# Bioreactor cultivations of polyhydroxybutyrate - producing halophilic bacteria: Effect of sterility conditions on the microbial population

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Thesis to obtain the Master of Science Degree in:

**Biotechnology**

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## DECLARATION

I declare that this document is an original work of my own authorship and that it fulfils all the requirements of the Code of Conduct and Good Practices of the Universidade de Lisboa.

## PREFACE

The work presented in this thesis was performed at the Institute for Bioengineering and Biosciences (IBB) of Instituto Superior Técnico in Lisbon, Portugal, during the period February 2022 – September 2022. The thesis was created under the supervision of Dr. Maria Teresa Ferreira Cesário Smolders and Dr. Pedro Carlos De Barros Fernandes.

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Thank you all for being a part of this journey and making it an enriching experience.

## ABSTRACT

Plastics have become an essential part of human life, with applications across multiple sectors due to their strength, flexibility, and low production costs. However, the environmental consequences of plastic production and incorrect disposal are severe. Microbial biopolyesters, or polyhydroxyalkanoates (PHAs), have emerged as a promising alternative to petroleum-based plastics, but high and complex production costs remain a significant challenge.

To enable large-scale PHA manufacturing, this project proposes using *Halomonas boliviensis* to produce the most common type of PHA, poly(3-hydroxybutyrate) (P3HB), as a model system. The strategy involves using halophilic bacteria to prevent cross-contamination in open fermentations, without compromising P3HB productivity. This approach yields cost savings associated with energy and water usage for bioreactor, tubing and media sterilization. Moreover, the project employs another cost-effective approach to reduce carbon source expenses by utilizing hydrolysates of the seaweed *Gelidium corneum* residues as a substrate for P3HB accumulation and effective growth.

The project successfully proves the effectiveness of using halophilic bacteria to prevent cross-contamination without compromising P3HB productivity. The proposed strategy offers a sustainable solution to the production of PHAs, paving the way for large-scale manufacturing and reducing the environmental impact of plastics. The findings have significant implications for the manufacturing economics, with a 37% reduction in the overall costs, as evaluated by the SuperPro Designer® modelling software. Furthermore, in the assay using hydrolysates of *G. corneum* as a substrate, *H. boliviensis* was shown to grow effectively on glucose and galactose. However, the low nutrient availability, such as phosphate and nitrogen, in the hydrolysate may have impeded biomass growth and consequently lower P3HB productivities. Under the operational conditions used, *H. boliviensis* consistently produced as by-product gluconic acid, a commodity with wide application in food, pharma, chemical and construction industries.

**Keywords:** polyhydroxyalkanoates; sustainable production; renewable feedstocks; non-sterile conditions; halophiles; *Halomonas boliviensis*;

## RESUMO

Os plásticos tornaram-se indispensáveis no quotidiano, com aplicações em diversos setores devido à sua resistência, flexibilidade e baixos custos de produção. No entanto, as consequências ambientais da produção de plástico e da sua incorreta eliminação são graves. Os biopolímeros microbianos, ou polihidroxialcanoatos, surgiram como uma alternativa promissora aos plásticos à base de petróleo, mas os elevados custos de produção continuam a ser um desafio.

Com o objetivo de permitir a fabricação de PHA em larga escala, este projeto propõe o uso de *Halomonas boliviensis* para produzir o tipo mais comum de PHA, o poli(3-hidroxibutirato) (P3HB), como um sistema modelo. A estratégia envolve o uso de bactérias halofílicas para prevenir a contaminação cruzada em fermentações abertas, sem comprometer a produtividade de P3HB e, deste modo, reduzir os custos associados à esterilização de equipamentos e meios. Além disto, o projeto emprega outra abordagem económica para reduzir os gastos com fontes de carbono, utilizando hidrolisados de resíduos da alga vermelha *Gelidium corneum*.

O projeto comprova a eficácia do uso de bactérias halofílicas para prevenir a contaminação cruzada sem comprometer a produtividade de P3HB, oferecendo uma solução sustentável para a sua produção e contribuindo para a redução do impacto ambiental dos plásticos. As descobertas têm ainda implicações significativas para a economia de fabricação, conforme avaliado pelo software de modelagem SuperPro Designer®. No ensaio com hidrolisados de *G. corneum* como substrato, *H. boliviensis* demonstrou um crescimento efetivo em glicose e galactose. Contudo, a baixa disponibilidade de nutrientes no hidrolisado, como fosfato e nitrogênio, pode ter limitado o crescimento da biomassa e, consequentemente, reduzido a produtividade de P3HB. Nas condições operacionais utilizadas, *H. boliviensis* produziu elevadas concentrações de ácido glicónico como subproduto, o qual tem uma ampla aplicação em diversas indústrias.



**Palavras-Chave:** polyhydroxyalkanoates; produção sustentável; indústria biotecnológica; matérias-primas renováveis; fermentações abertas; halófilos; *Halomonas boliviensis*;

# INDEX

## LIST OF FIGURES

## LIST OF TABLES

## LIST OF ABBREVIATIONS

**BPA -** Bisphenol A

**CAGR-** Compound annual growth rate

**FAME-** Fatty acid methyl esters

**LCL-** Long chain length polyhydroxyalkanoate

**LPS-** Lipopolysaccharides

**MCL-** Medium chain length polyhydroxyalkanoate

**MIDI-** Microbial Identification Incorporation

**MMC-** Mixed microbial communities

**NGIB-** Next Generation Industrial Biotechnology

**NPCM-** Non-cellular PHA mass

**PET-** Polyethylene terephthalate

**PHA-** Polyhydroxyalkanoates

**P3HB-** Poly-R-3-hydroxybutyrate

**PHBV-** Poly (3-hydroxybutyrate-co-3-hydroxyvalerate)

**PLA-** Polylactic acid

**SCL-** Short chain length polyhydroxyalkanoate

**SDS-** Sodium dodecyl sulphate

**HMF-** 5-Hydroxymethylfurfural

**ED-** Entner-Doudoroff Pathway

**PPP-** Pentose Phosphate Pathway

**GnuK-** Gluconate kinase

**EER-** Economic Evaluation Report

**ICR-** Itemized Cost Report

# 1. STATE OF THE ART

## 1.1. INTRODUCTION

Plastic has become one of the most controversial subjects due to its harmful impacts in today´s society. These include water pollution, high decomposition time, expensive recycling process, degradation of soils if directly disposed, and their combustible properties that may lead to the release of toxic materials into the environment (Alabi et al., 2019). With predecessors in the 19th century, plastic was invented just over a century ago, with the unfolding of the Second Industrial Revolution. This led to many transformations in the chemical industry, and more technologies were made available for manufacturing processes, enabling the production and distribution of plastic on a large scale (Meikle, 1997). Plastic has its name from the Greek “plastikos”, which means capable of being molded, and derives from a wide range of synthetic or semi-synthetic materials from nature such as natural gas, oil, coal, mineral and plants. Currently, there are countless types of plastics with various properties, applications, durability, low cost, and easy production (Gadre & Sangawar, 2013).

Additionally, since the end of 18$^{th}$ century, the world´s population has been consuming fossil fuels at a faster rate than ever, being largely contributed by plastic industry, as most of the plastics produced used to come from non-renewable sources such as oil, natural gas and coal. In recent research, it was reported that by 2030, plastic industry in the United States will emit more greenhouse gases than coal-fired power stations, contributing to global warming. Thus, the energy intensive process inherent to plastic production poses a threat on achieving the Paris climate goals (Rosenboom et al., 2022; *The New Coal: Plastics & Climate Change*, n.d.).

With the sustained growth of the world’s population, and consequent increase of industrialization, the global demand for petroleum-based plastics is rising exponentially. As seen in Figure 1, from 1950 a remarkable growth has been witnessed, and by 2015, it was estimated that 381 million tonnes of plastic were produced worldwide.

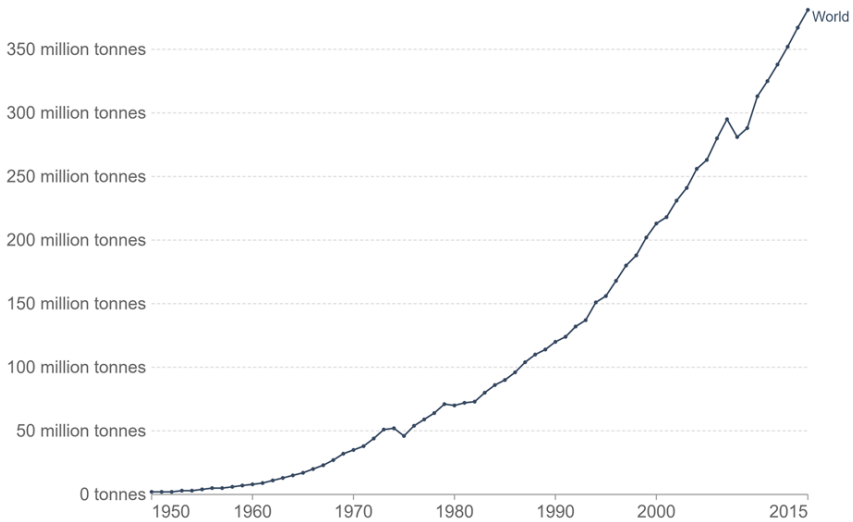



**Figure 1**. The increase of global plastic production, from 1950 through to 2015, measured in tonnes per year. Adapted from (Ritchie & Roser, 2018).

The alarming exponential increase in plastic manufacture, accompanied by ineffective waste management, has resulted in the leakage of such material into the environment, in particular the marine one. It is estimated that 80% to 85% of marine litter, in European Union, is plastic. With aim of lowering the adverse impacts associated with plastic, the European Parliament and the Council issued a directive on the reduction of the impact of certain plastic products on the environment, to tackle these matters while promoting a circular economy approach. Hence, it fosters plastic goods being produced, reused and recycled in more sustainable ways with focus on consumption reduction, restrictions and requirements of plastic products while increasing awareness for separate collection (Directive (EU) 2019/904).

To understand the seriousness of the problem, according to information released in 2016 at the World Economic Forum in Davos, it is possible that in 2050 the oceans will have “more plastic than fish” and twelve billion tons of plastic will be present in landfills and in the environment (MacArthur et al., 2016). The uncontrolled plastic dumping into the marine environment is translated into several phenomena like the ocean’s gyres, huge concentrations of marine debris and plastics restrained in the ocean (garbage patches). If located in bays near large cities and industrial centers, they have the designation of “ocean hotspots” and if found in inland waterways and on land they are nominated “plastic soup”. All these different plastic aggregations affect marine life, contaminate oceans, rivers, and soils with toxic chemicals, contributing to greenhouse gas emissions (Li et al., 2021; Li et al., 2016)

Plastic production has increased considerably in recent years, reaching exponential values with the recent COVID-19 pandemic mainly due to the increased demand of single-used plastics. Peng et al, demonstrated that more than 8 million tons of pandemic-associated plastic waste have been generated globally, with more than 25 000 tons entering the global ocean (Peng et al., 2021). A report also estimated that 1.56 million face masks entered the ocean in 2020 (Phelps Bondaroff & Cooke, 2020).

However, new strategies and approaches are emerging as a response to this problem. For instance, a group of scientists has successfully designed a bionic robot-fish with the capability of consuming microplastics. The Ocean Cleanup designed a system comprised of a long U-shaped barrier that guides the garbage patches plastic into a retention zone. According to projections, this company estimates that by 2040, The Ocean Cleanup projects will have been able to remove 90% of floating ocean plastic (*The Ocean Cleanup*, n.d.).

Even though a few years ago it was thought that plastic pollution was a faraway problem mainly ocean associated, it is now evident that it is present everywhere. As plastic does not perish but breaks down in small pieces, it was found that microplastics heavily contaminated agriculture land, food destined for human consumption and the atmospheric air (Suaria et al., 2016).

In the production of some plastics, to make them more flexible, substances as bisphenol A (BPA) and phthalates are used. Both chemicals are suspected of interfering with the human hormone responsible for balance (aldosterone), negatively impacting human’s health. As humans and animals are constantly exposed to plastics, almost everyone has BPA in their bodies in some form. Around eighty diseases

have been linked to this endocrine-disrupting substances, including testicular cancer, obesity, and reproductive disorders (Vandenberg et al., 2017).

## 1.2. BIO-BASED AND BIODEGRADABLE POLYMERS

In recent decades, several possible solutions were found and are being implemented to solve the plastic pollution. However, the current solutions - such as recycling, consumer behavior campaigns and greenwashing techniques (misleading advertising of the concept of bioplastics) used by some companies - are ineffective and not realistic at the present day (Maesen, 2018). For instance, although recycling processes have been highly publicized, of all plastic produced in the planet, only 9% is recycled (Schmidt et al., 2017). Therefore, taking all these matters into consideration, shifting towards more sustainable alternatives is mandatory. Switching to biodegradable polymers can help mitigate some of these issues (H. Liu et al., 2021).

One of the alternatives is bio-based polymers production, bio-based plastics can be subdivided mainly into three type such as agropolymers from agroresources (starch, cellulose derivatives and natural rubbers), bio-based monomers produced by fermentation followed by conventional chemistry polymerization (polylactic acid or PLA, polybutylene succinate, and polyethylene) and lastly, extracted biopolymers formed by microorganisms such as polyhydroxyalkanoates (PHAs) (Babu et al., 2013).

However, despite these various options and manufacturing methods, not all bio-based polymers are biodegradable. There is a widespread misconception among consumers about the notions of bio-based, compostable and biodegradable. The concept of bio-based (Figure 2) implies that materials are derived from biological resources, such as lignocellulose biomass or algae, instead of petroleum, not necessarily being compostable or biodegradable (Greene, 2021). Bioplastics are considered biodegradable if they are broken down with the combination of adequate environmental conditions and microorganisms, which in turn use them as a food source. Compostable plastics are a subset of biodegradable plastics, in which they are considered compostable if within 180 days, a complete microbial assimilation takes place in a compost environment (Ashter, 2016).

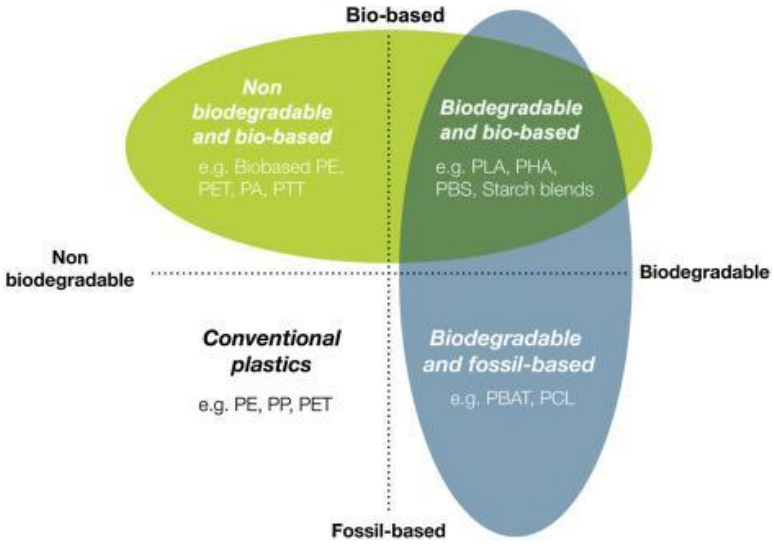



**Figure 2.** Bio-based plastics and conventional plastics classification based on their biodegradability and bio-based content. Retrieved from (*European Bioplastics e.V.*, n.d.).

Several companies have already started using Bio-PET labeling on their products, promoting the idea of a more sustainable product. Although its manufacture produces significantly fewer greenhouse gas emissions compared to traditional polyethylene terephthalate (PET), over its lifetime, it has the same properties as the fossil predecessors. Only in recent years, studies have focused on demonstrating the possibility of enzymatic degradation of PET to monomers, and such processes are still in development and research stages (Khairul Anuar et al., 2022; Sevilla et al., 2023).

From the bio-based alternatives, only PLAs and PHAs are entirely biodegradable under certain circumstances. PLA is compostable, under industrial composting conditions (at a high temperature, around 58 °C), but not marine biodegradable like PHAs. Therefore, PLAs alternatives are ineffective in addressing the issue of the rising pollution in marine life (DiGregorio, 2009). Moreover, PHAs are biocompatible as they occur naturally in human tissues and blood, making them viable to be used in medical applications (Bonartsev et al., 2019; Pulingam et al., 2022). All these features make PHAs a unique subset of polyesters, with great potential to contribute to a green industrial evolution.

The EU closed loop bioplastics economy plan is depicted in Figure 3. Essentially, just like fossil-based plastics, bio-based plastic end-products suffer a conversion process to applicable products. Since several bioplastics are biodegradable, they may be used as compost or as organic waste biomass. This leads to the production of renewable resources that through biotechnological and chemical processes are transformed into bio-based plastics, closing the circle. The Life Cycle Assessment (LCA) is a tool to measure environmental impacts of products or services and its analysis showed that bio-based plastics enable a significant carbon dioxide saving, almost up to carbon neutrality compared to conventional plastics (Rezvani Ghomi et al., 2021). According to European Bioplastics Association, substituting annual European demand in fossil-based polyethylene (PE) with bio-based PE would result in a 73 million tons of carbon dioxide emission reduction (*European Bioplastics e.V.*, n.d.).

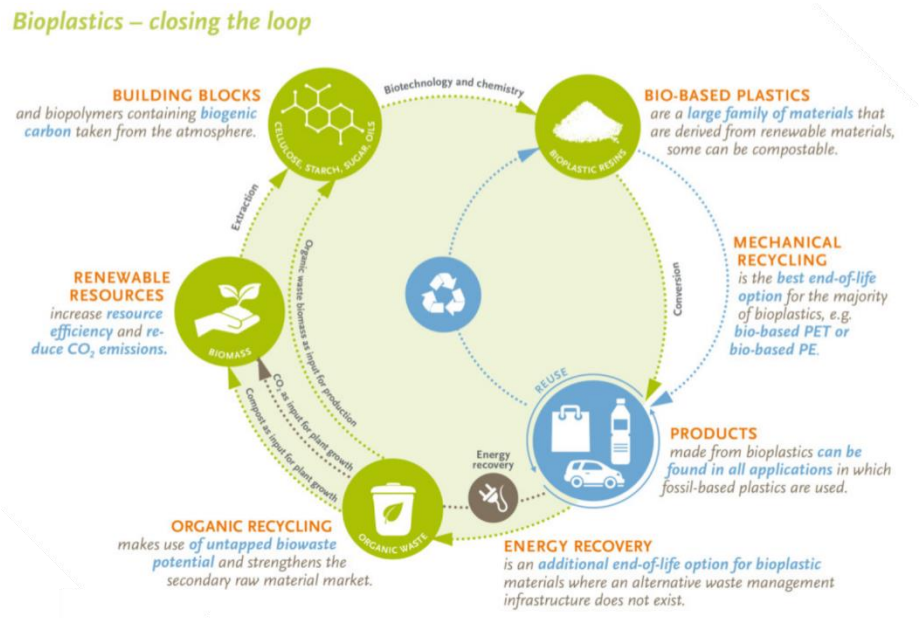



**Figure 3.** EU closed loop bioplastics economy plan: Bioplastics waste management and recovery alternatives. A broad range of end-of-life solutions, such as reuse, mechanical recycling, organic recycling, and energy recovery, are appropriate for bioplastics. Retrieved from (*European Bioplastics e.V.*, n.d.).

## 1.3. MARKET TRENDS AND KEY PLAYERS

A growing number of countries are adopting strategies to reduce plastic consumption and implementing policies regarding purchasing sustainable and environmental-friendly products. For instance, the European Union is paving the way for a worldwide plastics agreement, to support the global shift to a circular economy as outlined in the new circular economy action plan (*Circular Economy Action Plan*, n.d.). The EU has also recently updated the list of Food Contact Plastics Regulation on January 10, 2019, which will boost the use of PHA in the packaging and food services application (Commission Regulation (EU) 2019/37) .

In 2021, 2.42 million tons of bioplastics were produced worldwide. As seen in Figure 4, bio-based/non-biodegradable plastic has a significant share representing 36% of the total capacity. The main biodegradable plastics currently produced are polybutylene adipate terephthalate (PBAT), polylactic acid (PLA), starch blends and PHA, with shares of 19.2%, 18.9%, 16.4% and 1.8%, respectively. The deployment of new measures and growing awareness will translate into a higher bioplastic production, with estimations of reaching 7.59 million tons by 2026, as PHA may represent a share of 6.4% of global annual production. Asia stands out as the major key player to obtain these goals as they represent around 50% of total bioplastics being produced, with Europe and North America having a share of 24.1% and 16.5%, respectively (Table 1). Forecasts predict Asia will be responsible for 70% of bioplastic production by 2026 (*European Bioplastics e.V.*, n.d.).

