aeration/agitation pair. Finally, hydrochloride acid addition to the medium and the turbidity of the medium can be used as a proxy for the optical density. More parameters can be tested such as the combination of the micro sparger and marine blades to see how the microorganism reacts. Also, other fermentation strategy can be tested as the feast and famine strategy and see its impact on the productivity. Overall, the research was promising and the PHBV produced seemed to be an interesting alternative to fossil fuel-based plastics.

References

(1) United Nations. *Global Sustainable Development Report 2019: The Future Is Now – Science for Achieving Sustainable Development*; 2019. https://sustainabledevelopment.un.org/content/documents/24797GSDR_report_2019.pdf.

(2) United Nations Industrial Development Organization (UNIDO). *Addressing the Challenge of Marine Plastic Litter Using Circular Economy Methods: Relevant Considerations*; 2019. https://www.unido.org/sites/default/files/files/2019-06/UNIDO_Addressing_the_challenge_of_Marine_Plastic_Litter_Using_Circular_Economy.pdf

(3) World Health Organization (WHO). *Microplastics in Drinking-Water*; 2019. https://apps.who.int/iris/bitstream/handle/10665/326499/9789241516198-eng.pdf?ua=1.

(4) European Commission. *A European Strategy for Plastics in a Circular Economy*; 2018. http://www.europarc.org/wp-content/uploads/2018/01/Eu-plastics-strategy-brochure.pdf.

(5) Plastics Europe. Plastics — the Facts 2020: An Analysis of European Plastics Production, Demand and Waste Data, 2020. https://plasticseurope.org/wp-content/uploads/2021/09/Plastics_the_facts-WEB-2020_versionJun21_final.pdf.

(6) Jambeck, J. R.; Geyer, R.; Wilcox, C.; Siegler, T. R.; Perryman, M.; Andrady, A.; Narayan, R.; Law, K. L. Plastic Waste Inputs from Land into the Ocean. *Science* **2015**, *347* (6223), 768–771. https://doi.org/10.1126/science.1260352.

(7) Rosenboom, J.-G.; Langer, R.; Traverso, G. Bioplastics for a Circular Economy. *Nat Rev Mater* **2022**, *7* (2), 117–137. https://doi.org/10.1038/s41578-021-00407-8.

(8) Barnes, D. K. A.; Galgani, F.; Thompson, R. C.; Barlaz, M. Accumulation and Fragmentation of Plastic Debris in Global Environments. *Philosophical Transactions of the Royal Society B: Biological Sciences* **2009**, *364* (1526), 1985–1998. https://doi.org/10.1098/rstb.2008.0205.

(9) Bergmann, M.; Collard, F.; Fabres, J.; Gabrielsen, G. W.; Provencher, J. F.; Rochman, C. M.; van Sebille, E.; Tekman, M. B. Plastic Pollution in the Arctic. *Nat Rev Earth Environ* **2022**, *3* (5), 323–337. https://doi.org/10.1038/s43017-022-00279-8.

(10) Cózar, A.; Martí, E.; Duarte, C. M.; García-de-Lomas, J.; van Sebille, E.; Ballatore, T. J.; Eguíluz, V. M.; González-Gordillo, J. I.; Pedrotti, M. L.; Echevarría, F.; Troublè, R.; Irigoien, X. The Arctic Ocean as a Dead End for Floating Plastics in the North Atlantic Branch of the Thermohaline Circulation. *Science Advances* **2017**, *3* (4), e1600582. https://doi.org/10.1126/sciadv.1600582.

(11) Collard, F.; Ask, A. Plastic Ingestion by Arctic Fauna: A Review. *Science of The Total Environment* **2021**, *786*, 147462. https://doi.org/10.1016/j.scitotenv.2021.147462.

(12) Obbard, R. W.; Sadri, S.; Wong, Y. Q.; Khitun, A. A.; Baker, I.; Thompson, R. C. Global Warming Releases Microplastic Legacy Frozen in Arctic Sea Ice. *Earth's Future* **2014**, *2* (6), 315–320. https://doi.org/10.1002/2014EF000240.

(13) Peeken, I.; Primpke, S.; Beyer, B.; Gütermann, J.; Katlein, C.; Krumpen, T.; Bergmann, M.; Hehemann, L.; Gerdts, G. Arctic Sea Ice Is an Important Temporal Sink and Means of Transport for Microplastic. *Nat Commun* **2018**, *9* (1), 1505. https://doi.org/10.1038/s41467-018-03825-5.

(14) Halsband, C.; Herzke, D. Plastic Litter in the European Arctic: What Do We Know? *Emerging Contaminants* **2019**, *5*, 308–318. https://doi.org/10.1016/j.emcon.2019.11.001.