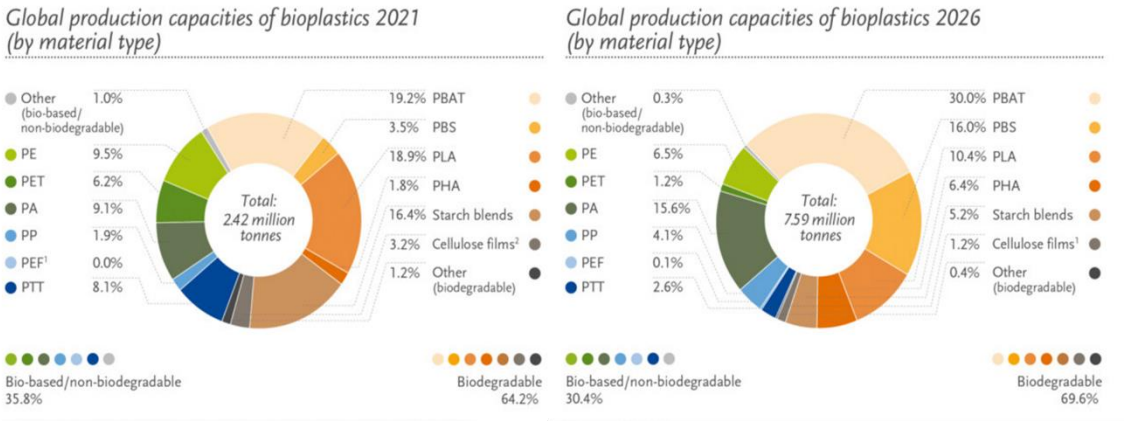



**Figure 4.** Global production capacities of bioplastics in 2021 and their expected evolution in 2026. The production of biodegradable plastics is estimated to increase to almost 5.3 million in 2026. Retrieved from (*European Bioplastics e.V.*, n.d.)

**Table 1.** Global annual production of bioplastics in 2021, by region, with a total global production of 2.42 million tonnes (*European Bioplastics e.V.*, n.d.).

| Global bioplastics annual production contribution | |
|---|---|
| Asia | 49.9% |
| Europe | 24.1% |
| North America | 16.5% |
| South America | 9.1% |
| Australia/ Oceania | 0.4% |

Due to these new action plans and consumer´s growth awareness about the toxicity of petroleum-based products, the market size of PHA is estimated to reach 121 million USD by 2025, at a CAGR (Compound annual growth rate) of 14.2% between 2020 and 2025 (*Polyhydroxyalkanoate (PHA) Market Global Forecast to 2025* | *MarketsandMarkets*, n.d.). However, one of the major limitations to continuous market growth is PHA superior production costs when comparing with conventional polymers. The cost of production is 20% to 80% higher than conventional plastics, as most of the methods are still in their early stages of development. Only a few companies currently produce PHAs at either pilot-scale or at industrial-scale levels. At present, many studies are being developed to optimize the production techniques and materials, to reduce costs and turn biopolymers as commercially attractive as petroleum polymers (Kourmentza et al., 2017; *Polyhydroxyalkanoate (PHA) Market Share, Size | 2022 - 2027 | MarketsandMarkets*, n.d.).

## 1.4. PHA

### 1.4.1. Discovery and function

Microbial polyhydroxyalkanoates (PHAs) are a family of aliphatic biopolyesters, synthesized by numerous microorganisms, as intracellular carbon, and energy reserve materials, stored in form of granules, as seen in Figure 5 (Koller, 2017; Surendran et al., 2020). Poly-R-3-hydroxybutyrate (P3HB) was the first PHAs described in 1927 (Lemoigne, 1927), but over 150 different polyhydroxyalkanoic acid

monomers that constitute PHAs family have been recorded and the number is continuously growing, due to several potential combinations of the monomer units (Mitra et al., 2020).

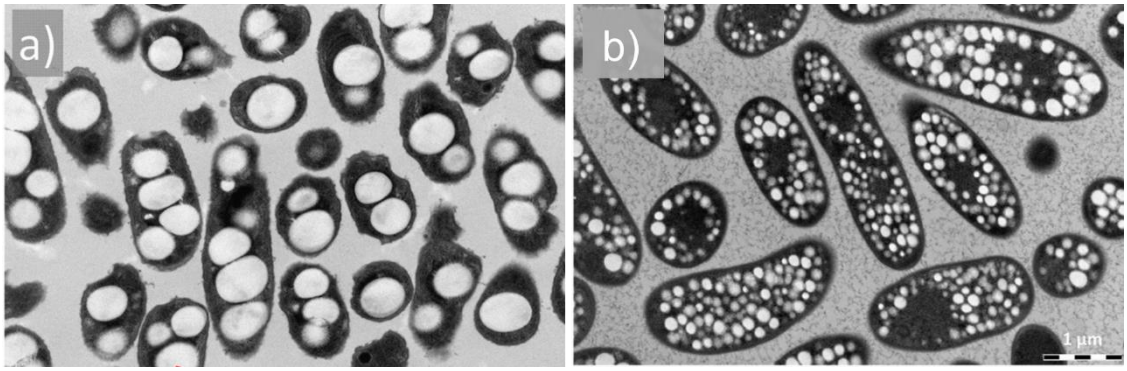


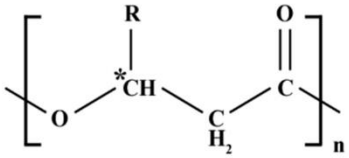

**Figure 5.** Morphology of PHAs granules in PHAs-producing bacteria. A) PHAs granules in the cells of the mesophilic PHAs producer *Cupriavidus necator* H16. B) PHAs granules in the cells of the halophilic PHAs producer *Halomonas hydrothermalis.* Retrieved from (Obruca et al., 2020).

PHAs granules are synthesized in the cytoplasm from a wide variety of microorganisms, both Gram-positive and Gram-negative, with the function of storing carbon and energy as a survival mechanism (Muhammadi et al., 2015). This happens when these microorganisms encounter stress conditions caused by a nutritional deficiency of magnesium, nitrogen, oxygen, and phosphorus, for instance, and simultaneously excess carbon source (Sagong et al., 2018). This will lead to an accumulation of PHAs granules that will be broken down to fuel the bacterial metabolism when the carbon supply is depleted. More than 300 different microorganisms have been identified to accumulate PHAs both aerobically and anaerobically (Steinbüchel & Füchtenbusch, 1998).

Plants can also be engineered to produce PHAs, however due to the negative impact that high levels of polymers have on plants growth, large scale production of PHAs is currently not feasible *via* plant cells (Bohmert et al., 2002).

### 1.4.2. Basic structure

A PHA molecule consists of monomer units of (R)-hydroxy fatty acids, that are linked via an ester bond between the hydroxyl and the carboxyl groups of neighboring monomers, as seen in Figure 6. Depending on the side chain R, several types of PHA can be formed. In the case of P3HB, the side chain corresponds to a methyl group (Naser et al., 2021).




Poly(3-hydroxyalkanoate)

| *R* group | Carbon no. | PHA polymer |
|---|---|---|
| methyl | $C_4$ | Poly(3-hydroxybutyrate) |
| ethyl | $C_5$ | Poly(3-hydroxyvalerate) |
| propyl | $C_6$ | Poly(3-hydroxyhexanoate) |
| butyl | $C_7$ | Poly(3-hydroxyheptanoate) |
| pentyl | $C_8$ | Poly(3-hydroxyoctanoate) |
| hexyl | $C_9$ | Poly(3-hydroxynonanoate) |
| heptyl | $C_{10}$ | Poly(3-hydroxydecanoate) |
| octyl | $C_{11}$ | Poly(3-hydroxyundecanoate) |
| nonyl | $C_{12}$ | Poly(3-hydroxydodecanoate) |
| decyl | $C_{13}$ | Poly(3-hydroxytridecanoate) |
| undecyl | $C_{14}$ | Poly(3-hydroxytetradecanoate) |
| dodecyl | $C_{15}$ | Poly(3-hydroxypentadecanoate) |
| tridecyl | $C_{16}$ | Poly(3-hydroxyhexadecanoate) |

**Figure 6.** Polyhydroxyalkanoate (PHA) monomer repeating unit. The nomenclature and carbon number for PHA compounds is determined by the functional R group. Adapted from (G. Y. A. Tan et al., 2014).

Generally, PHAs are classified based on the number of carbon atoms in the monomer units and these can be divided into three major groups. Short chain length polyhydroxyalkanoates (SCL) consist of monomeric building blocks of 3 to 5 carbon atoms. SCL-PHAs tend to be hard, crystalline, and brittle polymers with high melting points. They also lack the superior mechanical properties required for biomedical and packaging film applications (Lukasiewicz et al., 2018). The most well-known PHAs, P3HB and poly (3-hydroxybutyrate-co- 3-hydroxyvalerate) (PHBV) are both short chain PHAs and represent the most common forms commercially available (Sharma et al., 2021). P3HB has the particularity of being the most frequent PHA-type naturally synthesized by bacteria. It is regarded as promising for biotechnology and, therefore, it is the PHA most produced in industry, nowadays. It has properties attractive for biomedical application, is biodegradable within in the body and shares mechanical properties with PET having high strength and high rigidity (Alves et al., 2017).

Medium chain length polyhydroxyalkanoates (MCL) consist of monomeric units of 6 to 14 carbon atoms, for example, poly (3-hydroxyoctanoate), exhibit elastomeric properties as natural rubber but have very low mechanical strength which limits the application of these PHAs (Koller et al., 2010). Finally, long chain length polyhydroxyalkanoates (LCL), such as poly (3-hydroxypentadecanoate), contain monomer building blocks of 15 or more carbons (Sharma et al., 2021) and are being used for several medical applications like tissue engineering and biodegradable implants (Ray & Kalia, 2017).

The chemical and physical properties of PHAs vary considerably with monomer composition. Biodegradability is one of the most distinctive properties of PHA. Several microorganisms existing in nature produce PHA degrading enzymes, that break down the biopolymers into water and $CO_2$ if buried in soil, using it as a source of nutrition (Samrot et al., 2021).

### 1.4.3. PHA Applications and properties

PHA generally exhibits similar properties to petroleum-based polymers. Some physical properties of PHA include insolubility in water, good resistance to UV rays, stiffness, high degree of polymerization, biodegradability and thermo-plasticity. However, the physicochemical characteristics of PHAs differ

depending on the bacterial producer, growth conditions, and the number of carbon atoms in a monomer unit (Sharma et al., 2021).

The main target markets for this family of bioplastics are packaging, coatings, agriculture, pharmaceutical and medical application (G. Q. Chen, 2009; Sharma et al., 2021). PHAs have many possible agricultural applications, such as fertilizer encapsulation for gradual release, biodegradable plastic sheets for crop protection, biodegradable containers for green-house facilities and seed encapsulation (González et al., 2020). They can also be utilized as packaging films in containers, paper coatings, and shopping bags. Among the different applications of PHAs, much attention has been obtained in medical and pharmaceutical fields. The biodegradability and biocompatibility of PHAs for medical applications are appealing because they can be injected into the human body without the need to be scavenged as they result from cell metabolism. Nonetheless, in health industry, it is highly important to have particularly high-quality purification and extraction methods to ensure it does not contain harmful bacterial endotoxin, as PHA come in touch with blood cells, when administered (Ulery et al., 2011).

Some other medical applications of PHA are wound dressing, medical devices such as orthopedic pins, stents, nerve guides, implants, and bone marrow scaffolds (Gadgil et al., 2017). There have been reports on PHA degrading enzymes activity in a rat gastrointestine near a PHA insert. This deterioration of PHA paired with the liberation of a bioactive compound, indicates a great potential use in drug delivery systems (Löbler et al., 1999).

PHAs monomers can also be cleaved and reacted with methanol to form methyl esters to be used as a biofuel. Other applications include diapers, toners, and adhesives, as well as electronic devices (Muhamad et al., 2015).

### 1.4.4. Biosynthesis

There are three main pathways for PHA production, depicted in Figure 7, primarily dependent on the carbon source and the genomic structure of the bacterial strain. Short chain length PHA (SCL-PHA) are synthesized through a metabolic pathway consisting of three enzymatically catalyzed reactions. Firstly, in the metabolic pathway I, the enzyme β-ketothiolase (encoded by PhaA) condenses two acetyl-CoA molecules, resulting in the formation of acetoacetyl-CoA, which is subsequently reduced by acetoacetyl-CoA dehydrogenase (encoded by PhaB) to R-3-hydroxybutyrylCoA, regenerating the oxidized form of redox equivalent. Finally, PHA synthase (encoded by PhaC) catalyzes the polymerization of R-3-hydrobuytyryl-CoA into the long linear polyester chain of P3HB (Kessler & Witholt, 2001; C. Liu et al., 2021).

The synthesis of medium-chain-length PHAs monomers (MCL-PHA), can be obtained either by pathway II or III. Pathway II consists of fatty acid degradation by β-oxidation mechanisms and is predominantly reported in bacteria that can utilize fatty acids as a primary carbon feedstock. Pathway III consists in a fatty acid biosynthesis pathway (C. Liu et al., 2021).

PHA metabolism is frequently referred to as “PHA cycle”, as biosynthesis and degradation of PHA are interconnected and occur simultaneously. Thus, even with conditions favoring PHA biosynthesis, PHA

depolymerases are active to some extent, ensuring balanced flow between storage and usage of carbon and energy (Kadouri et al., 2005; Prieto et al., 2016).

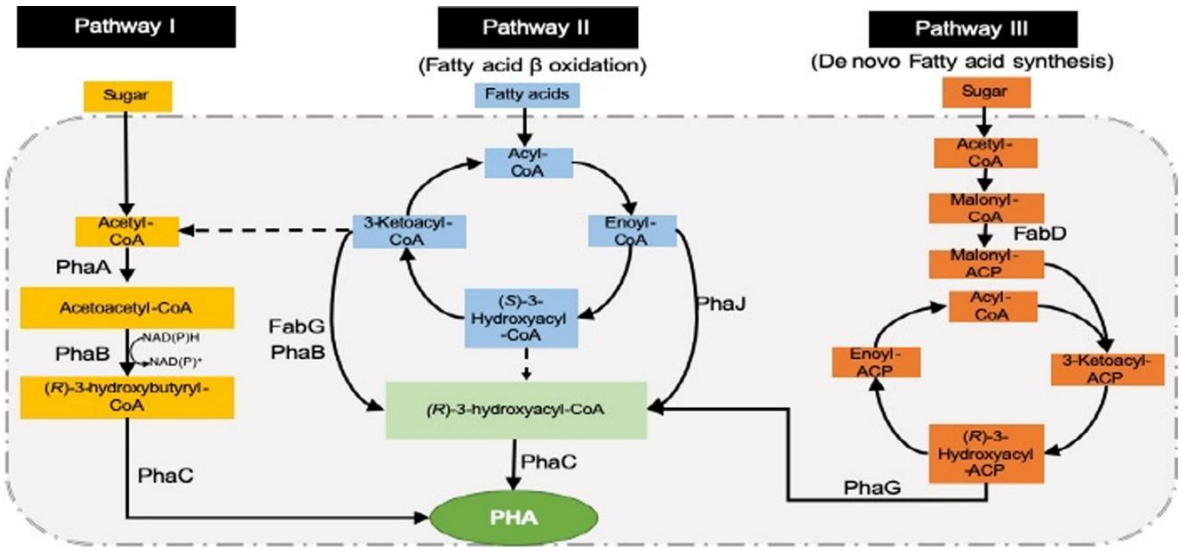



**Figure 7.** The 3 main metabolic pathways for PHA biosynthesis. PhaA is β-ketothiolase; PhaB is acetoacetyl coenzyme A(CoA) reductase: PhaC is PHA synthase; FabG is 3-ketoacyl acyl carrier protein (ACP) reductase; PhaG is acyl-ACP-CoA transacylase; PhaJ is enoyl-C.Retrieved from (Khatami et al., 2021).

## 1.5. SUSTAINABLE PRODUCTION OF PHA

As mentioned before, the current PHA industry is facing several challenges, particularly related to its high production costs. To address these drawbacks, it is crucial to develop new technologies that fall under the umbrella of "Next Generation Industrial Biotechnology" (NGIB). NGIB has the potential to overcome the limitations of the present industrial biotechnology approaches that hinder competitiveness (G. Q. Chen & Jiang, 2018), such as fresh water and heavy energy consumption, microbial contaminations, complexity of sterile operations, poor oxygen utilization in cultures, expensive substrates, low substrate to product conversion efficiency, complications regarding cells and broth separation, large amount of wastewater production, discontinuous processes, heavy labor involvements and expensive bioreactors (G. Q. Chen & Liu, 2021). Table 2 provides a summary of the current industry problems and their possible solutions.

In the following sections, possible solutions to problems associated with current biotechnology industry will be discussed, highlighting key factors that can make the bioplastics industry more efficient. These factors include the carbon source, strategies to avoid contamination in open processes and different fermentation modes.

**Table 2.** Comparison between the problems of the current PHA industry and the solutions of the future PHA industry. Adapted from (G. Q. Chen & Jiang, 2018; G. Q. Chen & Liu, 2021).

| | Current PHA Industry | Future PHA Industry | Notes |
|---|---|---|---|
| **Microbial strain** | Mesophilic bacteria such as *E. coli, Pseudomonas spp., Bacillus spp*. | Extremophilic bacteria, mixed microbial consortia | Additionally, using synthetic engineering to improve growth and uptake of low-cost substrates |
| **Sterilization** | Needed | Not needed | |
| **Contamination** | High costs and labor to prevent contamination | No | |
| **Fresh water** | Heavy consumption | Seawater or freshwater recycling | |
| **Growth Process** | Batch or fed batch | Open and continuous | |
| **Substrates** | Commercial sugar (mostly glucose and fatty acids) | Industrial and agriculture wastes | |
| **Bioreactor** | Stainless steel | Plastics, ceramics, cements | |
| **Wastewater** | Discharged and treated | Recycle | |
| **Downstream** | Complicated | Improved by morphology engineering | |
| **Labor** | Complicated | Simplified | |

### 1.5.1. Carbon source

The main carbon sources employed in traditional PHA manufacturing are typically raw materials, such as pure carbohydrates (glucose, sucrose, maltose, starch) and fatty acids, as well as their derivatives, including methanol and alkanes. However, these carbon sources can account for approximately half of all expenditures in the production process (Khosravi-Darani et al., 2013), making it important to develop low-cost alternative feedstocks that can increase PHA production sustainability while achieving commercial viability through fermentation. Substrates can generally be divided into three categories: simple sugars (monosaccharides), triacylglycerol and hydrocarbons. While most PHA-producing microorganisms can use simple sugars, the use of triacylglycerol has only been reported for some microorganisms (Jiang et al., 2016). The selection of an appropriate carbon source is crucial in PHA production, as it can affect the yield, productivity, and composition of the resulting biopolymer. Thus, the identification and optimization of alternative, low-cost carbon feedstocks are an important area of research in the field of PHA production.

#### *1.5.1.1. Requirements*

Inexpensive carbon sources, such as agricultural wastes and industrial by-products, may be considered as potential alternative. However, it is important to note that these sources can incur additional costs due to pre-treatment steps, extended cultivation times, and purification (Chanprateep, 2010). While the options mentioned below could be potential alternatives to cheaper carbon source, several factors need

to be considered for sustainable and effective commercial PHA production, as it greatly affects manufacturing economics (Kourmentza et al., 2017; Sirohi et al., 2020).

The substrate must ensure quality, as it affects the cell growth, redox potential of cell metabolism, productivity, molecular mass and carbon yield (Favaro et al., 2018). Moreover, the utilization of waste biomass resources is preferable, as it does not compete with food and feed supply destined for human consumption, unlike purified sugars, edible vegetable oils, and food crops (Kosseva & Rusbandi, 2018).

Furthermore, it is necessary to fulfill the minimum quality requirements and establish a stable logistics program to ensure a steady feedstock. This includes easy collection and production, transportation, storage, continuous availability and global price consistency. The choice of the microbial strains also affects the effective utilization of waste streams and their conversion into PHA (Bosworth, 2020; Chanprateep, 2010). Figure 8 illustrates a range of feedstocks and the corresponding pre-treatment processes employed in the production of PHAs.

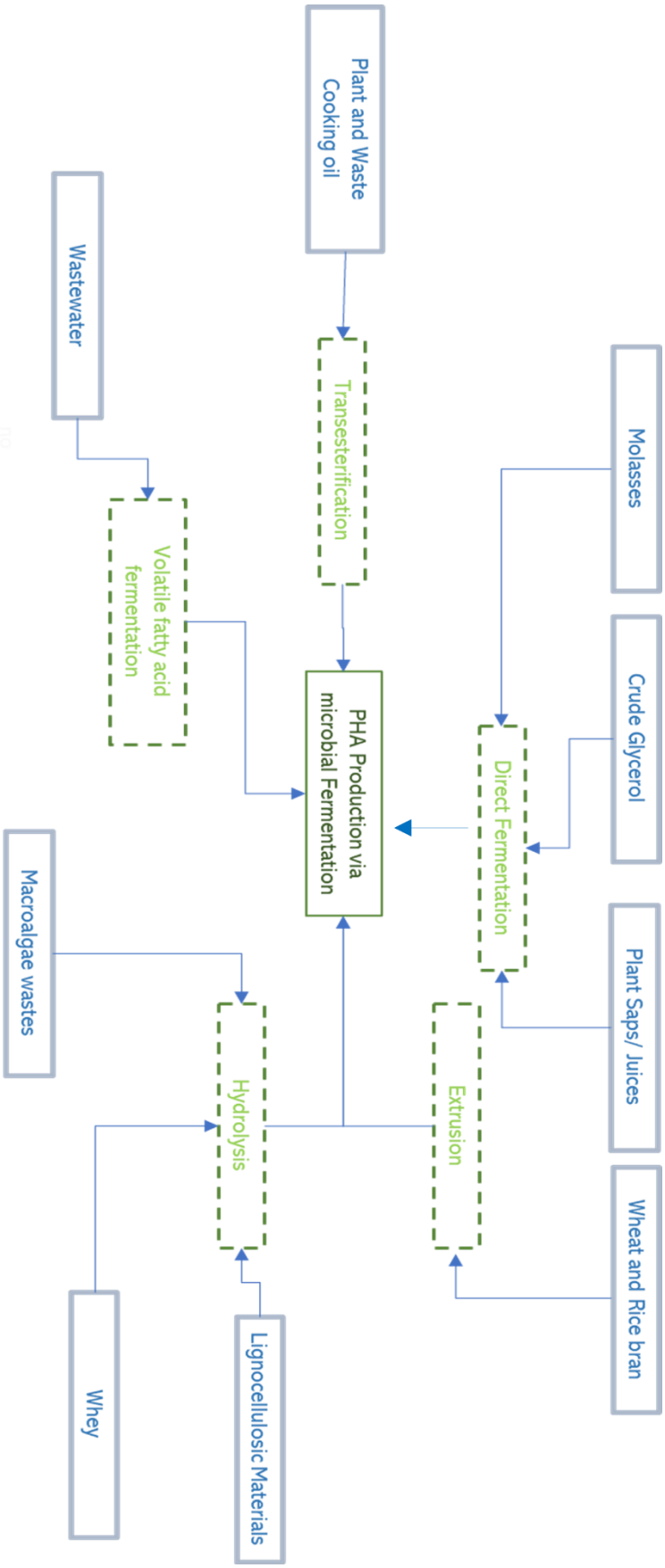



**Figure 8.** Various feedstocks and their respective pre-treatments steps for production of PHAs. Adapted from Aslan et al., 2016.

#### *1.5.1.2. Carbohydrates*

Carbohydrates can be classified into monosaccharides, oligosaccharides and polysaccharides which can be hydrolyzed to simple sugars (monosaccharides). Polysaccharides are polymerized carbohydrates, predominantly starch, cellulose, and hemicellulose. Most commonly, polysaccharides cannot be fermented without being hydrolyzed, monosaccharides and disaccharides can be metabolized directly to create PHAs (Jiang et al., 2016).

### Sucrose

Sucrose is usually obtained from sugar-bearing plants, such as sugar beet in temperate climates and sugar cane in tropical climates. Composed of one glucose unit connected by a glycoside bond to a fructose molecule, sucrose is hydrolyzed extracellularly into glucose and fructose by microorganisms in the early stages of cultivation, which are then used for cell development (Reid & Abratt, 2005).

Sugary by-products are also a common source of raw material for bioconversion into high-value products. Molasses, a by-product of the sugar industry, is currently most employed (as a carbon source) for food, feed, and industrial scale fermentations of ethanol, due to its abundance and low cost. The composition of molasses can vary depending on the source, but in general, it contains a mixture of different types of sugar. The main components of molasses, on average, are sucrose (30-35%), fructose and glucose (10-25%), non-sugar compounds (2-3%), mineral content, and moisture, with around 45-55% of total fermentable sugars (Solomon, 2011). However, the amount of molasses available is currently insufficient to meet the growing demand for PHA manufacturing, mainly due to improper management, such as storage, packaging and transportation (Jamir et al., 2021; WJ Page, 1993).

### Lignocellulosic-derived sugars

Considering that starch, sucrose, and lactose are essential food sources for humans and animals, the extraction of glucose and other simple sugars from lignocelluloses has attracted a lot of interest. Lignocellulose is the most common and long-lasting carbon resource, primarily composed of cellulose, hemicellulose, and lignin (Vicuna et al., 1987). Bagasse, wheat straw, rice straw, and wheat bran are examples of sources of agricultural residues that can be resourced into carbon substrates. However, they usually require pre-treatments to convert them to simpler sugars. During this process, inhibitory compounds can be produced, and a step of detoxification is needed to remove them. These treatment processes add complexity to the process and increase total production costs (Govil et al., 2020; Obruca et al., 2015).

### Seaweed and seaweed waste-derived sugars

The seaweed industry produces around 12 million tons per year, according to a 2018 report by the Food and Agriculture Organization of the United Nations (Fao, 2018). The main markets for seaweed are food, industrial specialties, fertilizers, cosmetics, pharmaceuticals and feed. Due to its high carbohydrate content, seaweed may also serve as a carbon platform for biological processes, namely in the production of bio-combustibles and bioplastics (Jung et al., 2013; Kartik et al., 2021). Additionally, as macroalgae have a low lignin content, harsh pre-treatments for biomass delignification may be avoided.

Seaweed wastes from phycocolloid and alginate extraction industries are carbohydrate-rich and are often underutilized (Cebrián-Lloret et al., 2022; Tůma et al., 2020). Among the red macroalgae, *Gelidium* is most frequently associated with commercial agar used in food, biological research, and pharmaceutical manufacturing. Agar is the main polysaccharide in *Gelidium* cell walls, accounting for 50% of the total seaweed dry weight, followed by cellulose (a structural component) and floridean starch (an energy reserve) (Gomes-Dias et al., 2020). Upon hydrolysis of the carbohydrate fraction, the main monosaccharides in *Gelidium* hydrolysates are glucose (from cellulose and starch) and galactose plus L-anhydrogalactose from agar. After agar extraction, *Gelidium* residues are still an interesting biomass to be used as a carbon platform in biological processes due to its high carbohydrate content, mainly cellulose (approximately 30%) (Tůma et al., 2020). Thus, the full valorization of the carbohydrate fraction in macroalgae helps to ensure the long-term viability of the seaweed industry (Araújo et al., 2021; Tůma et al., 2020).

#### *1.5.1.3. Triacylglycerols*

Triacylglycerols are the main components of animal fats and plant oils. Triacylglycerols are simply three fatty acids attached to a glycerol backbone. Waste animal fats from food processing and slaughtering industries have a huge potential as carbon source for PHA production but might be problematic during fermentation procedures due to their high melting point. Plant oils are relatively easy to ferment due to their liquid form (Jiang et al., 2016).

Crude glycerol gained more interest as feedstock as large quantities are produced from various industrial processes (Cavalheiro et al., 2009; Shay, 1993), particularly in biodiesel production. In the biodiesel industry, with every 100 tons of biodiesel produced via transesterification of vegetable oils or animal fats, about 10 tons of crude glycerol is produced (Andreesen et al., 2010). Thus, the conversion of crude glycerol into PHAs is a viable way to offset the expense of biodiesel production while also valorizing the crude glycerol.

Additionally, waste plant frying oil is also a low-cost, long-term source for PHA synthesis. The oils are labelled as industrial waste after several uses. The oils undergo several chemical reactions, such as hydrolysis, thermal oxidation and polymerization when repeatedly exposed for a long time at elevated temperatures. Thus, although the majority of waste frying oil is composed of triacylglycerol, the remainder consists of oil degradation products (Rincón et al., 2010).

#### *1.5.1.4. Hydrocarbons*

Hydrocarbons can be metabolized by many microorganisms. However, not all microorganisms that consume hydrocarbons can accumulate PHAs. In 1983, de Smet et al. reported low PHA productivity during growth on hydrocarbons, making these substrates an unlikely future choice for PHA production (de Smet et al., 1983). However, recent developments indicate the possibility of optimizing processes for hydrocarbon biodegradation and PHA accumulation with both terrestrial and marine hydrocarbon-degrading bacteria (Crisafi et al., 2022).

Hydrocarbon contamination is of serious concern because of its widespread effect on all forms of life, which occurs due to toxic organic substances, petroleum, and pesticides (Srivastava et al., 2019). The

environments with high levels of hydrocarbon present an imbalanced C:N ratio, which is a stress condition favorable for bacteria to produce PHAs. Current bioremediation research has focused on combining the ability of these bacteria to degrade hydrocarbons, whilst producing a high-value-added material, such as PHA. Numerous hydrocarbon-degrading bacteria, including *Pseudomonas, Alcanivorax, and Marinobacter* spp., have been identified among marine PHA-degrading bacteria (Crisafi et al., 2022).

#### *1.5.1.5. Substrates from residual streams*

Another potential substrate is wastewater from various industrial practices, such as biodiesel production, food processing, municipal sewage, and brewery waste. These wastewaters are typically rich in both organic and inorganic nutrients, making them highly accessible and cost-effective as a substrate for PHA production.

The first step in the PHA production process using wastewater involves the conversion of the organic carbon sources present in the wastewater into volatile fatty acids using anaerobic activated sludge. In the second step, mixed cell cultures are used to convert the volatile fatty acid into PHA (Aslan et al., 2016; Sukphun et al., 2021). It is worth noting that the use of wastewater as a substrate for PHA production has the added benefit of reducing environmental pollution by treating the wastewater and converting it into a valuable biopolymer. However, one of the main challenges is the heterogeneity of the substrate composition, which can vary depending on the type of industry and the process used and can affect the quality and quantity of PHA produced. Therefore, it is important to ensure proper management of any potential contaminants in the wastewater to avoid negative impacts on the final PHA product (Lam et al., 2017).

### 1.5.2. Sterile versus open PHA producing process

The amount of PHAs granules formation may vary between different organisms (Zhang et al., 2018) due to factors such as substrates used, the nature of polymerization, and the various metabolic pathways involved in its synthesis (G. Q. Chen & Jiang, 2018). In addition to the microorganism's production rates, the stability and biological safety of the microorganism, PHA extractability, and the molecular weight of the agglomerated PHA are also determinant factors for the selection of the most suitable type of microorganism, depending on the desired application, when designing the biological process (Naser et al., 2021).

Different mesophilic bacterial strains have been screened according to their polyhydroxyalkanoates production capability, but only a few are actively researched for their high efficiency and high production rates. *Burkholderia sacchari* DSM 17165 (Cesário et al., 2014), *Bacillus megaterium*, *Pseudomonas aeruginosa*, *Pseudomonas putida, Pseudomonas fluorescens, Pseudomonas oleovorans* (Chaudhry et al., 2011), *Cupriavidus necator* (Cavalheiro et al., 2009) and recombinant *Escherichia coli* are a few examples of highly used bacteria to produce PHAs (Koller, 2017; Nahar et al., 2019). Other examples can be found in recently published reviews, such as (Alvarez Chavez et al., 2022; Crisafi et al., 2022). Although being good PHA producers, these bacterial species are mesophilic and grow at conditions that

are prone to contamination. For this reason, sterile conditions must usually be used to assure high plastic yields (G. Q. Chen & Jiang, 2018).

Contamination is a common problem related with fermentation, regardless of the substrates or fermentation products, and can result in significant financial burden, especially at industrial-scale (D. Tan et al., 2021). Some sterilization methods include heating, filtration, irradiations, sonic vibration, and chemical agents (Berovič, 2011). Among these, heating is the most frequently used due to its efficiency and simplicity but consumes a substantial amount of energy, which accounts for a significant portion of the production cost, affecting the overall economy process (Moustogianni et al., 2015).

As mentioned before, the sterilization processes require extra caution in terms of labor, energy, equipment, all of which contribute to higher manufacturing costs and process complexity. To avoid extra costs, few fermentation procedures have been designed to be carried out under open conditions, eliminating the sterilization step, because microbial contamination is improbable (D. Tan et al., 2011). Some possible strategies to implement non-sterile fermentations include the starvation strategy (Z. Chen & Wan, 2017), substrate and product inhibition (Taskin et al., 2016), antimicrobial agents (Moustogianni et al., 2015), high salts concentration, extreme pH (Yen et al., 2015) and temperatures (Qin et al., 2009), inoculation size (W. H. Chen et al., 2013), mixed culture (Reungsang et al., 2013), and metabolic engineering (Shaw et al., 2016) . It is critical to develop efficient, cost-effective, and resilient fermentation methods to give competitive alternatives to petroleum-based biopolymers. Non-sterile fermentation may present a viable technique for cost reduction in fermentation procedures in future. However, conducting fermentation in non-sterile conditions without jeopardizing the bioreactor efficiency remains the main challenge (Z. Chen & Wan, 2017).

### Thermophilic bacteria

The thermophilic fermentation has received a lot of attention since it operates at 50–60ºC, which is a way to avoid contamination from mesophilic bacteria (Khatami et al., 2021). The procedure also has the benefits of eliminating costs associated with sterilization, can be used in both semi-continuous and continuous operation modes and requires less water consumption (G. Q. Chen & Jiang, 2018). Moreover, as thermostable enzymes are attractive candidates to breakdown lignocellulosic biomass, these microorganisms could improve the sustainable conversion of unprocessed lignocellulosic wastes to bioplastics (Koller, 2017). In the recent decade, a growing trend has been observed towards the discovery of various thermophilic and thermotolerant bacterial strains for industrial-scale PHA synthesis. Nonetheless, there is still a need for more research mainly due to lack of knowledge concerning these strains (Chavan et al., 2021).

### Mixed microbial communities

Another method for the production of PHAs is the usage of mixed microbial communities (MMC). This approach selects microorganisms capable of accumulating PHAs based on operational conditions imposed on the biological system, using ecological selection principles. The MMC strategy offers several advantages over processes based on axenic cultures, as it allows for the production under non-sterile conditions and on a variety of low-value substrates, such as industrial wastes (Kourmentza et al., 2017).

In addition, a diverse microbial community in MMC can increase the stability of the system and reduce the risk of unwanted genetic variations. The presence of multiple microbial species minimizes the impact of random mutations or genetic drift. Furthermore, the cooperation between the species can increase the efficiency of PHA production and the ability to withstand disruptions in the environment. This production method provides greater stability and robustness (Bhatia et al., 2018; Pagliano et al., 2021).

Over the last few decades, MMC processes have progressed from lab research to pilot prototypes for the production of PHA biopolymers using activated sludge as inoculum. Recently, pilot-scale prototypes have demonstrated PHA production from MMC integrated into both municipal and industrial wastewater treatment, improving the economic and environmental performance of PHA manufacturing (Arcos-Hernández, et al., 2015; Lorini et al., 2021). Working with mixed cultures or natural consortia under unsterile conditions on an industrial scale allows for continuous production, significantly lowering production costs. This approach offers a promising solution for producing environmentally-friendly plastic and has the potential to revolutionize the plastic industry (Morgan-Sagastume, 2016; Salehizadeh & van Loosdrecht, 2004).

### Halophilic and halotolerant bacteria

Halophiles are a unique and diverse group of microorganisms, found in all domains of life (Bacteria, Archaea, and Eukarya) and can thrive in diverse hypersaline environments such as saline lakes, salt pans, and salt marshes (Edbeib et al., 2016). Depending on their optimal salinity for growth, they can be classified as mild, growing between of 1–6% (w/v) of salt concentration, moderate at 7–15 %(w/v), and extreme halophiles enduring concentrations higher than 15% (w/v) (Scheuer et al., 2008). The adaptation of the surrounding hypersaline environment is maintained through a high osmotic pressure created by high accumulation of $K^+$ salts or compatible solutes (Youssef et al., 2014).

Compatible solutes are small organic molecules that act as osmoprotectants, with the ability of supporting cellular proteins, providing a hydration shell and stabilize their tertiary structures without interfering in cell metabolism (Kolp et al., 2006). They usually have low molecular weight and are highly water soluble allowing their accumulation in high concentration in the cytoplasm (Galinski, 1993). Due to these compatible solutes and their great biotechnological potential, halophiles have gained a lot of attention. Some examples include ectoine, an osmostress protectant used in cosmetic and food industry (Kolp et al., 2006), and hydrolytic enzymes which can be employed for industrial processes under hypersaline conditions (Delgado-García et al., 2012).

More recently, the inherent ability of these extremophile to accumulate PHA intracellularly created a growing commercial interest of bioplastic production. The high saline media has the advantage of reducing/eliminating cross-contamination issues, not allowing the growth of non-halophilic microorganisms. This results in cost savings associated with energy usage for sterilizing bioreactors and tubings, as production processes do not require rigorous and strict sterile conditions; decrease in freshwater consumption in medium preparation; and a more efficient continuous fermentation processes (Kourmentza et al., 2017). In addition, compared to the non-halophilic microbes, halophiles yield other advantages to become a viable candidate for large scale PHA production, as they can use a variety of

low-cost source substrates. Also, due to the high intracellular osmotic pressure, cells can easily be lysed in salt-deficient water, causing hypo-osmotic shock, facilitating the recovery of PHA (Obruca et al., 2018; Quillaguamán et al., 2010).

While there are advantages to using high salinity concentrations in fermentation processes, scaling up production can pose some challenges. One obstacle is corrosion caused by the high salt concentration medium in fermentation equipment, which can lead to costly repairs and equipment replacement. However, this issue can be overcome by using low-cost materials such as plastics, ceramics, or carbon steel in the design and construction of fermentation and piping systems, as sterilization procedures are not required. By using these alternative materials, corrosion can be avoided, and the equipment can be more durable and cost-effective (G. Q. Chen & Liu, 2021).

More recently, some researchers have turned their attention to members of the family *Halomonadaceae*, belonging to the class *Gammaproteobacteria*, that consists mostly of marine and moderately halophilic microorganisms with a wide range of phenotypes. Some species such as *Halomonas boliviensis* (Quillaguamán et al., 2008), *Halomonas* sp. TD01 ((D. Tan et al., 2011) and *Halomonas* sp. KM-1 (Kawata et al., 2013), have been found to accumulate large amounts of PHAs from various inexpensive carbon sources (de La Haba et al., 2014). More specifically, *H. boliviensis* has been reported in the literature to be able to convert diverse carbohydrates into P3HB. Table 3 illustrates some of the P3HB production studies with different carbon sources and their yields. These results support the potential of using *H. boliviensis* for the commercial production of P3HB. However, further studies under non-sterile conditions are required, in order to access the potential of this strain, in large scale, in a continuous and open conditions for biopolymer production. Additionally, other strains of the genus *Halomonas* have already been reported in “Non-Sterility” studies. For instance, in one study, *H. campaniensis* was reported to maintain 65 days of contamination-free growth in artificial seawater under open and continuous conditions (Yue et al., 2014). Tan et al. 2011 also achieved production of P3HB under an open unsterile with *Halomonas* TD01 for 14 days without contamination (D. Tan et al., 2011).

**Table 3.** Examples of PHA production by *Halomonas boliviensis* using various substrates. Retrieved from (Arcila-Echavarría, 2016; Mitra et al., 2020).

| Carbon Source | Fermentation Operation mode | PHA content (%) | References |
|---|---|---|---|
| EFB* hydrolysate | Shake flask | 30.8 | Arcila Echavarría, 2016 |
| Glucose | Shake flask | 58.3 | Arcila Echavarría, 2016 |
| Starch hydrolysate | 2-L fermenter | 35-49 | Quillaguamán et al., 2005 |
| Glucose | Shake flask | 70.6 | Arcila-Echavarría et al., 2021 |
| EFB hydrolysate | Shake flask | 35.7 | Arcila-Echavarría et al., 2021 |
| Maltose | Shake flask | 58.8 | Quillaguamán et al., 2005 |
| Butyric acid and sodium acetate | Shake flask | 54 | Quillaguamán et al., 2006 |
| Butyric acid and sodium acetate | 2-L fermenter | 88 | Quillaguamán et al., 2006 |
| Glucose or sucrose | 2-L fermenter | ±55 | Quillaguamán et al., 2006 |
| Wheat bran and digested potato waste | 2-L fermenter | 43 | Van-Thuoc et al., 2008 |
| Volatile fatty acid (VFA) | 2-L fermenter | 70 | García-Torreiro et al., 2016 |

*EFB (Empty Fruit Bunches) refers to the residual biomass produced from the palm oil industry.