(15) Protection of the Arctic Marine Environment (PAME). *Desktop Study on Marine Litter*; 2019. http://hdl.handle.net/11374/2389 (accessed 2022-10-14).

(16) *Plastics in the Arctic*. Arctic Council. https://www.arctic-council.org/explore/topics/ocean/plastics/ (accessed 2022-10-26).

(17) Kline, K. L.; Msangi, S.; Dale, V. H.; Woods, J.; Souza, G. M.; Osseweijer, P.; Clancy, J. S.; Hilbert, J. A.; Johnson, F. X.; McDonnell, P. C.; Mugera, H. K. Reconciling Food Security and Bioenergy: Priorities for Action. *GCB Bioenergy* **2017**, *9* (3), 557–576. https://doi.org/10.1111/gcbb.12366.

(18) Abdul Khalil, H. P. S.; Saurabh, C. K.; Tye, Y. Y.; Lai, T. K.; Easa, A. M.; Rosamah, E.; Fazita, M. R. N.; Syakir, M. I.; Adnan, A. S.; Fizree, H. M.; Aprilia, N. A. S.; Banerjee, A. Seaweed Based Sustainable Films and Composites for Food and Pharmaceutical Applications: A Review. *Renewable and Sustainable Energy Reviews* **2017**, *77*, 353–362. https://doi.org/10.1016/j.rser.2017.04.025.

(19) Anna, F. G. Bioprocess Development for the Robust Production of Polyhydroxyalkanoates, University of Manchester, 2018. https://www.research.manchester.ac.uk/portal/files/83387705/FULL_TEXT.PDF.

(20) Bhattacharyya, A.; Pramanik, A.; Maji, S. K.; Haldar, S.; Mukhopadhyay, U. K.; Mukherjee, J. Utilization of Vinasse for Production of Poly-3-(Hydroxybutyrate-Co-Hydroxyvalerate) by Haloferax Mediterranei. *AMB Expr* **2012**, *2* (1), 34. https://doi.org/10.1186/2191-0855-2-34.

(21) Koller, M. Biodegradable and Biocompatible Polyhydroxy-Alkanoates (PHA): Auspicious Microbial Macromolecules for Pharmaceutical and Therapeutic Applications. *Molecules* **2018**, *23* (2), 362. https://doi.org/10.3390/molecules23020362.

(22) Lagarón, J.-M. 1 - Multifunctional and Nanoreinforced Polymers for Food Packaging. In *Multifunctional and Nanoreinforced Polymers for Food Packaging*; Lagarón, J.-M., Ed.; Woodhead Publishing, 2011; pp 1–28. https://doi.org/10.1533/9780857092786.1.
(23) Utsunomia, C.; Ren, Q.; Zinn, M. Poly(4-Hydroxybutyrate): Current State and Perspectives. *Frontiers in Bioengineering and Biotechnology* **2020**, *8*.
(24) Tokiwa, Y.; Calabia, B.; Ugwu, C.; Aiba, S. Biodegradability of Plastics. *IJMS* **2009**, *10* (9), 3722–3742. https://doi.org/10.3390/ijms10093722.
(25) Ellen MacArthur Foundation. *The New Plastics Economy: Rethinking the Future of Plastics*; 2016; p 68. https://ellenmacarthurfoundation.org/the-new-plastics-economy-rethinking-the-future-of-plastics (accessed 2022-10-13).
(26) *Recycle codes – Enkay Industrial Supplies Inc.* https://enkay.ca/recycle-codes/ (accessed 2023-03-09).
(27) OECD. Global Plastics Outlook - Plastics Use in 2019. *OECD Environment Statistics (database)*. https://doi.org/10.1787/efff24eb-en.
(28) Evode, N.; Qamar, S. A.; Bilal, M.; Barceló, D.; Iqbal, H. M. N. Plastic Waste and Its Management Strategies for Environmental Sustainability. *Case Studies in Chemical and Environmental Engineering* **2021**, *4*, 100142. https://doi.org/10.1016/j.cscee.2021.100142.
(29) Shome, R. Role of Microbial Enzymes in Bioremediation. **2020**, *1* (1), 7.
(30) OECD. *Plastic pollution is growing relentlessly as waste management and recycling fall short, says OECD*. https://www.oecd.org/newsroom/plastic-pollution-is-growing-relentlessly-as-waste-management-and-recycling-fall-short.htm (accessed 2022-11-01).
(31) Kuhn, S.; Rebolledo, E. B.; Franeker, J. A. van. Deleterious Effects of Litter on Marine Life. *Marine Anthropogenic Litter* **2015**, 75–116. https://doi.org/10.1007/978-3-319-16510-3_4.
(32) Hartmann, N. B.; Hüffer, T.; Thompson, R. C.; Hassellöv, M.; Verschoor, A.; Daugaard, A. E.; Rist, S.; Karlsson, T.; Brennholt, N.; Cole, M.; Herrling, M. P.; Hess, M. C.; Ivleva, N. P.; Lusher, A. L.; Wagner, M. Are We Speaking the Same Language? Recommendations for a Definition and Categorization Framework for Plastic Debris. *Environ. Sci. Technol.* **2019**, *53* (3), 1039–1047. https://doi.org/10.1021/acs.est.8b05297.
(33) Cózar, A.; Echevarría, F.; González-Gordillo, J. I.; Irigoien, X.; Úbeda, B.; Hernández-León, S.; Palma, Á. T.; Navarro, S.; García-de-Lomas, J.; Ruiz, A.; Fernández-de-Puelles, M. L.; Duarte, C. M. Plastic Debris in the Open Ocean. *Proceedings of the National Academy of Sciences* **2014**, *111* (28), 10239–10244. https://doi.org/10.1073/pnas.1314705111.
(34) Ostle, C.; Thompson, R. C.; Broughton, D.; Gregory, L.; Wootton, M.; Johns, D. G. The Rise in Ocean Plastics Evidenced from a 60-Year Time Series. *Nat Commun* **2019**, *10* (1), 1622. https://doi.org/10.1038/s41467-019-09506-1.
(35) Parga Martínez, K. B.; Tekman, M. B.; Bergmann, M. Temporal Trends in Marine Litter at Three Stations of the HAUSGARTEN Observatory in the Arctic Deep Sea. *Frontiers in Marine Science* **2020**, *7*.
(36) Trevail, A. M.; Gabrielsen, G. W.; Kühn, S.; Van Franeker, J. A. Elevated Levels of Ingested Plastic in a High Arctic Seabird, the Northern Fulmar (Fulmarus Glacialis). *Polar Biol* **2015**, *38* (7), 975–981. https://doi.org/10.1007/s00300-015-1657-4.
(37) Technau, B.; Unnsteinsdóttir, E. R.; Schaafsma, F. L.; Kühn, S. Plastic and Other Anthropogenic Debris in Arctic Fox (Vulpes Lagopus) Faeces from Iceland. *Polar Biol* **2022**, *45* (8), 1403–1413. https://doi.org/10.1007/s00300-022-03075-8.
(38) European Parliament. *Circular economy: definition, importance and benefits | News | European Parliament*. European parliament. https://www.europarl.europa.eu/news/en/headlines/economy/20151201STO05603/circular-economy-definition-importance-and-benefits (accessed 2022-11-20).
(39) Geyer, R.; Jambeck, J. R.; Law, K. L. Production, Use, and Fate of All Plastics Ever Made. *Science Advances* **2017**, *3* (7), e1700782. https://doi.org/10.1126/sciadv.1700782.
(40) Mukherjee, A.; Koller, M. Polyhydroxyalkanoate (PHA) Biopolyesters - Emerging and Major Products of Industrial Biotechnology. *The EuroBiotech Journal* **2022**, *6* (2), 49–60. https://doi.org/10.2478/ebtj-2022-0007.
(41) Steinbüchel, A.; Lütke-Eversloh, T. Metabolic Engineering and Pathway Construction for Biotechnological Production of Relevant Polyhydroxyalkanoates in Microorganisms. *Biochemical Engineering Journal* **2003**, *16* (2), 81–96. https://doi.org/10.1016/S1369-703X(03)00036-6.
(42) Matarredona, L.; Camacho, M.; Zafrilla, B.; Bravo-Barrales, G.; Esclapez, J.; Bonete, M.-J. The Survival of Haloferax Mediterranei under Stressful Conditions. *Microorganisms* **2021**, *9* (2), 336. https://doi.org/10.3390/microorganisms9020336.

(43) Danis, O.; Ogan, A.; Tatlican, P.; Attar, A.; Cakmakci, E.; Mertoglu, B.; Birbir, M. Preparation of Poly(3-Hydroxybutyrate-Co-Hydroxyvalerate) Films from Halophilic Archaea and Their Potential Use in Drug Delivery. *Extremophiles* **2015**, *19* (2), 515–524. https://doi.org/10.1007/s00792-015-0735-4.

(44) Thomas, T.; Sudesh, K.; Bazire, A.; Elain, A.; Tan, H. T.; Lim, H.; Bruzaud, S. PHA Production and PHA Synthases of the Halophilic Bacterium Halomonas Sp. SF2003. *Bioengineering* **2020**, *7* (1), 29. https://doi.org/10.3390/bioengineering7010029.