### 1.5.3. Fermentation modes

In industrial microbiology production there are four major types of liquid fermentation processes used: batch fermentation, fed-batch fermentation, continuous fermentation, and semi-continuous fermentation. Most bioprocess are performed in batch, however, in recent years, fed-batch has gained relevance. Continuous processes are very appealing but present some limitations and, therefore, few processes are implemented on a large scale. The concept of semi-continuous is traditionally associated with fed-batch, but in this case, it refers to an approach where there is a periodic removal of a fraction of the culture and replacement with an equal volume of fresh culture medium (Rosa et al., 2019).

#### *1.5.3.1. Batch and Fed batch*

In a batch/fed-batch process, a seed culture is inoculated with a chosen substrate, and under proper conditions, cell growth is conducted over a short period of time. At the end of the cultivation phase, the

broth is collected, added to a fermentation media and after fermentation is ceased, the final products are harvested (Henley, 2019; Rosa et al., 2019). However, the substantial difference between these two processes is that a batch process is a closed system, meaning that all nutrients are provided at the beginning of the cultivation, an no additions occur during the entire bioprocess. Consequently, with microbial growth, the nutrients are gradually consumed, and by-products accumulate. The bioprocess ends when the nutrients are fully consumed. This strategy is often used to optimize conditions at early stages of experimental design (Rosa et al., 2019; Yang & Sha, 2019).

In contrast, a fed-batch process is a modification of the batch fermentation, being a partly open system, where nutrients are added aseptically. Microorganisms are inoculated and cultured in a batch system during an established time, with incremental nutrient and substrate addition so that fermentation performance is not affected during the time course. This process offers a wide range of control strategies and is also suitable for highly specialized applications. It is the most common mode of operation in the bioprocess industry (Yang & Sha, 2019).

The advantage of feeding during cultivation is that it allows achievement of higher product quantities overall. However, it may lengthen the processing time, allowing inhibitory substances to accumulate, and increase the risk of cross-contamination. Under a batch fermentation methodology, total process duration is reduced, resulting in an operational expenditures reduction. The global economy process is an important factor when establishing the operation mode (Henley, 2019; Rosa et al., 2019).

#### *1.5.3.2. Continuous fermentation*

In continuous fermentation, fresh medium is continuously fed to the fermenter, while exhaust medium and cells are extracted at the same time. Toxic metabolites are eliminated from the culture, and consumed nutrients are replenished. The culture volume remains constant when performed at a steady state, with bacterial cultures being able to endure such conditions for days, weeks, or even months, decreasing downtime and improving the process's economic competitiveness (Rosa et al., 2019; Yang & Sha, 2019).

Although current biotechnology industry is mainly based on continuous fermentation processes to attend market needs, there are some obstacles to be tackled. Lower concentration of final products, more complicated downstream processing, difficult process flow sheet design, and long-term process sterility maintenance present some of the barriers (G. Q. Chen & Liu, 2021).

#### *1.5.3.3. Semi-Continuous fermentation*

Semi-continuous or repeated fed batch operation is thought of a hybrid between batch and continuous operations. Differences regarding semicontinuous and continuous modes rely on feed concentration, replacement rate and time between replacements (Rosa et al., 2019; Zohri et al., 2017).

The feed solution is fed at regular intervals while the effluent is withdrawn discontinuously. The key advantage of this strategy is that intermittent feeding of the substrate prevents inhibition and catabolite repression. If the substrate has an inhibitory impact, fermentation productivity is enhanced by keeping the substrate concentration low. Semi continuous fermentation relies on several advantages of both

continuous and batch operations. There is no requirement for a separate inoculum vessel, cleaning and re-sterilization time is not used up in non-productive idle time, and not much control is required. However, due to long cultivation periods and frequent handling, there is a substantial risk of contamination. In addition, greater reactor volumes are required, translating into higher investment expenses (Zohri et al., 2017).

## 1.6. DOWNSTREAM PROCESS

Another major factor that significantly contributes to the high expenses of PHA production is the downstream processing, largely due to the use of great amounts of solvents and high energy requirements (Kosseva & Rusbandi, 2018). PHAs downstream processing (Figure 9) is commonly comprised by several steps: biomass separation, pre-treatment of biomass, PHA extraction, PHA separation, PHA purification (Mannina et al., 2020).


**Figure 9.** Schematic representation of the steps involved in PHA production, from the upstream section (bacterial fermentation) to the downstream. The downstream portion is, mainly, divided in five phases. Adapted from (Kosseva & Rusbandi, 2018).

### 1.6.1. Biomass separation and pre-treatment of biomass

In biomass physical separation, there are three main methods that can be used: centrifugation, (micro)filtration, and sedimentation. The following pretreatment step is employed to enhance the yield and purity in the extraction step, aiming at weakening the cell structure that protects and surrounds the PHA granules (Jacquel et al., 2008). The pre-treatment can be biological, chemical, physical method, or it can also be a combination of two or more methods.

A traditional biological pre-treatment consists in removing biomass water molecules through heat drying or lyophilization. However, these methods can have some drawbacks as they can be quite expensive and time-consuming. In addition, it can comprise some technical difficulties that reduce the potential for industrial application. Chemical pre-treatments consist in the use of chemicals such as sodium chloride (NaCl) and sodium hypochlorite (NaClO) to cause osmotic stress to the cells, leading them to dehydrate and shrink, which will facilitate the PHA granules liberation (Anis et al., 2013). Physical methods include high temperature usage (100–121 ◦C) and ultrasonication. The high temperature promotes PHA removal with microbial cell wall disruption by denaturation of proteins, DNA and RNA (Neves & Müller, 2012), whereas ultrasonication uses acoustic waves to damage the cell wall. Both treatments anteceded non-cellular PHA mass (NPCM) digestion methods. The advantages of these method are the lack of any previous cell harvesting method and the fast pace in which is performed (Mannina et al., 2020).

Pre-treatments can increase the recovery and purity yields of the PHA extracted from a fermentation broth, but its implementation is still not feasible at the industrial scale, unless the additional expense associated with adding this step to the purification line is offset by the increased yield and purity (Kourmentza et al., 2017). Moreover, the bacterial strain, fermentation broth properties, and subsequent

PHA application all influence the pre-treatment choice. As a result, each PHA procedure must be evaluated individually from an economic, environmental, and technical standpoint (Koller et al., 2013).

### 1.6.2. Extraction of PHA

After the pre-treatments, the PHA granules recovery can be grouped mainly into solubilizing the Non-cellular PHA mass (NCPM) through chemical digestion or by solubilizing the PHA through solvent extraction and mechanical disruption.

#### *1.6.2.1. NCPM digestion*

Extraction of PHAs through chemicals digestion is advantageous because the cells do not need to be dried before solvent extraction, and consequently the energy and time required for the downstream process is reduced (Mannina et al., 2020). Additionally, since chemicals convert NPCM into water-soluble substances, the PHA granules can be easily separated by centrifugation, filtration or flotation (Koller et al., 2013).

It's important to note that when utilizing oxidants and alkalis such as sulphuric acid or sodium hydroxide (NaOH), the concentration of the chemicals must be carefully monitored, as an excess might result in not only NPCM dissolving, but also PHA granule deterioration, resulting in lower recovery and product quality (Mannina et al., 2020). The fundamental problem of this approach is dissolving the NPCM while keeping the PHA granules intact. However, because PHA granules are normally enclosed by a membrane that protects them from chemical attack, deterioration is limited (Pérez-Rivero et al., 2019).

Surfactants are another chemical group that can be used for digestion since they can infiltrate lipid membranes and cause cell membranes to break down, allowing PHA granules to escape. Anionic sodium dodecyl sulphate (SDS) is the most frequent surfactant used in PHA extraction Enzymatic cell digestion is also a method of PHA extraction, which usually involves a cocktail of enzymes in combination with heat and surfactants to improve the quality and amount of PHA produced (Pérez-Rivero et al., 2019). However, the high cost of enzymes and the complexity of the extraction method are significant barriers to its use in current industry (Mannina et al., 2020).

#### *1.6.2.2. Solvent extraction*

Currently, the most used extraction method at the industrial scale is solvent extraction. The solvent's function is to change the permeability of the cell membrane, allowing PHA to be solubilized and extracted. The most common solvents used for PHA recovery are chlorinated solvents such as chloroform, compounds such as propylene and ethylene, and mixtures of, for example, trichloroethane with water or chloroform either with ethanol, methanol, hexane or acetone (Mannina et al., 2020).

Chloroform is one of the most used solvents for polymer recovery, given that it lowers the endotoxin levels of numerous gram-positive bacteria, and consequently the extracted PHA can be employed for medical purposes (Kunasundari & Sudesh, 2011). However, because chlorinated solvents create a hazard for the environment, researchers are now focusing on green solvents that are reusable, more economic and environmentally friendly, such as dimethyl carbonate, cyclohexanone, and anisole (Koller et al., 2013; Rosenboom et al., 2022; Samorì et al., 2015).

#### *1.6.2.3. Mechanical disruption*

Bead milling and high-pressure homogenization are the most often utilized mechanical procedures for PHA extraction, as it causes little damage to the biopolymer, is an eco-friendly approach and does not require chemicals usage (Mannina et al., 2020). Mechanical digestion, even without any prior treatment, present a high yield of disintegration (Koller et al., 2013). However, it has some disadvantages, as high costs and high process time associated difficult the transition to an industrial scale (Kunasundari & Sudesh, 2011). Mechanical cell disruption can also be combined with other methods such as solvents, surfactants, or chemicals addition (Kosseva & Rusbandi, 2018).

### 1.6.3. PHA separation

After extraction, PHA is recovered from the solution through basic operations such as evaporation; precipitation, addition of precipitating agents (ethanol or methanol), water, or even by changing temperature or pH to a range where the polymer is not soluble anymore, filtration, centrifugation, sedimentation and flotation (Koller et al., 2013), which separates PHA granules from the debris according to their different affinities to air/liquid interface. Additionally, for higher-value products the biopolymer can also be retrieved with liquid-liquid extraction (Mannina et al., 2020; Pérez-Rivero et al., 2019).

### 1.6.4. PHA purification

In the final step of PHA downstream process, PHA should be purified from residues, contaminants, and endotoxins to obtain the marketable product suitable for its application. For instance, gram negative bacteria, such as *H. boliviensis*, have lipopolysaccharides (LPS) present in their outer membrane, that act as endotoxins and, in contact with blood, they induce adverse effects. Thus, medical products need to have stricter requirements on biopolymer purification. Repeated dissolution and precipitation of the polymer is a standard purification procedure for this polymer grade (Kunasundari & Sudesh, 2011; Pérez-Rivero et al., 2019).

To further reduce the endotoxin content, a bleaching step with hydrogen peroxide or sodium hypochlorite can take place. Moreover, ozone or activated charcoal treatments can solubilize contaminants and reduce the risks of working with hydrogen peroxide, as well as the biopolymer degradation caused by sodium hypochlorite (Pérez-Rivero et al., 2019). Future research should focus on purification methods that are less expensive, more effective, and have a lower environmental impact than traditional procedures (Mohammadi et al., 2012).

As the downstream process and costs are affected by intracellular accumulation of PHAs, numerous criteria should be addressed when choosing a suitable PHA recovery strategy: cell wall fragility, the type of PHA and quantity produced (high intracellular PHA concentration can increase the fragility of the cell and facilitate their release), the required purity, the impact of the extraction method over the PHAs' final molecular mass, the overall costs (including production and downstream processing), and environmental impacts (Mannina et al., 2020).

## 2. OBJECTIVES

As discussed in the previous chapters, it is crucial to examine potential answers for issues within the current biotechnology sector, while outlining factors to make the bioplastics sector more effective. The global aim of this project was to explore different strategies to lower the production costs of P3HB. To this end, the halotolerant bacteria *Halomonas boliviensis,* was chosen because it is able to convert different simple monosaccharides and some disaccharides into P3HB, combined with its ability to grow in high salinity conditions. This characteristic potentially allows the use of open cultivations, with a consequent reduction of sterility costs. Furthermore, the use of crude seaweed and residual seaweed hydrolysates as carbon source, was evaluated.

This thesis is divided in four chapters:

1. Feasibility of performing open cultivations of self-aseptic halophilic bacteria – *H. boliviensis* - and consequent effect on P3HB productivities,
2. Study of the microbial population of the open cultivations above mentioned,
3. Study *of* P3HB productivities using *Gelidium corneum* and its industrial wastes as the main carbon source in cultivations of *H. boliviensis.*
4. Industrial scale simulation and economic evaluation of P3HB productions by *H. boliviensis* under different sterility conditions,

# 3. MATERIAS AND METHODS

## 3.1. MATERIALS

**Reagents:** D(+)-glucose anhydrous 99.5 % (Thermo Fisher Scientific), D(+)-galactose ≥ 98 % (Carl Roth Chemicals, Germany), magnesium sulfate heptahydrate ≥ 99.5 % (LabChem), L-glutamic acid monosodium salt monohydrate ≥ 98% (Sigma-Aldrich), potassium di-hydrogen phosphate ($K_2HPO_4$) ≥ 98 % (PanReac Quimica SA), ammonium chloride (PanReac Quimica SA), Tris (Eurobio), Ferrous sulfate heptahydrate (Merck), sodium chloride (Sigma-Aldrich), Tryptic Soy Agar (Sigma-Aldrich), marine agar (PanReac Quimica SA), agar (LabChem), glycerol 86 - 88 % (Acros Organics), anti-foam solution (Simethicone Emulsion USP, Dow Corning), sodium Carbonate (Sigma-Aldrich), triton X 100 (Merck).

**Organic Solvents**: Sulfuric acid 96 % (Acros Organics), hydrochloric acid 37 % (Riedel-de Haën™), Sodium Hydroxide (Fisher Chemical), Ethanol absolute for HPLC (Acros Organics), Methanol (VWR Chemicals), Hexanoic acid (Acros Organics), Ethylenediamine-Tetraacetic acid (EDTA, Panreac)).

**Enzymes:** Lysozyme (Roche)

**The equipment:** Autoclave (Uniclave 88, AJC), Laminar Flow chamber (BioAir Instruments aura 2000 MAC 4 NF, BIOAIR Instruments), Orbital Shaker (Agitorb 200, Aralab), Centrifuge (Sigma), Spectrophotomer (UH 5300, Hitachi), Analytical Scale (AG204, Mettler Toledo), Incubator (Memmert), Oven (Memmert), Vortex (VELP Scientifica), pH meter (Metrohm-780), Gas Chromatography system (Hewlett-Packard, HP 5890), HPLC System (Hitachi LaChrom Elite), Gas Chromatography system (Agilent Technologies 6890N), 2 L STR Bioreactor (New Brunswick Bioflow 115), Spectrophotometer (Hitachi U-2000), NanoDrop One (Thermo Scientific).

**Computer programs:** SuperPro Designer® V.12 (Intelligen, Inc.) and BioCommand Batch Control software.

**KITs:** GeneJeT Genomic DNA Purification Kit (Thermofisher), Kit GOPOD (Megazyme) and the MIDI Inc. Instant FAME Kit

## 3.2. MICROORGANISMS HANDLING AND STORAGE

*Halomonas boliviensis* DSM 15516 was stored at -80ºC in 2 mL sterile cryotubes containing 300 µL of pure sterilized glycerol and 1500 µL of a previously grown liquid culture in the late exponential phase prepared with seeding medium (see subsection 3.4.1.1), supplemented with 20 $gL^{-1}$ of glucose and incubated at 30ºC in an orbital at 170 rpm for 16h.

## 3.3. SEAWEED BIOMASS AND SEAWEED HYDROLYSATES PRODUCTION

Crude red algae *Gelidium corneum* and industrial waste products of agar-agar extraction were supplied by Iberagar SA-Sociedade Luso-Espanhola de Colóides Marinhos (Coina, Portugal). Prior to use, the algae were oven dried at 40ºC to obtain a moisture content inferior to 10% and undergone an acid hydrolysis to convert the polysaccharides of the algae into simple sugars. Hydrolysates of the

macroalgae *G. corneum* (liquid fraction after hydrolysis) were used as substrate in the batch phase of fermentation, with its composition previously determined: 42.6 $gL^{-1}$ galactose, 13.1 $gL^{-1}$ glucose, 2.4 $gL^{-1}$ of nitrogen and 0.23 $gL^{-1}$ of phosphorus, 5-Hydroxymethylfurfural (HMF) < 0.1 $gL^{-1}$ (Table 4). Following the acid pretreatment, the residual biomass remaining after the is minimal.

**Table 4.** Previously determined composition of the hydrolysates

| Components | *Crude G. corneum* hydrolysate ($gL^{-1}$) | *G. corneum* industrial waste hydrolysate ($gL^{-1}$) |
|---|---|---|
| Galactose | 42.6 | - |
| Glucose | 13.1 | 358 |
| Nitrogen | 2.4 | 2.1 |
| Phosphorus | 0.23 | 0.4 |
| HMF | < 0.1 | 0.28 |

Pretreated industrial G. *corneum* residues (remaining solid fraction after agar extraction) were also used as a carbon source for the production of P3HB in the fed-batch phase of fermentation. The composition of the seaweed residues hydrolysates was previously determined: 358 $gL^{-1}$ glucose, 2.1 $gL^{-1}$ of nitrogen and 0.4 $gL^{-1}$ of phosphorus (Table 4). The general process workflows used to produce both hydrolysates (Crude *Gelidium* and industrial *Gelidium* residues) are illustrated in Figure 10 and 11.

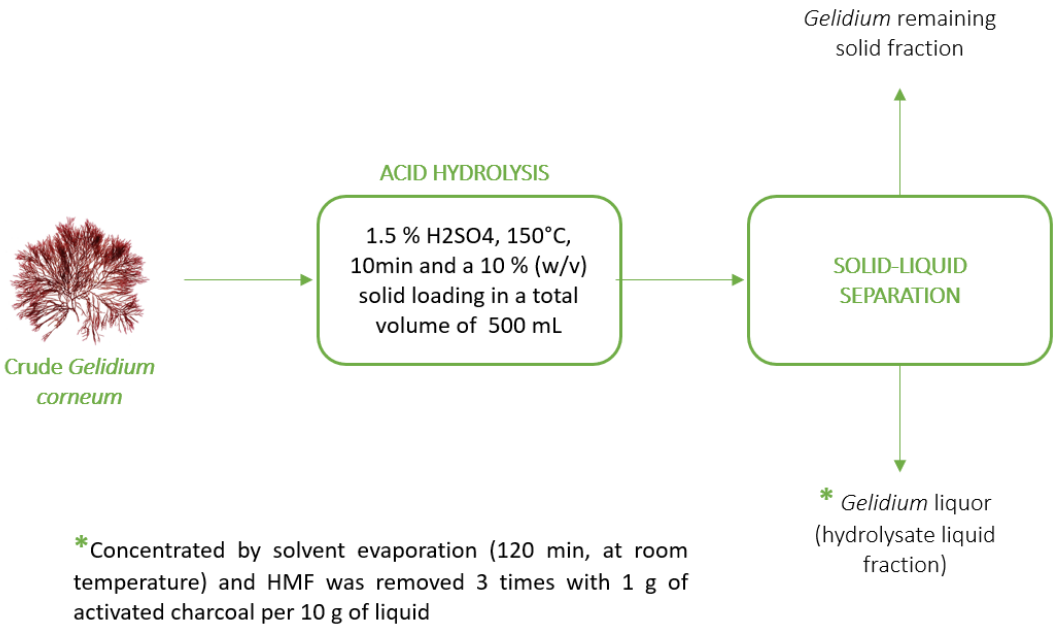



**Figure 10.** Crude *G. corneum* hydrolysates general process workflow. An acid pre-treatment was the first step in the process to fractionate the agarophyte red seaweed, followed by a solid-liquid separation. The liquid fraction after the pre-treatment was concentrated by solvent evaporation and HMF (microbial inhibitory component) was removed by processing with activated charcoal for 3 cycles. Adapted from (Gomes-Dias et al., 2020).