(45) Lillo, J. G.; Rodriguez-Valera, F. Effects of Culture Conditions on Poly(β-Hydroxybutyric Acid) Production by *Haloferax Mediterranei*. *Appl Environ Microbiol* **1990**, *56* (8), 2517–2521. https://doi.org/10.1128/aem.56.8.2517-2521.1990.

(46) Pantazaki, A. A.; Tambaka, M. G.; Langlois, V.; Guerin, P.; Kyriakidis, D. A. Polyhydroxyalkanoate (PHA) Biosynthesis in Thermus Thermophilus: Purification and Biochemical Properties of PHA Synthase. *Mol Cell Biochem* **2003**, *254* (1–2), 173–183. https://doi.org/10.1023/a:1027373100955.

(47) Miyake, M.; Erata, M.; Asada, Y. A Thermophilic Cyanobacterium, Synechococcus Sp. MA19, Capable of Accumulating Poly-β-Hydroxybutyrate. *Journal of Fermentation and Bioengineering* **1996**, *82* (5), 512–514. https://doi.org/10.1016/S0922-338X(97)86995-4.

(48) Cui, B.; Huang, S.; Xu, F.; Zhang, R.; Zhang, Y. Improved Productivity of Poly (3-Hydroxybutyrate) (PHB) in Thermophilic Chelatococcus Daeguensis TAD1 Using Glycerol as the Growth Substrate in a Fed-Batch Culture. *Appl Microbiol Biotechnol* **2015**, *99* (14), 6009–6019. https://doi.org/10.1007/s00253-015-6489-1.

(49) Tribelli, P. M.; López, N. I. Poly(3-Hydroxybutyrate) Influences Biofilm Formation and Motility in the Novel Antarctic Species Pseudomonas Extremaustralis under Cold Conditions. *Extremophiles* **2011**, *15* (5), 541–547. https://doi.org/10.1007/s00792-011-0384-1.

(50) Choi, T.-R.; Park, Y.-L.; Song, H.-S.; Lee, S. M.; Park, S. L.; Lee, H. S.; Kim, H.-J.; Bhatia, S. K.; Gurav, R.; Choi, K.-Y.; Lee, Y. K.; Yang, Y.-H. Fructose-Based Production of Short-Chain-Length and Medium-Chain-Length Polyhydroxyalkanoate Copolymer by Arctic Pseudomonas Sp. B14-6. *Polymers* **2021**, *13* (9), 1398. https://doi.org/10.3390/polym13091398.

(51) Sathiyanarayanan, G.; Bhatia, S. K.; Song, H.-S.; Jeon, J.-M.; Kim, J.; Lee, Y. K.; Kim, Y.-G.; Yang, Y.-H. Production and Characterization of Medium-Chain-Length Polyhydroxyalkanoate Copolymer from Arctic Psychrotrophic Bacterium Pseudomonas Sp. PAMC 28620. *International Journal of Biological Macromolecules* **2017**, *97*, 710–720. https://doi.org/10.1016/j.ijbiomac.2017.01.053.

(52) Lee, H.-J.; Rho, J.-K.; Yoon, S.-C. Growth Temperature-Dependent Conversion of De Novo-Synthesized Unsaturated Fatty Acids into Polyhydroxyalkanoic Acid and Membrane Cyclopropane Fatty Acids in the Psychrotrophic Bacterium Pseudomonas Fluorescens BM07. *Journal of Microbiology and Biotechnology* **2004**, *14* (6), 1217–1226.

(53) Verlinden, R. A. J.; Hill, D. J.; Kenward, M. A.; Williams, C. D.; Radecka, I. Bacterial Synthesis of Biodegradable Polyhydroxyalkanoates. *J Appl Microbiol* **2007**, *102* (6), 1437–1449. https://doi.org/10.1111/j.1365-2672.2007.03335.x.

(54) Obruca, S.; Sedlacek, P.; Krzyzanek, V.; Mravec, F.; Hrubanova, K.; Samek, O.; Kucera, D.; Benesova, P.; Marova, I. Accumulation of Poly(3-Hydroxybutyrate) Helps Bacterial Cells to Survive Freezing. *PLOS ONE* **2016**, *11* (6), e0157778. https://doi.org/10.1371/journal.pone.0157778.

(55) Chen, G.-Q.; Hajnal, I.; Wu, H.; Lv, L.; Ye, J. Engineering Biosynthesis Mechanisms for Diversifying Polyhydroxyalkanoates. *Trends in Biotechnology* **2015**, *33* (10), 565–574. https://doi.org/10.1016/j.tibtech.2015.07.007.

(56) Ayub, N. D.; Tribelli, P. M.; López, N. I. Polyhydroxyalkanoates Are Essential for Maintenance of Redox State in the Antarctic Bacterium Pseudomonas Sp. 14-3 during Low Temperature Adaptation. *Extremophiles* **2009**, *13* (1), 59–66. https://doi.org/10.1007/s00792-008-0197-z.