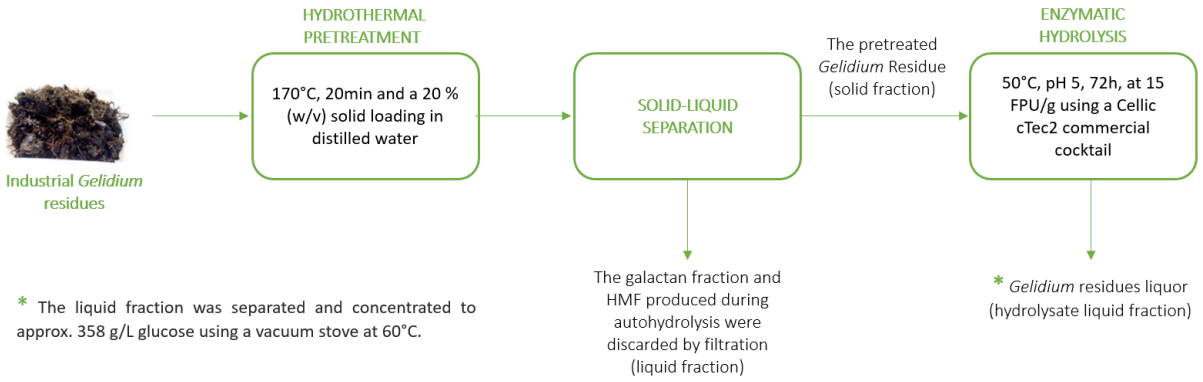



**Figure 11.** Industrial *G. corneum* residues general process workflow. A hydrothermal pre-treatment was the first step in the process to fractionate the agarophyte red seaweed *G. corneum*, followed by a solid-liquid separation. The liquid fraction after the pre-treatment was concentrated by solvent evaporation and HMF (microbial inhibitory component) was removed by processing with activated charcoal for 3 cycles. Adapted from (Gomes-Dias et al., 2020).

The hydrolysates of both the whole algae and the industrial residue, mentioned above, were prepared by the partner, University of Minho, prior to the commencement of this study.

## 3.4. BIOREACTOR CULTIVATIONS IN FED-BATCH

The bioreactor cultivations operating in fed-batch mode were carried out in a 2 L stirred-tank reactor (STR) to follow the growth and P3HB production of *H. boliviensis* culture under excess carbon and phosphate limitation. Glucose and *G. corneum* hydrolysates were used as carbon sources.

### 3.4.1. Seed medium and Inoculum preparation

The *H. boliviensis* inoculum medium was prepared based on the seed medium described by Quillaguamán et al. 2006: 45 $gL^{-1}$ NaCl, 2.5 $gL^{-1}$ $MgSO_4 \cdot 7H_2O$, 20 $gL^{-1}$ glucose, 0.55 $gL^{-1}$ $K_2HPO_4$, 2.3 $gL^{-1}$ $NH_4Cl$, 15 $gL^{-1}$ Tris, 3 $gL^{-1}$ Monosodium glutamate (MSG) and 0.005 $gL^{-1}$ $FeSO_4 \cdot 7H_2O$.

Solutions of NaCl (300 $gL^{-1}$), glucose (500 $gL^{-1}$), $MgSO_4 \cdot 7 H_2O$ (100 $gL^{-1}$) and $FeSO_4 \cdot 7H_2O$ (50 $gL^{-1}$) were prepared and sterilized separately to avoid precipitation or degradation. The $FeSO_4 \cdot 7H_2O$ (50 $gL^{-1}$) solution was prepared by dissolving first in $H_2SO_4$ (10 M) and then syringe filtered. The pH of the medium was adjusted to 7.5 using concentrated HCl 37 % (w/w) and sterilized in the autoclave at 121ºC, 1 bar for 20 min.

The inoculum for the bioreactor was prepared by transferring the contents of 2 cryovials (4 mL) in two 500 mL shake flasks with 65 mL volume each (13% capacity), representing 10% v/v of the initial fermentation working volume (1300 mL). Growth of the inoculum was carried out in an orbital incubator 30 °C and a shaking frequency of 170 rpm during 16 h until an exponential phase was reached. This corresponded, approximately, to an optical density at 600 nm of 5. The total shake flasks content was then transferred to the bioreactor.

### 3.4.2. Bioreactor production medium: Glucose as carbon source

The medium used, in the bioreactor assays, to trigger polymer production by imposing phosphate

limitation had the following composition: 45 $gL^{-1}$ NaCl, 5 $gL^{-1}$ $MgSO_4 \cdot 7\ H_2O$; 1.5 $gL^{-1}$ $K_2HPO_4$, 6 $gL^{-1}$ $NH_4Cl$, 0.005 $gL^{-1}$ $FeSO_4 \cdot 7\ H_2O$, 20 $gL^{-1}$ MSG and 25 $gL^{-1}$ glucose.

The bioreactor production medium (without NaCl, $MgSO_4 \cdot 7H_2O$, $FeSO_4 \cdot 7H_2O$ and glucose) was sterilized inside the bioreactor at 121°C for 20 minutes in an autoclave. The remainder medium components were prepared and sterilized separately, and then transferred to the bioreactor with a manual pump: 195 mL of a NaCl solution (300 $gL^{-1}$), 65 mL of a $MgSO_4 \cdot 7H_2O$ solution (100 $gL^{-1}$), 65mL glucose (500 $gL^{-1}$) and 130 µl $FeSO_4 \cdot 7H_2O$ (50 $gL^{-1}$). The $FeSO_4 \cdot 7H_2O$ (50 $gL^{-1}$) solution was prepared by dissolving first in $H_2SO_4$ (10 M) and then syringe filtered to avoid precipitation.

After 16h of inoculum growth, which corresponds to the end of the exponential phase, the content of two shake flasks (130 mL), as described above, were transferred to the bioreactor containing the entire production medium. The initial volume of the fed-batch cultivations was 1.3 L, including all medium components and inoculum.

Additionally, the feed used during the fed-batch mode was also prepared and autoclaved separately at 121°C for 20 minutes. The composition of the feed included: 45 $gL^{-1}$ NaCl, 5 $gL^{-1}$ $MgSO_4 \cdot 7\ H_2O$, 600 $gL^{-1}$ glucose and 0.125 $gL^{-1}$ $FeSO_4 \cdot 7H_2O$.

### 3.4.3. Bioreactor production medium: *Gelidium corneum* hydrolysate as carbon source

In these assays, crude *Gelidium* hydrolysates were used as a source of sugar and of N and P nutrients. The total sugars in the hydrolysate were calculated as the sum of galactose and glucose concentrations. The hydrolysate was diluted to attain the initial sugar concentration as in the assays with glucose (25 $gL^{-1}$). Then, concentrated solutions of $NH_4Cl$ and $K_2HPO_4$ were supplemented to attain the concentrations used in the batch phase describe in 3.4.2 taking into account the total N- and P- content of the hydrolysate.

The added medium had the following composition: 45 $gL^{-1}$ NaCl, 5 $gL^{-1}$ $MgSO4 \cdot 7H_2O$; 0.9 $gL^{-1}$ $K_2HPO_4$, 0.98 $gL^{-1}$ $NH_4Cl$, 0.005 $gL^{-1}$ $FeSO_4 \cdot 7\ H_2O$, 20 $gL^{-1}$ MSG and 580 mL of the *G. corneum* hydrolysate. The composition of the hydrolysate, described above, has been taken into account so that the final composition of the medium has the same amounts of phosphorus, nitrogen and sugar as the glucose assays.

580 ml of *G. corneum* hydrolysate was first neutralized with 10 mL NaOH (10 M) and autoclaved inside the bioreactor at 121°C for 20 minutes with 220 mL of distilled water. The remainder medium components were prepared, sterilized, and transferred to the bioreactor with a manual pump: 100mL of the production medium (without NaCl, $MgSO_4 \cdot 7H_2O$ and $FeSO_4 \cdot 7H_2O$), 195mL of a NaCl solution (300 $gL^{-1}$), 65 mL of a $MgSO_4 \cdot 7H_2O$ solution (100 $gL^{-1}$), and 130µl $FeSO_4 \cdot 7H_2O$ (50 $gL^{-1}$). The initial volume of the fed-batch cultivations was 1.3 L, including all medium components and inoculum. The $FeSO_4 \cdot 7H_2O$ (50 $gL^{-1}$) solution was prepared by dissolving first in $H_2SO_4$ (10 M) and then syringe filtered to avoid precipitation.

Additionally, the feed used during the fed-batch mode was prepared and autoclaved separately at 121°C for 20 minutes. The final volume of the feed was 500 mL, consisting of 425 mL of industrial

residue G. corneum hydrolysate and 75 mL of a NaCl solution (300 $gL^{-1}$). The production medium, *G. corneum* hydrolysate and the hydrolysate from *Gelidium* residues composition are describe above.

### 3.4.4. Bioreactor cultivation conditions

Fed-batch cultivations were carried out in 2 L STRs operated using the BioCommand Batch Control software, which enabled control, monitoring and data acquisition. The pH of the cultivation medium was controlled at 7.5 using a 30% $NH_3$ solution and 2M $H_2SO_4$ solution. The aeration rate used was set to 1.3 $L.min^{-1}$, under a temperature of 30°C. The dissolved oxygen (DO%) set point was set at 5% saturation and the agitation speed (max. 1200 rpm) was set in cascade with the DO (%).

After 10 h of cultivation, feeding was triggered using the pulse strategy, with each pulse adding 22 mL of feed to the reactor. The feed strategy was always programmed depending on the glucose consumption during bacteria fermentation, aiming to maintain the concentration above 25 $gL^{-1}$. Culture samples were periodically harvested, to analyze biomass, sugars, and polymer (P3HB) concentration. 10 mL culture samples were retrieved with a syringe through a nonreturn valve to maintain aseptic conditions.

Under the glucose assays, three conditions were tested, regarding the level of sterility. In the first condition, used as control, the entire procedure was carried out aseptically to ensure sterility within the bioreactor and in the entire assay. In the second condition the entire procedure was conducted to guarantee asepsis, but with an unsterilized feed, which was supplemented to the reactor from 10 h to the end of the assay. The third condition was performed under total non- septic circumstances. In all three approaches the inoculum was prepared aseptically. Throughout each assay samples were collected, aseptically and stored at -80ºC with 17% glycerol for further microbial identification.

## 3.5. ANALYTICAL METHODS

### 3.5.1. Optical density

To monitor bacterial growth, optical density (OD) measurements of samples were taken, at 600 nm in a double beam spectrophotometer, using 1 mL glass cuvettes with an optical path length of 1 cm. For the OD determination, an aliquot of the culture sample was diluted with deionized water to obtain an absorbance value lower than the threshold (ca.0.5-0.6). Deionized water was used as blank solution.

### 3.5.2. Cell dry weight determination

The cell dry weight (CDW) was determined by centrifuging at 9168 g for 5 minutes. The supernatant was rejected, and the pellet washed with deionized water, and then dried at 60°C, until constant weight. 1.2 mL aliquots of dried culture samples were collected then weighed. The CDW was determined dividing the weight difference after drying the pellets by the collected aliquots volume. The analysis was done in duplicate.

### 3.5.3. Sugar and gluconic acid determination

#### *3.5.3.1. HPLC vials preparation*

Samples for HPLC analysis were prepared by mixing 300 µL of supernatant aliquots with 300 µL of a 50 mM solution of $H_2SO_4$ in a microtube. After vortexing, these solutions were centrifuged at 9168 g for 5 minutes. Next, 100 µL of the supernatant of the previous 1:2 diluted samples were transferred to

appropriated HPLC vials with 900 µL of the 50 mM $H_2SO_4$ solution, giving a final dilution of 1:20.

#### *3.5.3.2. High-Performance Liquid Chromatography (HPLC)*

High-Performance Liquid Chromatography (HPLC) was used to monitor the sugars (glucose and galactose) concentration during the time course of fermentation. For the bioreactor assays, daily samples were run on HPLC, to program the feed pulses, and consequently to maintain glucose concentrations above 25 $gL^{-1}$. Additionally, HPLC was also used to monitor gluconic acid production throughout the fermentations.

HPLC apparatus is equipped with a Rezex ROA-Organic acid H+ 8% (300 mm_x7.8 mm) column, an autosampler (Hitachi LaChrome Elite L-2200), an HPLC pump (Hitachi LaChrome Elite L-2130), a Hitachi L-2490 refraction index (RI) detector for sugar and a Hitachi L-2420 UV-Vis detector for organic acids. A column heater for large columns (Croco-CIL 100-040-220P, 40 cm x 8 cm x 8 cm, 30-99°C) was connected externally to the HPLC system. The injection volume was 20 µL and elution was achieved using a 5 mM solution of $H_2SO_4$ as mobile phase. The column was kept at 65°C under a pressure of 26 bar, and the pump operated at a flow rate of 0.5 $mL.min^{-1}$.

### 3.5.4. P3HB determination

#### *3.5.4.1. Sample preparation - Methanolysis*

To determine the concentration of P3HB produced, the polymer was converted into stable and volatile hydroxycarboxylic acid methyl esters, through acidic methanolysis and the esters were further analyzed by gas chromatography.

To perform P3HB quantification ,1.2 mL aliquots of culture sample were centrifuged, at 9168 g for 5 minutes to remove the supernatant. The pellets were washed with deionized water and frozen for storage prior to acidic methanolysis. The cells were re-suspended in 1 mL of chloroform and transferred to Pyrex hermetic test tubes with Teflon lined caps and 1 mL of a “solution A” containing (per 100 mL solution): 97 mL of methanol, 3 mL of $H_2SO_4$ (96%) and 330 µL of hexanoic acid as the internal standard (IS) was added. After vortexing for 1 minute, these mixtures were incubated for 5 hours at 100°C. Halfway through the incubation process, the tubes were vortexed again, and placed back in the oven. After cooling, 1 mL of $Na_2CO_3$ (60 $gL^{-1}$) was added to the tubes for neutralization, and the samples were vortexed for 1 min and centrifuged at 1324g for 5 minutes. Finally, 200 µL of the organic phase from each hermetic tube were withdrawn to appropriate gas chromatography (GC) vials and kept at -20°C until GC analysis was performed.

#### *3.5.4.2. Gas Chromatography (GC)*

Offline determination of P(3HB) concentration was carried out using a GC apparatus (Agilent Technologies 5890 series II) equipped with an FID detector and a 7683B injector. The capillary column was a HP-5 from Agilent J&W Scientific, 30 m in length and 0.32 mm of internal diameter. The oven, injector, and detector were kept at constant temperatures of 60°C, 120°C, and 150°C, respectively. Data acquisition and integration were performed by a Shimadzu CBM-102 communication Bus Module and Shimadzu GC solution software (version 2.3), respectively. Peak identification was achieved using as standard 3-methyl hydroxybutyrate. P3HB content (weight percent) was obtained as the percentage

of the ratio of P3HB concentration to cell dry weight.

## 3.6. MICROBIAL IDENTIFICATION

### 3.6.1. Phenotypic Method: Morphology

To assess the existence of possible contaminations various methods for microbiological identification were employed, both phenotypically and genomically. For phenotypic identification samples were aseptically taken in each fermentation condition, and subsequently plated in various solid media: *H. boliviensis* production medium (described in section 3.4.1.2), marine agar, TSA (trypticase soy agar) and LB (Luria-Bertani) agar. The samples were plated in petri dishes, with the different media under sterilized conditions in a laminar flow chamber and incubated at 30ºC until sufficient growth of colonies is observed. The plates were then stored at 4ºC for storage.

### 3.6.2. Phenotypic Method: Physiological / Biochemical Characteristics

#### *3.6.2.1. Sample Preparation*

Another phenotypic identification approach concerns Sherlock® Microbial ID System from MIDI, Inc. This analysis system requires bacteria to be grown in TSA (Tryptic soy agar) culture media. The software used is optimized for TSA medium, so samples were withdrawn from different fermentation conditions, plated on a TSA media, and incubated at 30ºC for 24h. Because of the difference in salt concentration between the medium in the bioreactor (45 $gL^{-1}$) and the TSA plates (5 $gL^{-1}$), intermediate plates were prepared, before transferring the colonies into TSA media plates. Aiming at this, production media with less NaCl (20 $gL^{-1}$) was used as intermediate plate, as illustrated in Figure 12. A quadrant streak method was used to achieve the growth of well-isolated colonies.

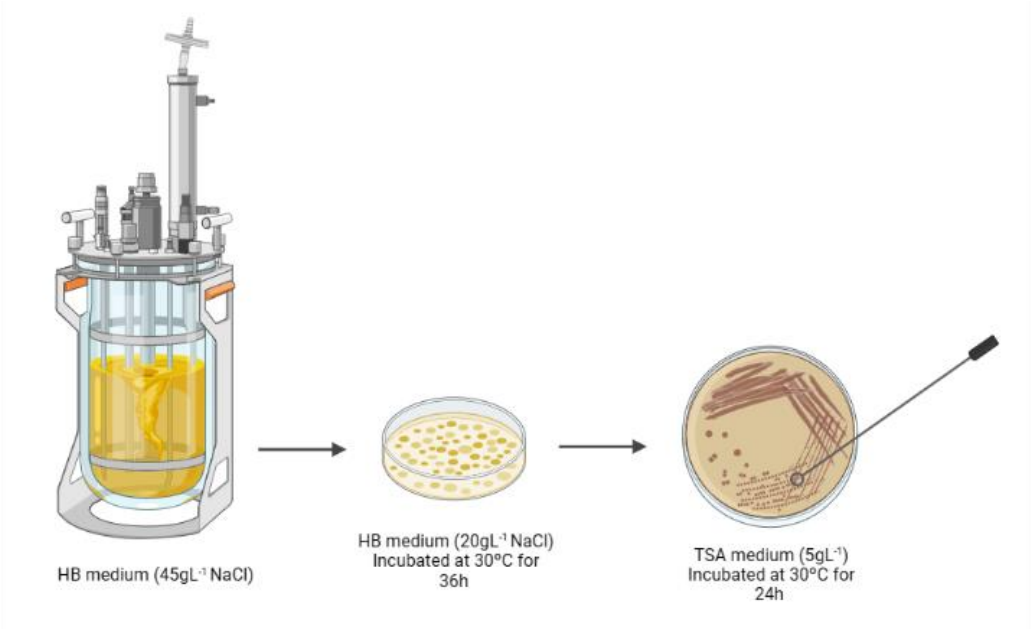



**Figure 12.** Schematic representation of the cultivation process of bioreactor samples on solid TSA agar medium. Intermediate plates were employed before colony transfer onto TSA media plates, due to the difference in salt concentration between the bioreactor medium (45 $gL^{-1}$) and TSA plates (5 $gL^{-1}$) The intermediate plate medium consisted in HB production media with lower NaCl concentration (20 $gL^{-1}$).

After 24h, cells from exponentially growing colonies were collected separately and their fatty acids were extracted and methylated to fatty acid methyl esters (FAMEs). This was done using a four-step procedure (saponification, methylation, extraction, and base wash) of the Instant FAME method from MIDI (*Fatty Acid Analysis* | *Bacterial ID* | *MIDI Inc.*, n.d.).

#### *3.6.2.2. Gas Chromatography (GC)*

The FAMEs obtained were analyzed using a GC apparatus. An Agilent Technologies 6890N gas chromatograph (Palo Alto, CA, USA), equipped with a flame ionization detector (FID), a 7683 B series injector and a 25 m long Agilent J&W Ultra 2 capillary column from Agilent was used. The software, Sherlock® MIS (version 6.2) from MIDI form, Inc, used for bacterial identification is based on the FAME profile of each bacterium using the ITSA1 method. The software's 2D plot function was used to conduct principal component analysis on the FAMEs profiles and examine correlations among the several isolated strains.

### 3.6.3. Genotypic Method: 16S amplicon sequencing

#### *3.6.3.1. Sample Preparation: DNA extraction and purification*

To identify the bacteria of each fermentation condition, the samples previously collected and stored at -80ºC with 17% glycerol underwent a DNA purification procedure. The samples analyzed were taken from the end of the fermentation process, and the duplicate samples for each condition were pooled and analyzed as a single sample.

Two different protocols of the GeneJET Genomic DNA Purification Kit for gram-negative and gram-positive were employed to induce lysis of the bacterial cell wall and purification of the DNA. For the gram-negative extraction protocol, an additional lysis buffer was prepared: 20 mM Tris-HCl, pH 8.0, 2 mM EDTA, 1.2% Triton X-100 and lysozyme to 20 mg/mL. The kit makes use of a silica-based membrane technology in the form of a convenient spin column. After the procedure, the purified DNA was quantified, using an NanoDrop UV-Vis Spectrophotometer, and stored at -20ºC. Identification of bacterial species were carried out by StabVida, Lda.