(57) Kamravamanesh, D.; Lackner, M.; Herwig, C. Bioprocess Engineering Aspects of Sustainable Polyhydroxyalkanoate Production in Cyanobacteria. *Bioengineering* **2018**, *5* (4), 111. https://doi.org/10.3390/bioengineering5040111.

(58) Troschl, C.; Meixner, K.; Drosg, B. Cyanobacterial PHA Production—Review of Recent Advances and a Summary of Three Years' Working Experience Running a Pilot Plant. *Bioengineering* **2017**, *4* (4), 26. https://doi.org/10.3390/bioengineering4020026.

(59) Christensen, M.; Jablonski, P.; Altermark, B.; Irgum, K.; Hansen, H. High Natural PHA Production from Acetate in Cobetia Sp. MC34 and Cobetia Marina DSM 4741T and in Silico Analyses of the Genus Specific PhaC2 Polymerase Variant. *Microb Cell Fact* **2021**, *20* (1), 225. https://doi.org/10.1186/s12934-021-01713-0.

(60) Madkour, M. H.; Heinrich, D.; Alghamdi, M. A.; Shabbaj, I. I.; Steinbüchel, A. PHA Recovery from Biomass. *Biomacromolecules* **2013**, *14* (9), 2963–2972. https://doi.org/10.1021/bm4010244.

(61) Meng, D.-C.; Shi, Z.-Y.; Wu, L.-P.; Zhou, Q.; Wu, Q.; Chen, J.-C.; Chen, G.-Q. Production and Characterization of Poly(3-Hydroxypropionate-Co-4-Hydroxybutyrate) with Fully Controllable

Structures by Recombinant Escherichia Coli Containing an Engineered Pathway. *Metabolic Engineering* **2012**, *14* (4), 317–324. https://doi.org/10.1016/j.ymben.2012.04.003.

(62) Panchal, B.; Bagdadi, A.; Roy, I. Polyhydroxyalkanoates: The Natural Polymers Produced by Bacterial Fermentation. In *Advances in Natural Polymers*; Thomas, S., Visakh, P. M., Mathew, Aji. P., Eds.; Advanced Structured Materials; Springer Berlin Heidelberg: Berlin, Heidelberg, 2013; Vol. 18, pp 397–421. https://doi.org/10.1007/978-3-642-20940-6_12.

(63) Guo, W.; Song, C.; Kong, M.; Geng, W.; Wang, Y.; Wang, S. Simultaneous Production and Characterization of Medium-Chain-Length Polyhydroxyalkanoates and Alginate Oligosaccharides by Pseudomonas Mendocina NK-01. *Appl Microbiol Biotechnol* **2011**, *92* (4), 791–801. https://doi.org/10.1007/s00253-011-3333-0.

(64) Jenkins, A. D.; Kratochvíl, P.; Stepto, R. F. T.; Suter, U. W. Glossary of basic terms in polymer science (IUPAC Recommendations 1996). *Pure and Applied Chemistry* **1996**, *68* (12), 2287–2311. https://doi.org/10.1351/pac199668122287.

(65) Sharma, S.; Dua, A.; Malik, A. Polyaspartic Acid Based Superabsorbent Polymers. *European Polymer Journal* **2014**, *59*, 363–376. https://doi.org/10.1016/j.eurpolymj.2014.07.043.

(66) Jendrossek, D.; Schirmer, A.; Schlegel, H. G. Biodegradation of Polyhydroxyalkanoic Acids. *Appl Microbiol Biotechnol* **1996**, *46* (5–6), 451–463. https://doi.org/10.1007/s002530050844.

(67) Crawford, C. B.; Quinn, B. 4 - Physiochemical Properties and Degradation. In *Microplastic Pollutants*; Crawford, C. B., Quinn, B., Eds.; Elsevier, 2017; pp 57–100. https://doi.org/10.1016/B978-0-12-809406-8.00004-9.

(68) *Basic Polymer Structure | MATSE 81: Materials In Today's World*. https://www.e-education.psu.edu/matse81/node/2210 (accessed 2022-11-22).

(69) Callister Jr, W. D.; Rethwisch, D. G. *Fundamentals of Materials Science and Engineering: An Integrated Approach, 5th Edition | Wiley*; 2018.

(70) Li, Z.; Yang, J.; Loh, X. J. Polyhydroxyalkanoates: Opening Doors for a Sustainable Future. *NPG Asia Mater* **2016**, *8* (4), e265–e265. https://doi.org/10.1038/am.2016.48.

(71) Asefa, T.; Dubovoy, V. 9.09 - Ordered Mesoporous/Nanoporous Inorganic Materials via Self-Assembly. In *Comprehensive Supramolecular Chemistry II*; Atwood, J. L., Ed.; Elsevier: Oxford, 2017; pp 157–192. https://doi.org/10.1016/B978-0-12-409547-2.12649-6.

(72) Shrivastava, A. Introduction to Plastics Engineering. In *Introduction to Plastics Engineering*; Elsevier, 2018; pp 1–16. https://doi.org/10.1016/B978-0-323-39500-7.00001-0.

(73) Rai, R.; Keshavarz, T.; Roether, J. A.; Boccaccini, A. R.; Roy, I. Medium Chain Length Polyhydroxyalkanoates, Promising New Biomedical Materials for the Future. *Materials Science and Engineering R: Reports* **2011**, *72* (3), 29–47. https://doi.org/10.1016/j.mser.2010.11.002.

(74) Sudesh, K.; Abe, H.; Doi, Y. Synthesis, Structure and Properties of Polyhydroxyalkanoates: Biological Polyesters. *Progress in Polymer Science* **2000**, *25* (10), 1503–1555. https://doi.org/10.1016/S0079-6700(00)00035-6.

(75) Mezzina, M. P.; Manoli, M. T.; Prieto, M. A.; Nikel, P. I. Engineering Native and Synthetic Pathways in Pseudomonas Putida for the Production of Tailored Polyhydroxyalkanoates. *Biotechnology Journal* **2021**, *16* (3), 2000165. https://doi.org/10.1002/biot.202000165.

(76) Djukic, S.; Bocahut, A.; Bikard, J.; Long, D. R. Mechanical Properties of Amorphous and Semi-Crystalline Semi-Aromatic Polyamides. *Heliyon* **2020**, *6* (4), e03857. https://doi.org/10.1016/j.heliyon.2020.e03857.

(77) Pal, T.; Pramanik, S.; Verma, K. D.; Naqvi, S. Z.; Manna, P. K.; Kar, K. K. 6 - Fly Ash-Reinforced Polypropylene Composites. In *Handbook of Fly Ash*; Kar, K. K., Ed.; Butterworth-Heinemann, 2022; pp 243–270. https://doi.org/10.1016/B978-0-12-817686-3.00021-9.

(78) Djafari Petroudy, S. R. Physical and Mechanical Properties of Natural Fibers. In *Advanced High Strength Natural Fibre Composites in Construction*; Elsevier, 2017; pp 59–83. https://doi.org/10.1016/B978-0-08-100411-1.00003-0.

(79) Shrivastav, A.; Kim, H.-Y.; Kim, Y.-R. Advances in the Applications of Polyhydroxyalkanoate Nanoparticles for Novel Drug Delivery System. *BioMed Research International* **2013**, *2013*, e581684. https://doi.org/10.1155/2013/581684.

(80) Khanna, S.; Srivastava, A. K. Recent Advances in Microbial Polyhydroxyalkanoates. *Process Biochemistry* **2005**, *40* (2), 607–619. https://doi.org/10.1016/j.procbio.2004.01.053.

(81) Koller, M. Recycling of Waste Streams of the Biotechnological Poly(Hydroxyalkanoate) Production by *Haloferax Mediterranei* on Whey. *International Journal of Polymer Science* **2015**, *2015*, 1–8. https://doi.org/10.1155/2015/370164.

(82) Koller, M.; Gasser, I.; Schmid, F.; Berg, G. Linking Ecology with Economy: Insights into Polyhydroxyalkanoate-Producing Microorganisms. *Engineering in Life Sciences* **2011**, *11* (3), 222–237. https://doi.org/10.1002/elsc.201000190.

(83) Higuchi-Takeuchi, M.; Morisaki, K.; Toyooka, K.; Numata, K. Synthesis of High-Molecular-Weight Polyhydroxyalkanoates by Marine Photosynthetic Purple Bacteria. *PLOS ONE* **2016**, *11*, e0160981. https://doi.org/10.1371/journal.pone.0160981.

(84) Senior, P. J.; Beech, G. A.; Ritchie, G. A.; Dawes, E. A. The Role of Oxygen Limitation in the Formation of Poly- -Hydroxybutyrate during Batch and Continuous Culture of Azotobacter Beijerinckii. *Biochem J* **1972**, *128* (5), 1193–1201. https://doi.org/10.1042/bj1281193.

(85) Urmeneta, J.; Mas-Castella, J.; Guerrero, R. Biodegradation of Poly-(Beta)-Hydroxyalkanoates in a Lake Sediment Sample Increases Bacterial Sulfate Reduction. *Applied and Environmental Microbiology* **1995**, *61* (5), 2046–2048. https://doi.org/10.1128/aem.61.5.2046-2048.1995.