## 3.7. SUPERPRO DESIGNER® MODELLING

The SuperPro Designer® v12 functional evaluation edition (Intelligen, Inc., Scotch Plains, NJ, USA) software enables a simulation of industrial scale processes through modelling, evaluation, and optimization tools (Petrides & Harrison, 2003). Two case scenarios for industrial production of P3HB were established to assess the bioprocess's effectiveness and its economic viability. The experimental data attained as well as several assumptions permitted the establishment of the pilot project.

# 4. RESULTS AND DISCUSSION

Five main topics are covered in this chapter. In the first part P3HB production under three levels of sterility and operating in fed-batch will be studied. The second part focuses on the uptake of glucose and its effect on the production of gluconic acid. The third, on the study of the microbial population dynamics, of the three conditions, with different microbial identification methodologies. The fourth part consists of other approaches to improve the economic viability of P3HB production: algal biomass

hydrolysates as the carbon substrate. Lastly, the final section focuses the economic evaluation and comparison of the scenarios using three levels of sterility.

### 4.1. P3HB PRODUCTION IN FED-BATCH BIOREACTOR CULTIVATIONS OF *HALOMONAS BOLIVIENSIS*

Fed-batch experiments were carried out aiming to understand the P3HB production and growth by *H. boliviensis* under different levels of sterility: “Full-Sterility”, “Semi-Sterility”, and “Non-Sterility”.

As previously explained in chapter 3.4.4., in the “Full-Sterility” condition, which was used as the control, the entire procedure was executed aseptically to avoid cross-contamination not only within the bioreactor, but also throughout the entire assay. In the “Semi-Sterility” regime, the entire procedure was conducted to ensure asepsis, with the exception of the unsterilized feed that was supplemented into the reactor from 10 hours until the end of the assay. The “Non-Sterility” approach was carried out under completely non-aseptic conditions. In all scenarios, the inoculum was prepared aseptically.

To reach higher yields and P3HB productivities, controlled cultivation conditions such as $DO$ (%); pH and supply of substrate were assessed in 2L stirred tank (STR) bioreactors. P3HB production by *H. boliviensis* was triggered by P limitation. The results of fed-batch cultivations under the different levels of sterility are shown in Figure 13 (“Full-Sterility”), Figure 14 (“Semi-Sterility”) and Figure 15 (“Non-Sterility”).

Regarding the cultivation parameters examined below, the residual biomass, expressed as XR ($gL^{-1}$), is used to measure the amount of cellular biomass except P3HB. This value is obtained by subtracting the concentration of P3HB ($gL^{-1}$) from that of CDW ($gL^{-1}$). The P3HB content (weight percent) was calculated as the ratio of the P3HB concentration to the cell dry weight, expressed as a percentage.

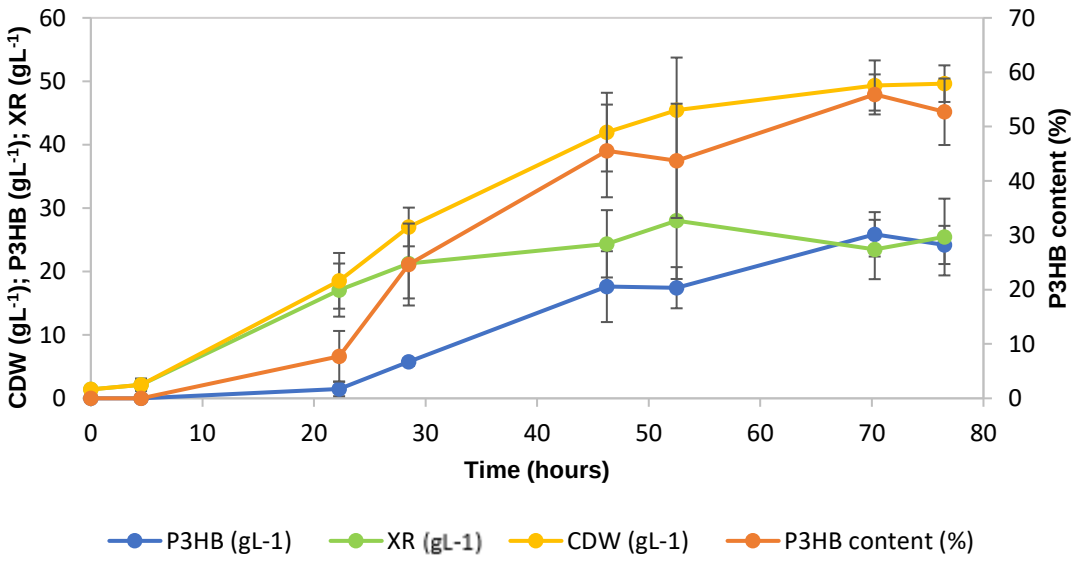



**Figure 13.** Graphic representation of *H. boliviensis* growth and P3HB accumulation in “Full-Sterility” conditions. All values are Mean with SD (Standard Deviation); n=4; Legend: P3HB $gL^{-1}$(o); XR $gL^{-1}$(o); CDW $gL^{-1}$ (o); P3HB Content (w/w) % (·).

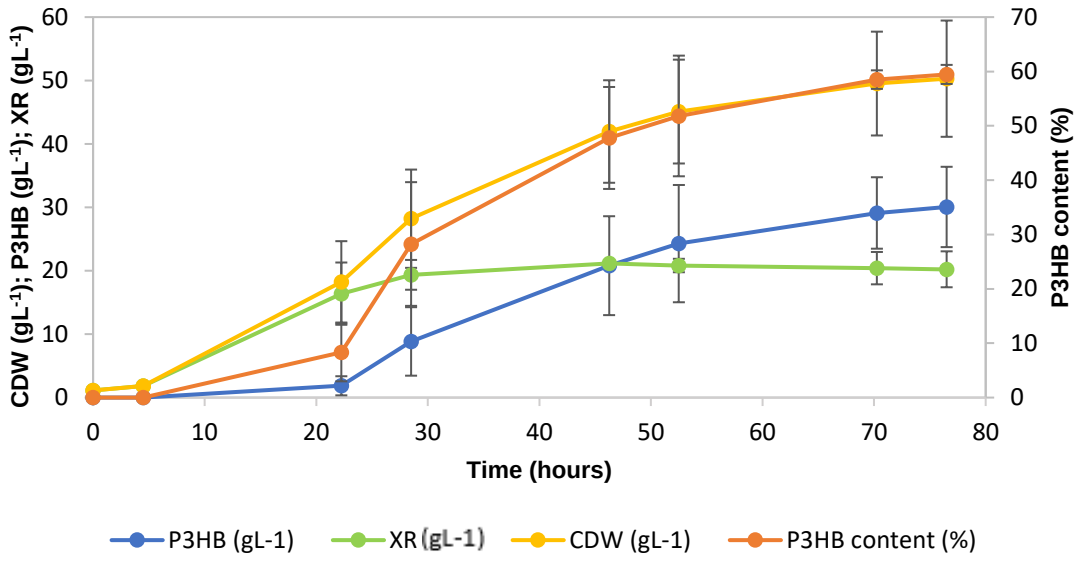



**Figure 14.** Graphic representation of *H. boliviensis* growth and P3HB accumulation in "Semi-Sterility" conditions. All values are Mean with SD (Standard Deviation); n=2; Legend: P3HB $gL^{-1}$(o); XR $gL^{-1}$(o); CDW $gL^{-1}$ (o); P3HB Content (w/w) % (·).

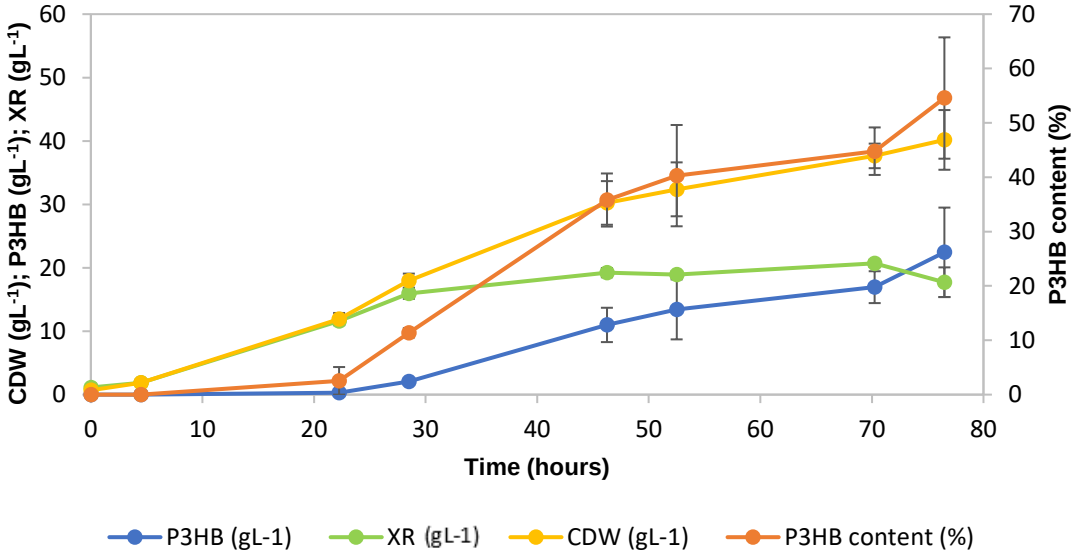



**Figure 15.** Graphic representation of *H. boliviensis* growth and P3HB accumulation in "Non-Sterility" conditions. All values are Mean with SD (Standard Deviation); n=3; Legend: P3HB $gL^{-1}$(o); XR $gL^{-1}$(o); CDW $gL^{-1}$ (o); P3HB Content (w/w) % (o)

The results are the means of four replicas on "Full-Sterility", two on "Semi-Sterility" and two on "Non-Sterility" conditions with the respective standard deviation displayed. The significance of the differences between the means were calculated using a one-way analysis of variance (ANOVA). Differences were

considered statistically insignificant (p-value of <0.05) between the three conditions for all the parameter evaluated.

A first look on the results (Figure 13,14 and 15) obtained from the fed-batch cultivation of *H. boliviensis* suggests similar bacterial behavior and P3HB production under the different conditions. The different cultivation parameters are compared in the figures below for a more thorough examination. Regarding biomass growth, dry weights and absorbance were monitored during the course of each cultivation. As presented in Figure 16, both, “Full-Sterility” and “Semi-Sterility” conditions, showed remarkably equivalent growth rates, attaining around 50 $gL^{-1}$ of dry weight after 76.5 hours of cultivation. In contrast, in non-sterile conditions, a mild decrease of the biomass growth is observed with a maximum of 40 ± 4,71 $gL^{-1}$.

The less promising results in biomass growth observed in non-sterility assays could also be attributed to the effect of heat sterilization on the compounds present in the medium. This can lead to various consequences. Heat sterilization has the potential to affect the stability and integrity of certain compounds. These compounds may undergo chemical changes or degradation when exposed to high temperatures during sterilization, potentially altering their bioavailability and impacting the growth of biomass in the experiments. It is possible that the sterilization process may have led to favorable changes, such as the breakdown of complex structures of compounds into simpler and easily usable forms by the organisms present in the assay. In addition, it is crucial to consider the potential impact of the Maillard reaction on compounds in the bacterial medium. The Maillard reaction, a chemical process involving amino acids and reducing sugars like glucose, leads to the production of brown pigments and flavor compounds. Within the context of bacterial growth, the Maillard reaction may affect the accessibility and utilization of glucose and MSG, which are vital nutrients for bacterial metabolism. Assessing the extent of Maillard reaction products and their influence on bacterial growth is pivotal in evaluating the overall consequences of sterilization on the composition of the medium (Marounek & Brezina, 1993; Pontes Eliodório et al., 2023).

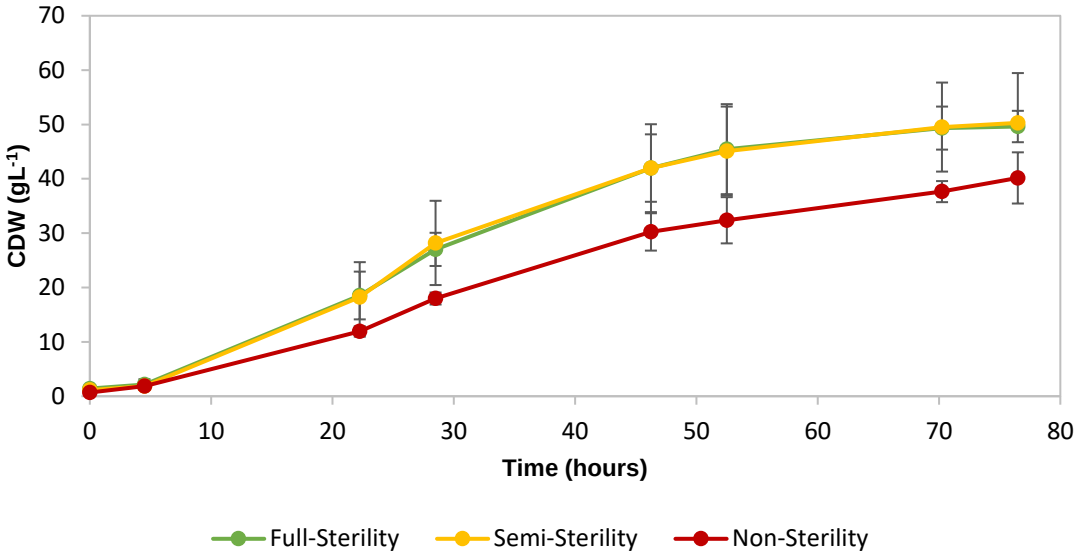



**Figure 16.** Evaluation of biomass growth of three sterility conditions, through the dry weight measurements. All values are Mean with SD (Standard Deviation); ("Full-Sterility") n=4, ("Semi-Sterility") n=2, ("Non-Sterility") n=3, n=number of samples. Legend: "Full-Sterility" (o); "Semi-Sterility" (o); "Non-Sterility" (o).

Concerning the P3HB production, represented in Figure 17, the highest P3HB concentration of 30.06 ± 6.33 $gL^{-1}$ was achieved on the "Semi-Sterility" assays, next, 25.9 ± 3.5 $gL^{-1}$ on the control assay. The unsterile assays had the lowest production of 22.4 ± 7.05 $gL^{-1}$. Additionally, in all assays, the P3HB content was higher than 50%, the highest content, 59.4 ± 1.75 %, was achieved in the "Semi-Sterility" tests while sterile and unsterile assays attained similar contents of circa 52% (Figure 18).

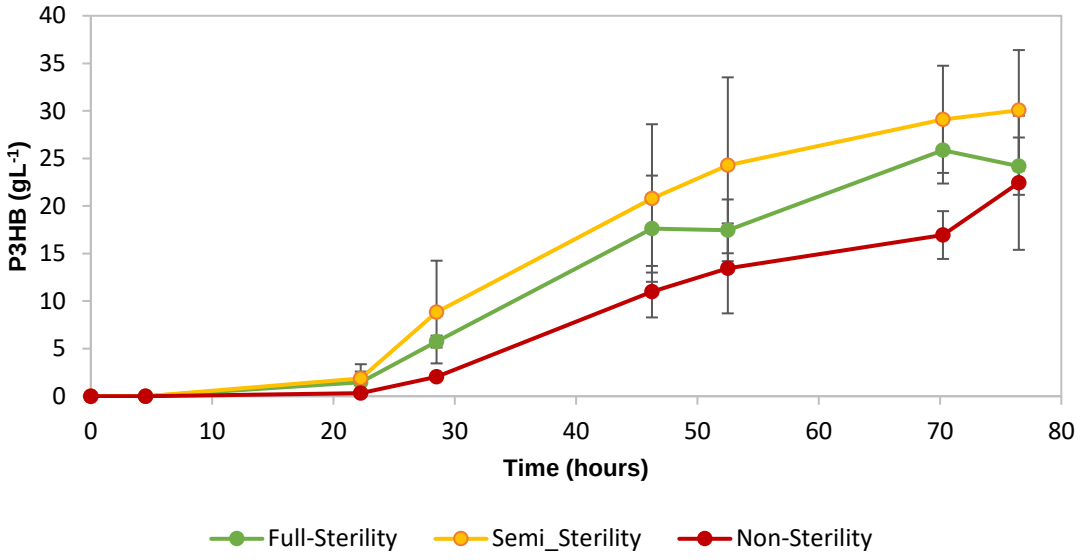



**Figure 17.** Comparison of P3HB ($gL^{-1}$) production regarding the three different sterility scenarios. All values are Mean with SD (Standard Deviation); ("Full-Sterility") n=4, ("Semi-Sterility") n=2, ("Non-Sterility") n=3; n=number of samples. Legend: "Full-Sterility" (o); "Semi-Sterility" (o); "Non-Sterility" (o).

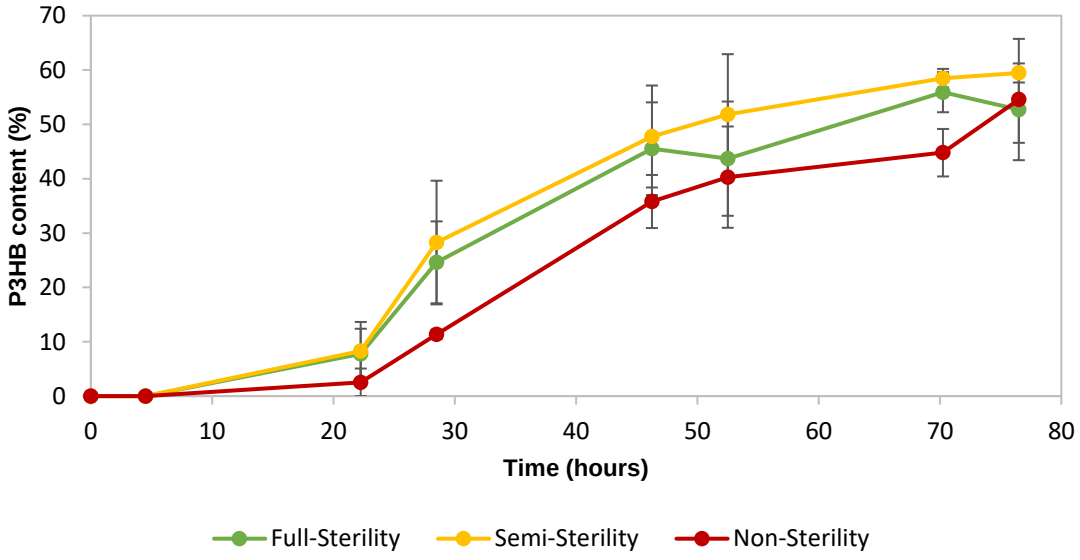



**Figure 18.** Comparison of P3HB content (weight percent) regarding the three different sterility scenarios. All values are Mean with SD (Standard Deviation); ("Full-Sterility") n=4, ("Semi-Sterility") n=2, ("Non-Sterility") n=3; n=number of samples. Legend: "Full-Sterility" (o); "Semi-Sterility" (o); "Non-Sterility" (o).

To maintain the optimal conditions for P3HB accumulation, the carbon source, glucose, was monitored using off-line analysis. Due to some technical HPLC limitations, in certain fermentations, it was not possible to maintain a constant glucose concentration. This could explain the different productivities

(Table 5) between the three conditions, as the P3HB synthesis is directly influenced by the carbon source concentration.

In Table 5, the results are summarized and compared to the results of Quillaguamán et al., 2008 Although the P3HB production results were less appealing than those obtained by Quillaguamán et al. (2008), the values obtained in the three tested conditions are very similar, although growth and productivity were slightly lower in the non-sterile assays. This could be attributed to variations in feeding strategies, specifically, the varying amounts of glucose that were added throughout the fermentations.

**Table 5.** Comparison of the cultivation's parameters from the fed-batch P3HB production of the three tested conditions with Quillaguamán et al., 2008. CDW ($gL^{-1}$) and XR ($gL^{-1}$) are appropriate from the maximum P3HB ($gL^{-1}$).

| Fermentation | *CDW ($gL^{-1}$)* | *P3HB max ($gL^{-1}$)* | *% P3HB max* | *Xr ($gL^{-1}$)* | *Prod vol max ($gPL^{-1}h^{-1}$)** | *Y (g PHA/g glucose consumed)* |
|---|---|---|---|---|---|---|
| **"Full-Sterility"** | 49.33 | 25.86 | 52.42 | 23.47 | 0.468 | 0.152 |
| **"Semi-Sterility"** | 50.29 | 30.06 | 59.45 | 20.23 | 0.462 | 0.154 |
| **"Non-Sterility"** | 40.17 | 22.44 | 54.57 | 17.72 | 0.293 | 0.182 |
| **Quillaguamán et al. (2008)** | 43.70 | 35.40 | 81.00 | Not reported | 1.10 | Not reported |

* The maximum volumetric productivity was determined by assessing the productivity of each sample harvesting time and selecting the highest value.

## 4.2. GLUCONIC ACID CO-PRODUCTION

The substantial accumulation of gluconic acid by *H. boliviensis* was one of the main challenges encountered to the P3HB production, as it competes for glucose as a substrate. The above-mentioned HPLC restrictions resulted in a variety of glucose concentrations and, as a result, diverse gluconic acid productions by different fermentations.

The variation of glucose and production of gluconic acid of a fermentation carried out with complete sterility is shown in Figure 19. Although glucose was not exhausted, it was maintained between 10 $gL^{-1}$ and 20 $gL^{-1}$, at relatively low concentrations, for most of the experiment. After 20 hours of cultivation, a substantial increase of the gluconic acid concentration, up to 91.0 $gL^{-1}$ can be observed.

Contrarily, in the cultivation represented in Figure 20, the concentration of glucose was always higher than 20 $gL^{-1}$ through the assay, attaining values of 130 $gL^{-1}$ towards the end of the cultivation. Gluconic acid, however, was not synthesized in considerable amounts, only producing 5.4 $gL^{-1}$ after 76.5 hours of cultivation. In the fermentation displayed in Figure 21, the glucose concentration ranged between 40$gL^{-1}$ and 60$gL^{-1}$, while gluconic acid concentration peaked at 33.6 $gL^{-1}$ around 76.5 hours of cultivation.

In the assay represented in Figure 22, when the concentration of glucose was under 40$gL^{-1}$, the gluconic concentration had a sharp increase, up to 120 $gL^{-1}$. The decline of gluconic acid at 76.5 hours could be

explained by the low concentrations of glucose between 70.5 hours and 76.5 hours, causing the metabolism to shift, and bacteria consuming the gluconic acid as the carbon source.

The fermentation represented in Figure 23 also supports the gluconic acid production trends mentioned above. A rather small amount of gluconic acid was produced during the first 45 hours of cultivation. An exponential rise in the synthesis of gluconic acid can be observed once glucose levels fall to levels close to 40 $gL^{-1}$.

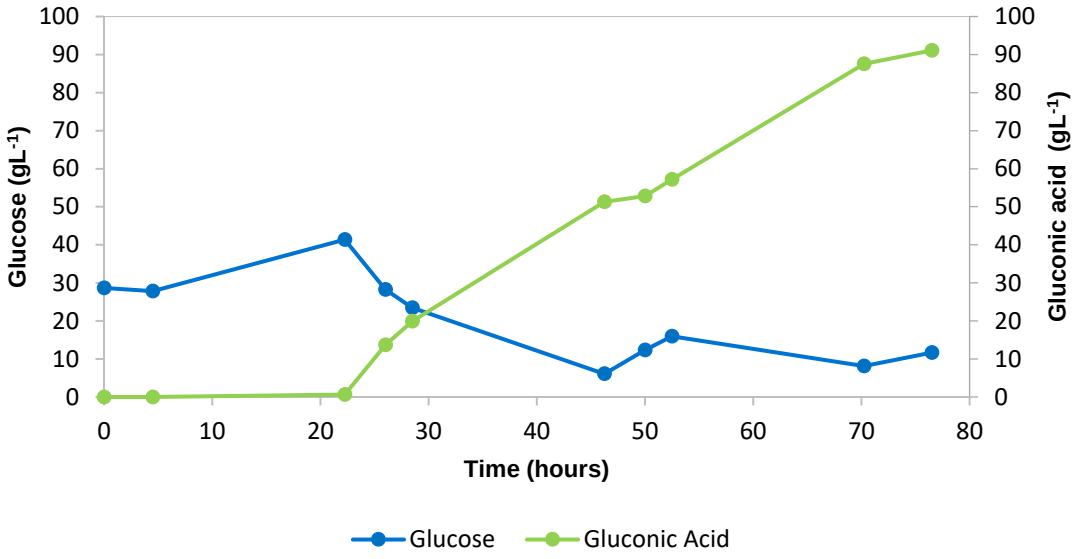



**Figure 19.** *H. boliviensis* glucose uptake and gluconic acid accumulation throughout 76.5 hours of a "Full-Sterility" cultivation. Both glucose and gluconic acid concentrations were monitored using off-line analysis by high performance liquid chromatography. Legend: Gluconic acid $gL^{-1}$ (o); Glucose $gL^{-1}$ (o).

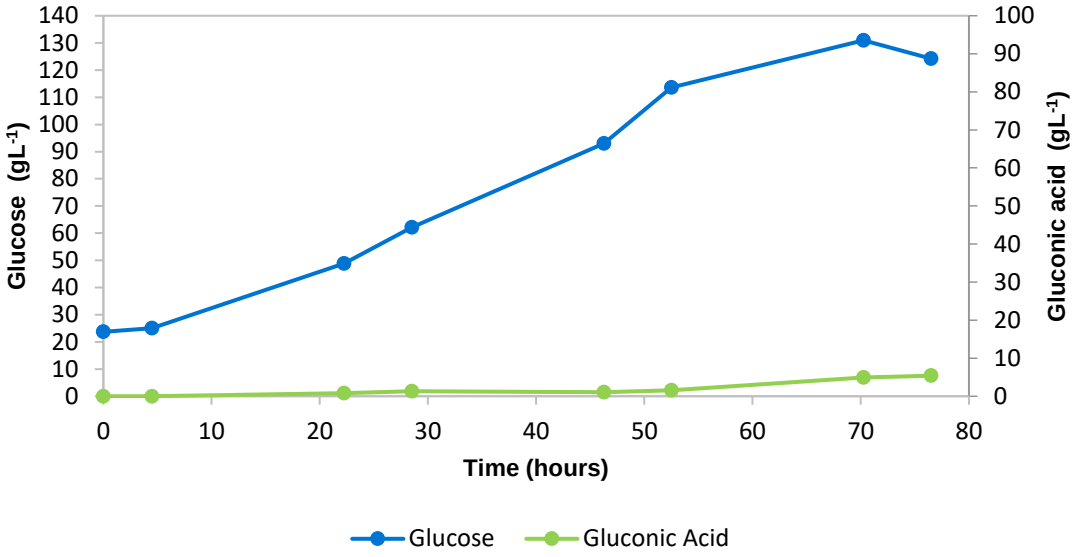



**Figure 20.** *H. boliviensis* glucose uptake and gluconic acid accumulation throughout 76.5 hours of a "Non-Sterility" cultivation. Both glucose and gluconic acid concentrations were monitored using off-line analysis by high performance liquid chromatography. Legend: Gluconic acid $gL^{-1}$ (o); Glucose $gL^{-1}$ (o).

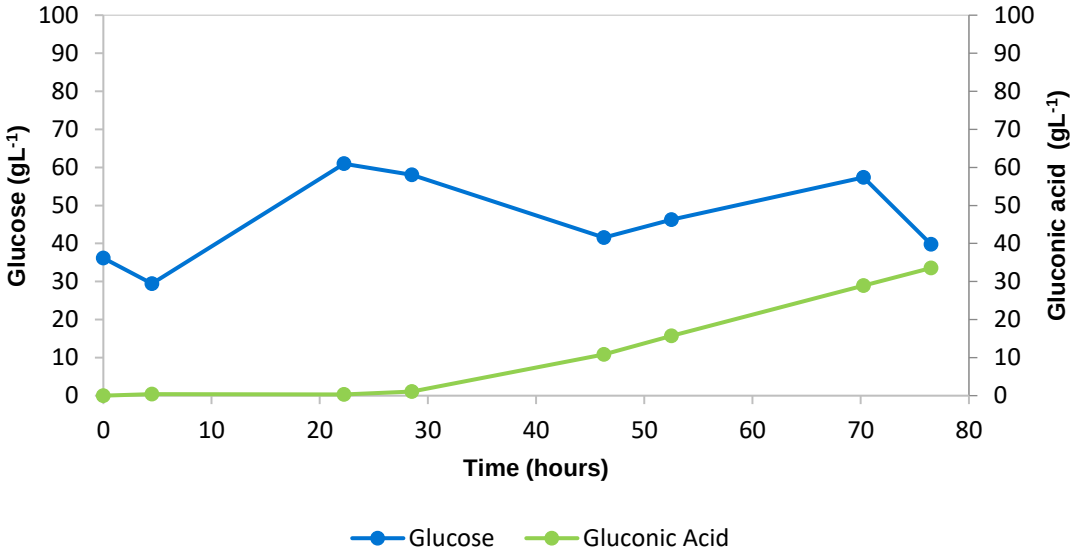



**Figure 21.** *H. boliviensis* glucose uptake and gluconic acid accumulation throughout 76.5 hours of a "Non-Sterility" cultivation. Both glucose and gluconic acid concentrations were monitored using off-line analysis by high performance liquid chromatography. Legend: Gluconic acid $gL^{-1}$ (o); Glucose $gL^{-1}$ (o).

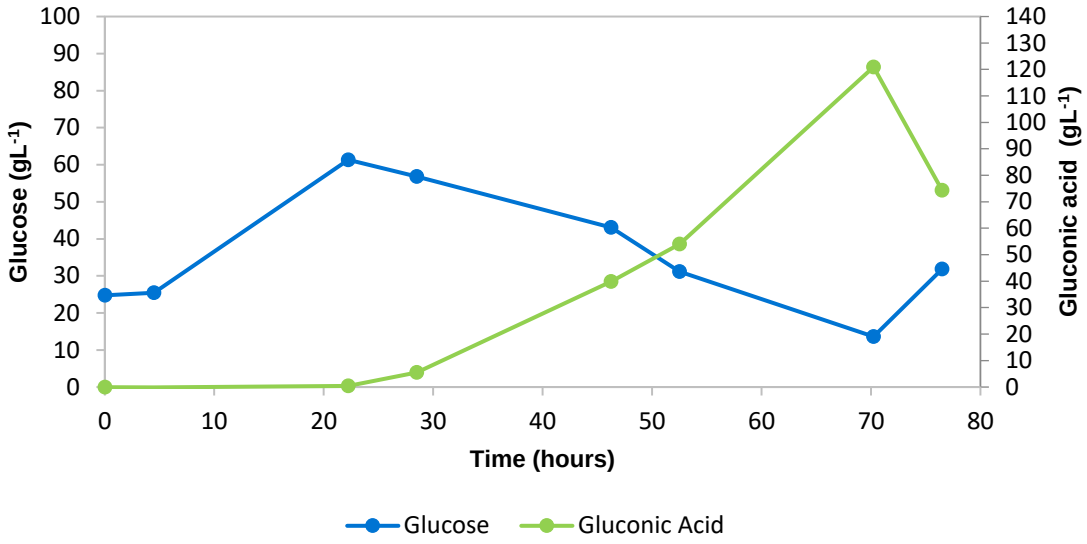



**Figure 22.** *H. boliviensis* glucose uptake and gluconic acid accumulation throughout 76.5 hours of a "Semi-Sterility" cultivation. Both glucose and gluconic acid concentrations were monitored using off-line analysis by high performance liquid chromatography. Legend: Gluconic acid $gL^{-1}$ (o); Glucose $gL^{-1}$ (o).

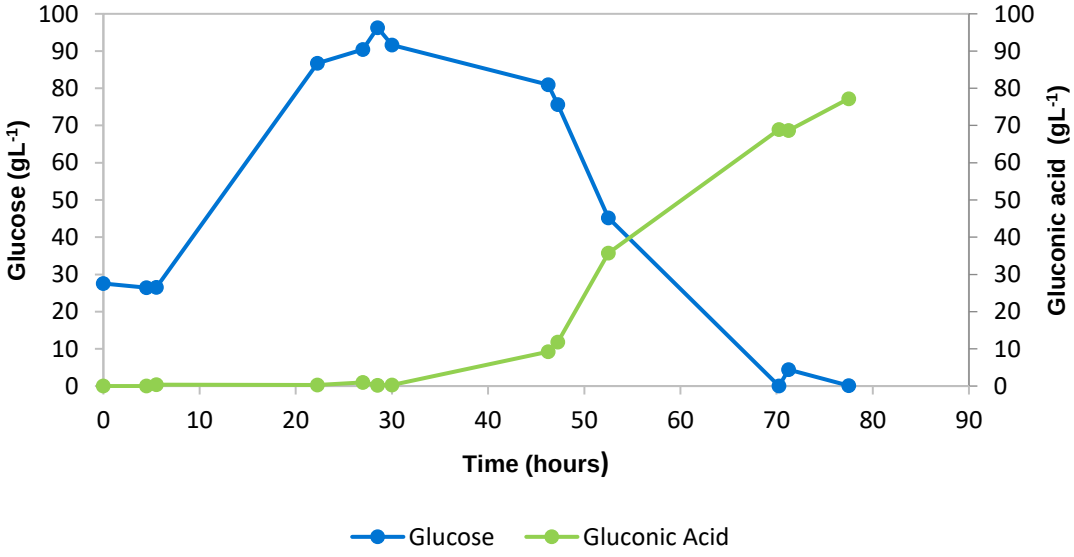



**Figure 23.** *H. boliviensis* glucose uptake and gluconic acid accumulation throughout 76.5 hours of a "Semi-Sterility" cultivation. Both glucose and gluconic acid concentrations were monitored using off-line analysis by high performance liquid chromatography. Legend: Gluconic acid $gL^{-1}$ (o); Glucose $gL^{-1}$ (o).

By analyzing the patterns of the influence of glucose on gluconic acid production, it can be suggested, by observation, that concentrations below 40 $gL^{-1}$ induce the metabolism of gluconic acid production, where concentrations around and above 50 $gL^{-1}$ and 60 $gL^{-1}$ repress the glucose oxidation pathway. This phenomenon could be explained by the inhibitory glucose threshold of the pathway.

In order to maximize P3HB synthesis, it is critical to understand the primary metabolic pathways involved in glucose intake. A nutrient concentration and presence or absence of stressor could affect the derivation of glucose between the metabolic alternatives, pentose phosphate pathway or glycolysis (Embden Meyerhof Parnas pathway) (Kornecki et al., 2020). However, another glycolytic pathway, Entner-Doudoroff (ED), and the pentose phosphate pathways (PPP) were deduced from an evolutionary analysis of the genome *H. boliviensis* reported in Rivera-Terceros et al., 2015. This could indicate that the assimilation of glucose is mainly done through the ED pathway or the PPP. The pentose phosphate metabolic pathway stimulates the production of gluconic acid, as gluconate (the form in which D-gluconic acid is present at physiological pH) is phosphorylated by the action of gluconate kinase (GnuK) to produce 6-phosphogluconate, which is the second intermediate of the PPP (Kornecki et al., 2020). Gluconic acid produced is imported to the cell and further catabolized via the reactions in PPP or the ED pathway.

In addition, a recent study conducted on *Halomonas elongata*, another species within the genus *Halomonas*, have suggested that the pathways from glucose to 6-phosphogluconate are likely carried out through the ED and PPP pathways, as depicted in Figure 24 (Leandro et al., 2023).

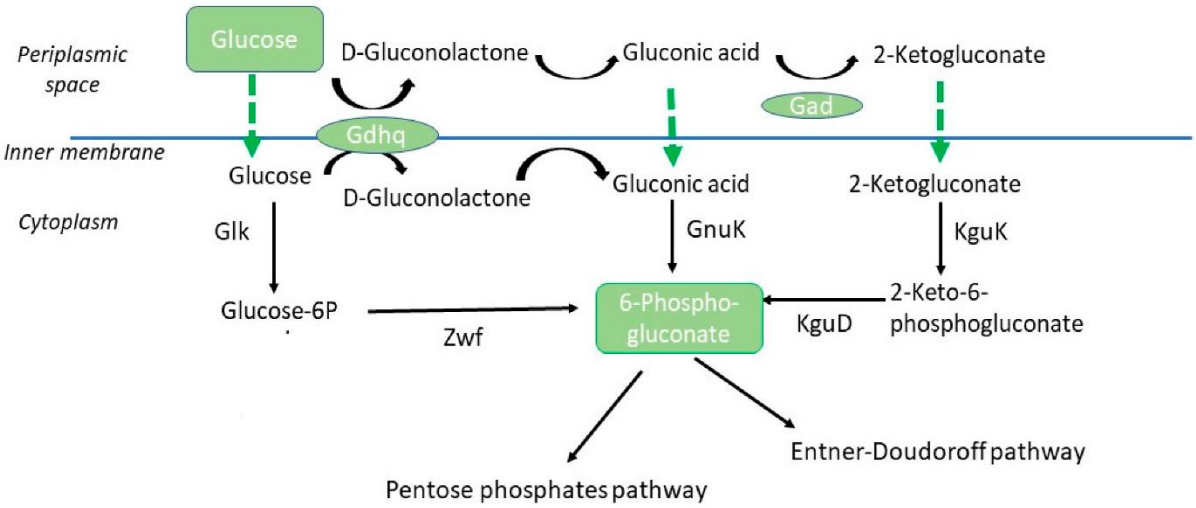



**Figure 24.** Pathways leading from glucose to 6-phosphogluconate in *Halomonas elongata* 1H9T. Abbreviations used are as follows: Gad, gluconate dehydrogenase; Gdhq, glucose dehydrogenase; Glk, glucokinase; GnuK, gluconokinase; KguD, 2-keto-6-phosphogluconate reductase; KguK, 2-ketogluconatekinase; Zwf, glucose-6-phosphate dehydrogenase. Retrieved from Leandro et al., 2023.

Through the results obtained above, it can be suggested that under stress conditions (phosphorous limitation) and concentrations below 40 $gL^{-1}$, the pathway of pentose phosphate (or the ED pathway) is repressed, and thus gluconic acid accumulation takes place. However, the conclusions reached are merely conjecture since there is limited metabolic literature on *H. boliviensis.* Additional research is required in order to determine which pathways are triggered or suppressed upon certain conditions.

An alternative to further optimize the economic viability of the process, could be the implementation of co-production of intracellular P3HB combined with gluconic acid, an extracellular product. Previous research has demonstrated that P3HB co-productions with other chemicals can lower production costs

and enable an economically viable downstream processing (T. Li et al., 2017). Gluconic acid is a noncorrosive, nontoxic, mild organic acid, used in the food, metal, leather and pharmaceutical industries. Often used in the food industry, as imparts a refreshing sour taste in items, such as wine and fruit juices. Sodium gluconate accounts for around 80% of the gluconic acid market, as it has a strong chelator capacity, at alkaline pH. Additionally, many detergents use this salt as a sequestering agent, and could also be added to cement to speed up the hardening process (Goldberg & Rokem, 2009; Yadav et al., 2022). The annual global market of gluconic acid is, currently, between $50 million and $80 million, and is expected to surpass $1.2\times10^5$ tonnes by 2024 (Ahuja & Bayas, 2018). In spite of this, the physiological functions of gluconic acid accumulation in bacteria are still unclear. Some reports have suggested it contributes to the organism's competitiveness by eliminating glucose from its immediate surroundings (Goldberg & Rokem, 2009).

Another HPLC constraint prevents accurate determination of the glucose concentration at high concentrations of gluconic acid because glucose and gluconic acid peaks overlap on the RI spectrum. Although a more accurate approximation of the glucose concentration is made through quantification of the gluconic acid peak in the UV-VIS spectrum, it is necessary to consider the potential uncertainties associated. As such, when gluconic acid reached significant concentrations, the analysis of glucose was performed using the Megazyme GOPOD Kit.

## 4.3. STUDY OF THE MICROBIAL POPULATION DYNAMICS- MICROBIAL IDENTIFICATION

### 4.3.1. Phenotypic Method: Morphology

To assess the microbial population dynamics of the three types of sterility scenarios, samples from each type of cultivation were plated in different solid media. As expected, colony phenotypic morphology differs between each medium, correlating to different medium-induced responses (van Teeseling et al., 2017).

Samples from all three conditions were plated in *H. boliviensis* production medium (HB medium), that is, a medium with the same composition as in the bioreactor, in order to present a more realistic representation of the microbial dynamics during the assay. Additionally, plates with more generic mediums were used, to examine whether some contamination could be present but in a state of dormancy, due to the nutrient restrictions of the medium used, or to the presence of high salt concentrations.

Concerning the plates of the full sterility assays, colonies had a similar surface appearance and color through all the types of solid media. The sizes appear to differ a little but not enough to indicate the presence of contamination. In Figure 25 a HB medium and a marine agar plate cultured with samples from an “Full-Sterility” fermentation are shown. The colonies grown in each plate have a similar appearance and thus no indication of the presence of different species could be observed.