(86) Obruca, S.; Sedlacek, P.; Mravec, F.; Samek, O.; Marova, I. Evaluation of 3-Hydroxybutyrate as an Enzyme-Protective Agent against Heating and Oxidative Damage and Its Potential Role in Stress Response of Poly(3-Hydroxybutyrate) Accumulating Cells. *Appl Microbiol Biotechnol* **2016**, *100* (3), 1365–1376. https://doi.org/10.1007/s00253-015-7162-4.

(87) Rehm, B. H. A. Polyester Synthases: Natural Catalysts for Plastics. *Biochem J* **2003**, *376* (Pt 1), 15–33. https://doi.org/10.1042/BJ20031254.

(88) Sudesh, K.; Gan, Z.; Maehara, A.; Doi, Y. Surface Structure, Morphology and Stability of Polyhydroxyalkanoate Inclusions Characterised by Atomic Force Microscopy. *Polymer Degradation and Stability* **2002**, *77* (1), 77–85. https://doi.org/10.1016/S0141-3910(02)00081-2.

(89) Sudesh, K.; Maehara, A.; Gan, Z.; Iwata, T.; Doi, Y. Direct Observation of Polyhydroxyalkanoate Granule-Associated-Proteins on Native Granules and on Poly(3-Hydroxybutyrate) Single Crystals by Atomic Force Microscopy. *Polymer Degradation and Stability* **2004**, *83* (2), 281–287. https://doi.org/10.1016/S0141-3910(03)00273-8.

(90) Pötter, M.; Steinbüchel, A. Biogenesis and Structure of Polyhydroxyalkanoate Granules. 28.

(91) Kunasundari, B.; Sudesh, K. Isolation and Recovery of Microbial Polyhydroxyalkanoates. *Express Polym. Lett.* **2011**, *5* (7), 620–634. https://doi.org/10.3144/expresspolymlett.2011.60.

(92) Zhao, D.; Cai, L.; Wu, J.; Li, M.; Liu, H.; Han, J.; Zhou, J.; Xiang, H. Improving Polyhydroxyalkanoate Production by Knocking out the Genes Involved in Exopolysaccharide Biosynthesis in Haloferax Mediterranei. *Appl Microbiol Biotechnol* **2013**, *97* (7), 3027–3036. https://doi.org/10.1007/s00253-012-4415-3.

(93) Cavalheiro, J. M. B. T.; de Almeida, M. C. M. D.; Grandfils, C.; da Fonseca, M. M. R. Poly(3-Hydroxybutyrate) Production by Cupriavidus Necator Using Waste Glycerol. *Process Biochemistry* **2009**, *44* (5), 509–515. https://doi.org/10.1016/j.procbio.2009.01.008.

(94) Pérez-Pantoja, D.; De la Iglesia, R.; Pieper, D. H.; González, B. Metabolic Reconstruction of Aromatic Compounds Degradation from the Genome of the Amazing Pollutant-Degrading Bacterium Cupriavidus Necator JMP134. *FEMS Microbiol Rev* **2008**, *32* (5), 736–794. https://doi.org/10.1111/j.1574-6976.2008.00122.x.

(95) Loo, C.-Y.; Sudesh, K. Biosynthesis and Native Granule Characteristics of Poly(3-Hydroxybutyrate-Co-3-Hydroxyvalerate) in Delftia Acidovorans. *International Journal of Biological Macromolecules* **2007**, *40* (5), 466–471. https://doi.org/10.1016/j.ijbiomac.2006.11.003.

(96) Sheu, D.-S.; Chen, W.-M.; Yang, J.-Y.; Chang, R.-C. Thermophilic Bacterium Caldimonas Taiwanensis Produces Poly(3-Hydroxybutyrate-Co-3-Hydroxyvalerate) from Starch and Valerate as Carbon Sources. *Enzyme and Microbial Technology* **2009**, *44* (5), 289–294. https://doi.org/10.1016/j.enzmictec.2009.01.004.

(97) Koller, M.; Rittmann, S. K.-M. R. Haloarchaea as Emerging Big Players in Future Polyhydroxyalkanoate Bioproduction: Review of Trends and Perspectives. *Current Research in Biotechnology* **2022**, *4*, 377–391. https://doi.org/10.1016/j.crbiot.2022.09.002.

(98) Koller, M. A Review on Established and Emerging Fermentation Schemes for Microbial Production of Polyhydroxyalkanoate (PHA) Biopolyesters. *Fermentation* **2018**, *4* (2), 30. https://doi.org/10.3390/fermentation4020030.

(99) Raza, Z. A.; Tariq, M. R.; Majeed, M. I.; Banat, I. M. Recent Developments in Bioreactor Scale Production of Bacterial Polyhydroxyalkanoates. *Bioprocess Biosyst Eng* **2019**, *42* (6), 901–919. https://doi.org/10.1007/s00449-019-02093-x.

(100) Lorantfy, B.; Ruschitzka, P.; Herwig, C. Investigation of Physiological Limits and Conditions for Robust Bioprocessing of an Extreme Halophilic Archaeon Using External Cell Retention System. *Biochemical Engineering Journal* **2014**, *90*, 140–148. https://doi.org/10.1016/j.bej.2014.06.004.

(101) Ziv, N.; Brandt, N. J.; Gresham, D. The Use of Chemostats in Microbial Systems Biology. *JoVE (Journal of Visualized Experiments)* **2013**, No. 80, e50168. https://doi.org/10.3791/50168.

(102) Jiang, X.; Ramsay, J. A.; Ramsay, B. A. Acetone Extraction of Mcl-PHA from Pseudomonas Putida KT2440. *Journal of Microbiological Methods* **2006**, *67* (2), 212–219. https://doi.org/10.1016/j.mimet.2006.03.015.

(103) Chen, G.-Q.; Wu, Q. The Application of Polyhydroxyalkanoates as Tissue Engineering Materials. *Biomaterials* **2005**, *26* (33), 6565–6578. https://doi.org/10.1016/j.biomaterials.2005.04.036.
(104) Berger, E.; Ramsay, B. A.; Ramsay, J. A.; Chavarie, C.; Braunegg, G. PHB Recovery by Hypochlorite Digestion of Non-PHB Biomass. *Biotechnol Tech* **1989**, *3* (4), 227–232. https://doi.org/10.1007/BF01876053.
(105) Ramsay, J. A.; Berger, E.; Ramsay, B. A.; Chavarie, C. Recovery of Poly-3-Hydroxyalkanoic Acid Granules by a Surfactant-Hypochlorite Treatment. *Biotechnol Tech* **1990**, *4* (4), 221–226. https://doi.org/10.1007/BF00158833.
(106) Merrick, J. M.; Doudoroff, M. DEPOLYMERIZATION OF POLY-β-HYDROXYBUTYRATE BY AN INTRACELLULAR ENZYME SYSTEM. *Journal of Bacteriology* **1964**, *88* (1), 60–71. https://doi.org/10.1128/jb.88.1.60-71.1964.
(107) Suzuki, D. V.; Carter, J. M.; Rodrigues, M. F. A.; da Silva, E. S.; Maiorano, A. E. Purification of Polyhydroxybutyrate Produced by Burkholderia Cepacia IPT64 through a Chemical and Enzymatic Route. *World J Microbiol Biotechnol* **2008**, *24* (6), 771–775. https://doi.org/10.1007/s11274-007-9537-x.
(108) Jacquel, N.; Lo, C.-W.; Wei, Y.-H.; Wu, H.-S.; Wang, S. S. Isolation and Purification of Bacterial Poly(3-Hydroxyalkanoates). *Biochemical Engineering Journal* **2008**, *39* (1), 15–27. https://doi.org/10.1016/j.bej.2007.11.029.
(109) Kellerhals, M. B.; Kessler, B.; Witholt, B.; Tchouboukov, A.; Brandl, H. Renewable Long-Chain Fatty Acids for Production of Biodegradable Medium-Chain-Length Polyhydroxyalkanoates (Mcl-PHAs) at Laboratory and Pilot Plant Scales. *Macromolecules* **2000**, *33* (13), 4690–4698. https://doi.org/10.1021/ma000655k.
(110) Lo, C.-W.; Wu, H.-S.; Wei, Y.-H. Optimizing Acidic Methanolysis of Poly(3-Hydroxyalkanoates) in Gas Chromatography Analysis. *Asia-Pacific Jrnl of Chem. Eng* **2009**, *4* (4), 487–494. https://doi.org/10.1002/apj.260.
(111) Mironescu, M.; Ignatova, M.; Posten, C. Investigations on Exopolysaccharide Production by the Extreme Halophylic Microorganism Haloferax Mediterranei. *Comptes Rendus de L'Academie Bulgare des Sciences* **2012**, *65*, 463–472.
(112) Singh, R.; Kaur, N. Microbial Biopolymers for Edible Film and Coating Applications; 2015; pp 187–216.
(113) Wang, K.; Zhang, R. Production of Polyhydroxyalkanoates (PHA) by *Haloferax Mediterranei* from Food Waste Derived Nutrients for Biodegradable Plastic Applications. *J. Microbiol. Biotechnol.* **2021**, *31* (2), 338–347. https://doi.org/10.4014/jmb.2008.08057.