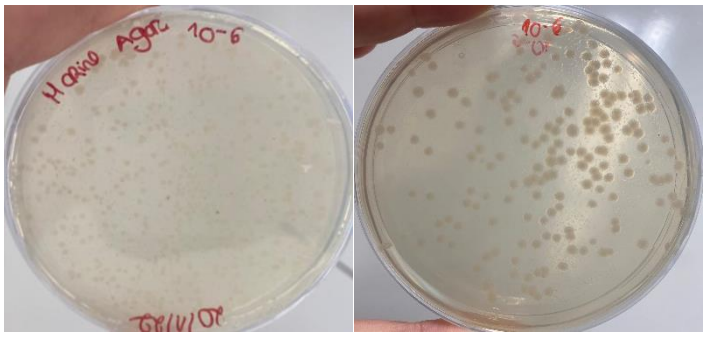


**Figure 25.** Marine agar plates cultured with samples from a "Full-Sterility" fermentation broth (left); HB medium (right). The dilution factor was 1:$10^6$ and the plates were incubated at 30ºC for 36 hours.

Regarding the assays where only the feed solution wasn't sterilized, the same method for microbial identification was applied, and different solid medium plates were analyzed. In Figure 26, a plate with HB medium (left) and a plate with HB medium with less NaCl (20 $gL^{-1}$) (center) are shown, both present a homogeneous appearance, although different between one another. Both size and surface appearance seem to be similar, except for the color.

**Figure 26.** Solid media cultured with samples from a "Semi-sterility" fermentation broth: HB medium (left) with a dilution factor of 1:$10^{-6}$; HB production medium with 20$gL^{-1}$ NaCl (center) with a dilution factor of 1:$10^{-7}$; and marine agar medium (right) with a dilution factor of 1:$10^{-6}$. The plates were incubated at 30ºC for 36 hours.

In Figure 26 (right) the marine agar plate has colonies with a uniform aspect, and similar with the control assay ("Full-Sterility"), mentioned above, in Figure 25. Finally, the plates of the unsterile cultivations are shown in Figure 27. All plates appear to have only one type of bacterial population. Upon closer analysis, although the size of some colonies may vary on the same plates, the remaining morphological features are similar and appear to belong to the same species.

**Figure 27.** Solid mediums cultured with samples from a “Non-Sterility” fermentation broth: HB medium (left); Marine agar medium (center); TSA medium (right). The dilution factor was 1:$10^6$ and the plates were incubated at 30ºC for 36 hours.

### 4.3.2. Phenotypic Method: Physiological / Biochemical Characteristics

To provide a more thorough examination of the microbial dynamics, the Sherlock® Microbial ID System from MIDI, Inc. was also used as a phenotypic identification method. After optimizing the growth of samples taken from the bioreactor on solid TSA medium, with the resource of intermediate plates, colonies that were apparently different were collected, as shown in Figure 28. The extracted colonies were submitted to methylation (see section 3.6.2.) and further analyzed with a GC apparatus, however the results were inconclusive since no match was found in the databank.

**Figure 28.** TSA solid media plated with quadrant streak method to achieve the growth of well-isolated colonies. The dilution factor was 1:$10^6$. The plates were incubated at 30ºC for 24 hours.

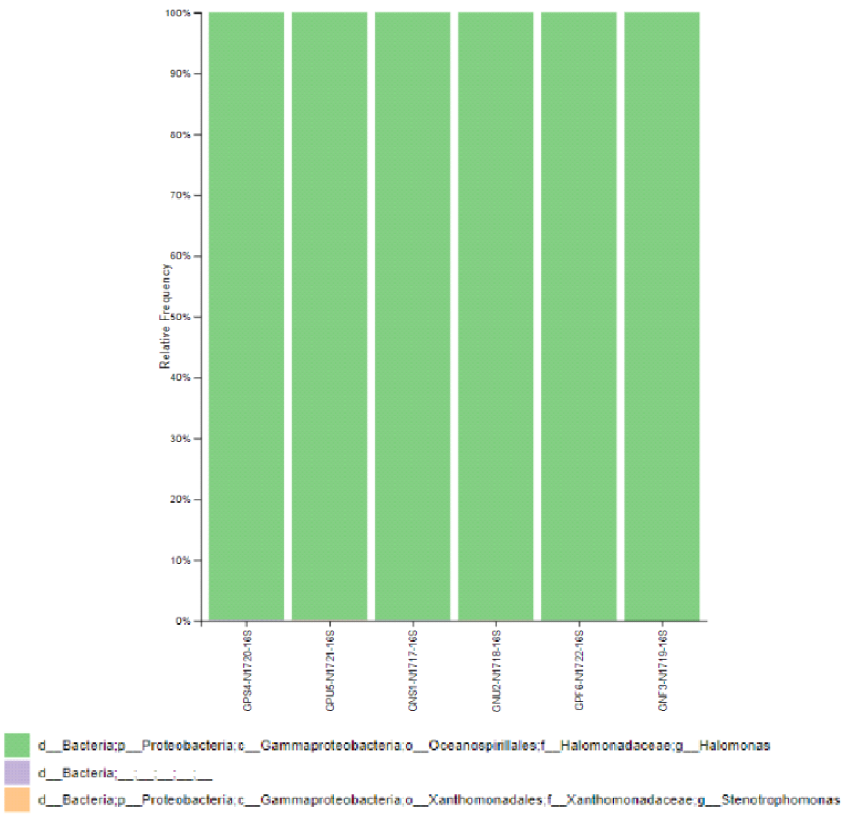
### 4.3.3. Genotypic Method: 16S amplicon sequencing

Due to the ambiguous results provided by the Sherlock® Microbial ID System, the samples were sent for 16S amplicon sequencing. Before the samples were sent for sequencing, their DNA was extracted and purified using two different procedures: Gram-positive and Gram-negative extraction protocols. Both protocols were used in order to prevent the Gram-positive extraction protocol, which is more aggressive, from damaging the DNA integrity of Gram-negative bacteria.

The NGS sequencing (Next-generation sequencing) project on StabVida began with quality control of all the samples, which included an examination of the DNA's concentration and consistency to ensure that the raw material was in the best possible shape for the library construction procedure. Prior to sequencing, the fragment size distribution and concentration of each library were evaluated using a set of primers for library creation. Sequencing of the DNA fragments (DNA libraries) through lllumina MiSeq platform, was conducted, and the resultant raw data underwent quality control.

As summarized in Figure 29, three taxon were identified, the taxa labeled as Bacteria (purple), lacked identification beyond the domain. Even though two unexpected different taxon were identified, as their relative frequencies are lower than 0.1%, they can be disregarded as negligible. The sequencing analysis yielded expected results, aligning with the phenotypic analysis conducted on the aforementioned plates, indicating the absence of any contamination.

Despite the lack of evidence for contamination in the phenotypic analysis, it is worth mentioning that the sequencing analysis was only able to identify the presence of the genus *Halomonas*. There is a possibility that contamination by the bacterium *Halomonas enlogata* occurred, considering that this particular bacterium was also under investigation in the same laboratory. This possible contamination could potentially explain the somewhat less favorable results obtained from the non-sterility tests. It is important to emphasize, however, that even if this were the case, it would not undermine the initial claim of the study, which stated that only halophilic bacteria could thrive in an environment with high salinity concentrations.




| | GNS1 | GNU2 | GNF3 | GPS4 | GPU5 | GPF6 |
|---|---|---|---|---|---|---|
| Genus: Halomonas | 99.943% | 99.945% | 100% | 99.902% | 99.905% | 99.968% |
| Genus: Stenotrophomonas | 0% | 0.026% | 0% | 0% | 0.039% | 0% |
| Domain: Bacteria | 0.057% | 0.029% | 0% | 0.098% | 0.056% | 0.032% |

**Figure 29.** Taxonomic summary results, at the genus level provided by StabVida. Six samples were sent for genomic analysis, the DNA fragments (DNA libraries) were sequenced and identified through an Illumina MiSeq platform. The samples were labeled according to the type of fermentation and the method used for DNA extraction: GNS1 (Gram-Negative DNA extraction protocol and “Full-Sterility” fermentation); GNU2 (Gram-Negative DNA extraction protocol and “Non-Sterility” fermentation); GNF3 (Gram-Negative DNA extraction protocol and “Semi-Sterility” fermentation); GPS4 (Gram-Positive DNA extraction protocol and “Full-Sterility” fermentation); GPU5 (Gram-Positive DNA extraction protocol and “Non-Sterility” fermentation); GPF6 (Gram-Negative DNA extraction protocol and “Semi-Sterility” fermentation); Taxonomic Results: Green (genus Halomonas ); Purple (domain Bacteria); Orange (Genus Stenotrophomonas).

Understanding the microbial composition of the three conditions examined in this study is crucial to determine the impact of non-sterile conditions on the population dynamics of fermentation. Performing fermentations for P3HB production under non-sterile conditions can have a significant impact on the microbial population compared to sterile conditions. Non-sterile conditions can introduce a wide range of microorganisms, including bacteria and fungi, into the fermentation environment. These microorganisms can compete with the P3HB-producing bacteria for nutrients and resources, potentially

reducing the yield of P3HB (Chatterjee & Abraham, 2018; T. Li et al., 2014). Additionally, the introduction of foreign microorganisms can lead to the formation of biofilms, which can further reduce the availability of nutrients for P3HB production. As a result, the population dynamics of the P3HB-producing bacteria can be greatly affected, leading to changes in growth rates and overall fermentation performance (Z. Chen & Wan, 2017). However, this study suggests that using a saline medium can alleviate the need for maintaining strict sterilization protocols, and the two non-sterile conditions ("Non-Sterility" and "Semi-Sterility") do not appear to have an impact on P3HB productivity, while still ensuring consistent fermentation performance.

## 4.4. APPROACHES TO IMPROVE THE ECONOMIC VIABILITY OF P3HB PRODUCTION

### 4.4.1. Algae waste stream as the carbon substrate

To reduce the price impact of the carbon source on manufacturing costs, cultivation parameters of *H. boliviensis* were assessed using *G. corneum* hydrolysates as the substrate. The batch phase started with an algal hydrolysate from crude *Gelidium* biomass while as feed a hydrolysate prepared from industrial *Gelidium* residues was used. Dry weights measurements were employed to monitor the evolution of the biomass. Figure 30 illustrates the cultivation parameters (**A**), the uptake of glucose and galactose and the consequent production of gluconic acid (**B**) and the data acquired automatically during the cultivation (**C**). A more comprehensive examination of each parameter is presented in the Figures 31, 32 and 33. When comparing the results, it is important to have in consideration that the hydrolysate itself adds to the dry weight measurements, as can be seen in Figure 30A at t=0.

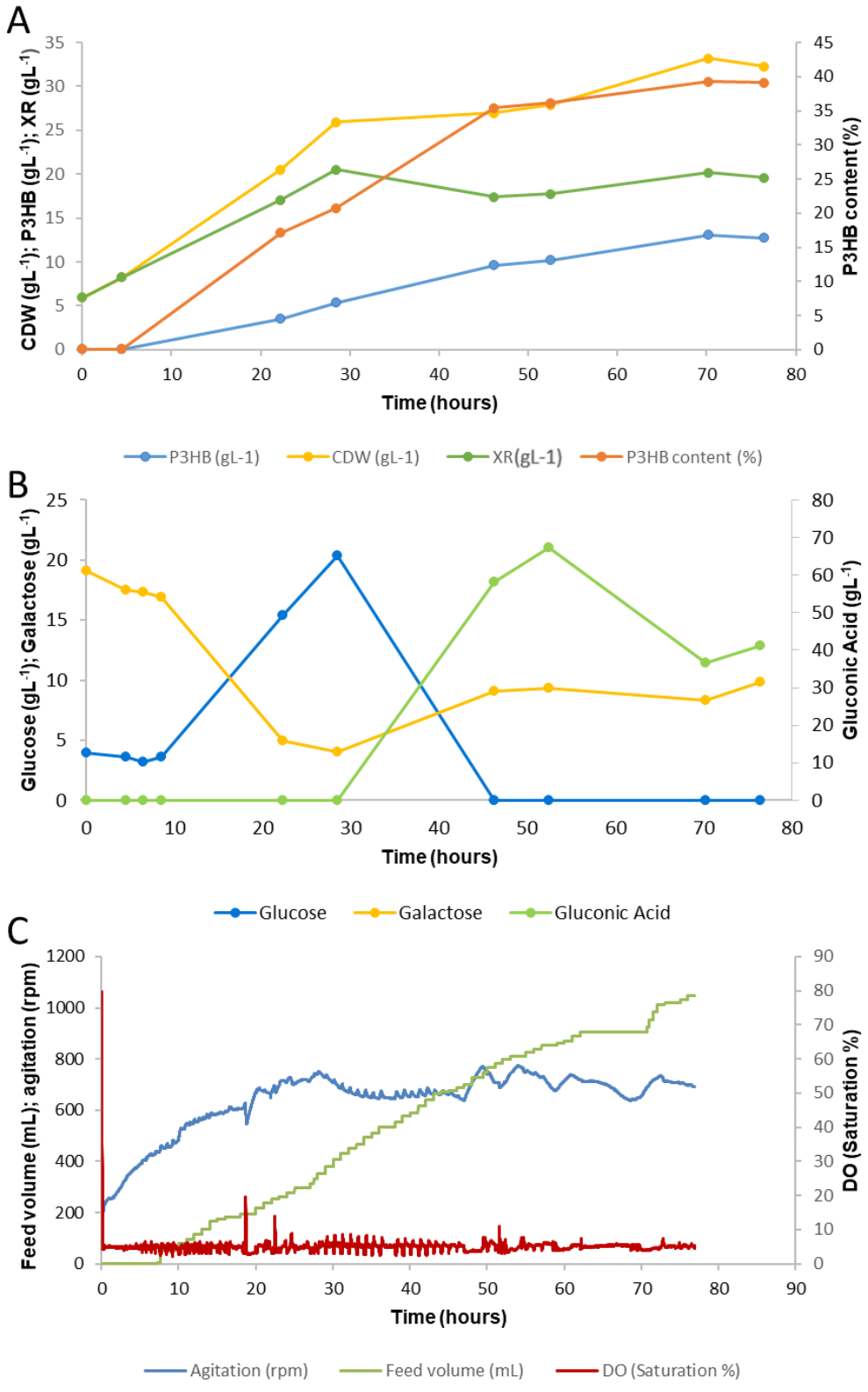



**Figure 30.** (**A**) Graphic representation of *H. boliviensis* growth and P3HB accumulation with crude *G. corneum* hydrolysate (Batch phase) and its industrial residues hydrolysate (Fed-batch phase) as a carbon source and under nitrogen limiting conditions; Legend: P3HB $gL^{-1}$(o); XR $gL^{-1}$(o); CDW $gL^{-1}$ (o); P3HB Content (w/w) % (o). (**B**) Glucose and galactose consumption and gluconic acid production; Legend: Gluconic acid $gL^{-1}$ (o); Glucose $gL^{-1}$ (o); Galactose $gL^{-1}$ (o). (**C**) Data acquired automatically during the cultivation, namely feed volume, agitation, and DO (% sat); Legend: Feed volume (mL) (o); Agitation (rpm) (o); DO (Saturation %) (o).

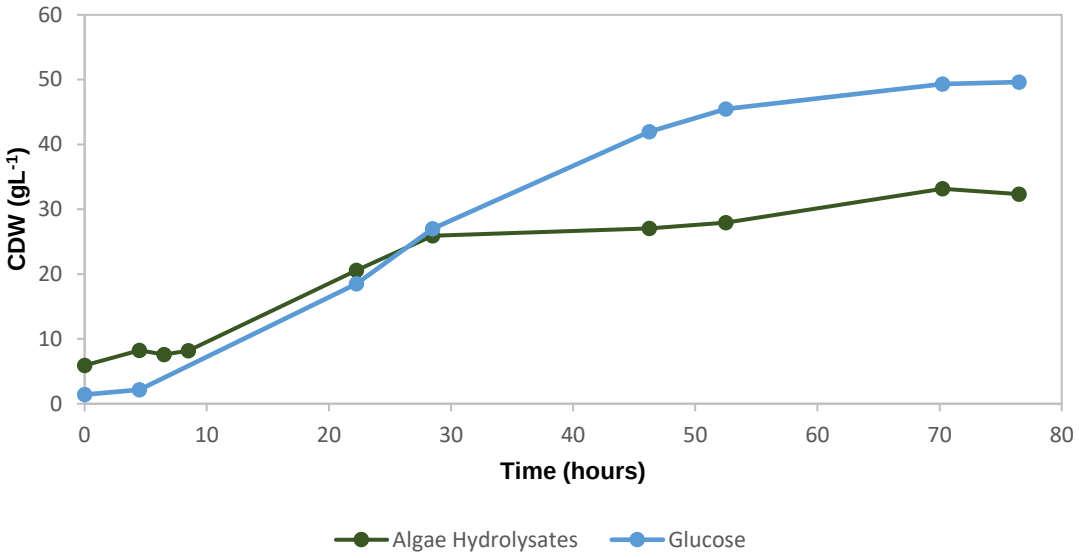



**Figure 31.** Comparison of biomass growth of *H. boliviensis* with different carbon sources, algae hydrolysates, and glucose, through dry weights (600nm) profiles. Legend: Algae hydrolysate (**o**); Glucose (**o**).

After 30 hours of cultivation in the hydrolysates assay, the growth stabilized, achieving a dry weight of 33gL$^{-1}$. Contrasted to the results attained with glucose, a significant difference is observed. Analyzing the growth phases, it can be concluded that in the batch phase (until 10h of cultivation), which corresponds to the lag phase and part of the log phase, the bacteria behave similarly as in the assay using glucose as substrate, as demonstrated in Figure 31. This could indicate that the sugars, mainly galactose, present in the hydrolysates of the macroalgae *G. corneum* have been assimilated efficiently. This result corroborates previous studies indicating that galactose, as glucose, is an effective substrate for P3HB production by *H. boliviensis* (Mesquita Marques, 2017).

However, the short duration of the log phase could indicate that the phosphate and nitrogen components in the hydrolysate from the batch phase may have been inaccessible for assimilation. Consequently, due to the comparatively lower nutrient concentration compared to the glucose assay, these essential elements likely became limiting factors sooner, preventing biomass growth and favoring P3HB accumulation, as observed in Figure 32. In accordance with the lower biomass growth, the outcome of polymer production also declines, yielding approximately 13.02 gL$^{-1}$, which corresponds to 39.26% P3HB accumulation in cells (Figure 32 and 33). However, as stated earlier, the quantification of P3HB (%) is consistently impacted by the presence of hydrolysate residues in the overall CDW, in addition to biomass. It is crucial to consider this factor when comparing this parameter with glucose assays.

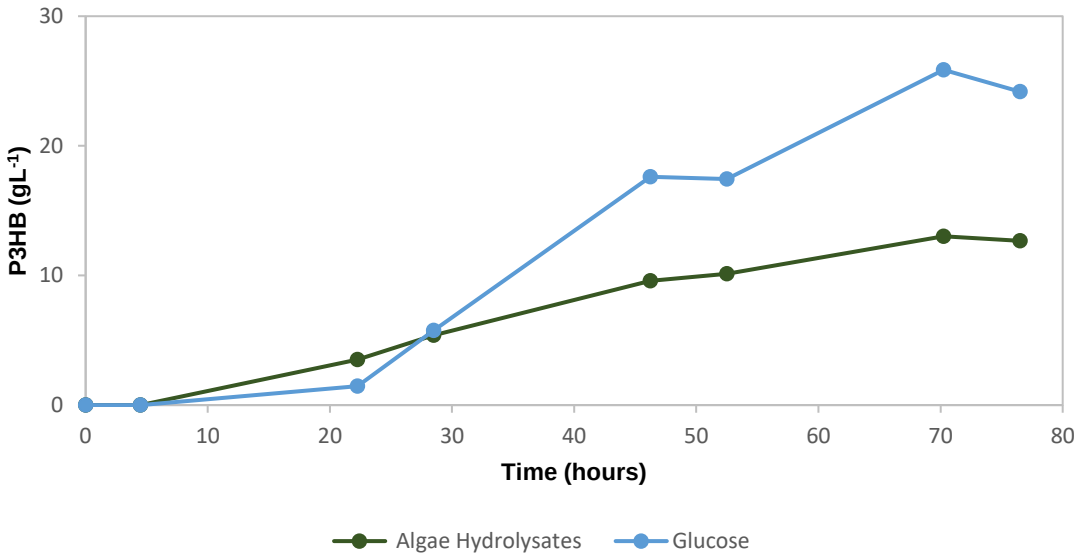



**Figure 32.** P3HB ($gL^{-1}$) production regarding different carbon sources scenarios, glucose (blue) and algae hydrolysates (green). Legend: Algae hydrolysate (o); Glucose (o).

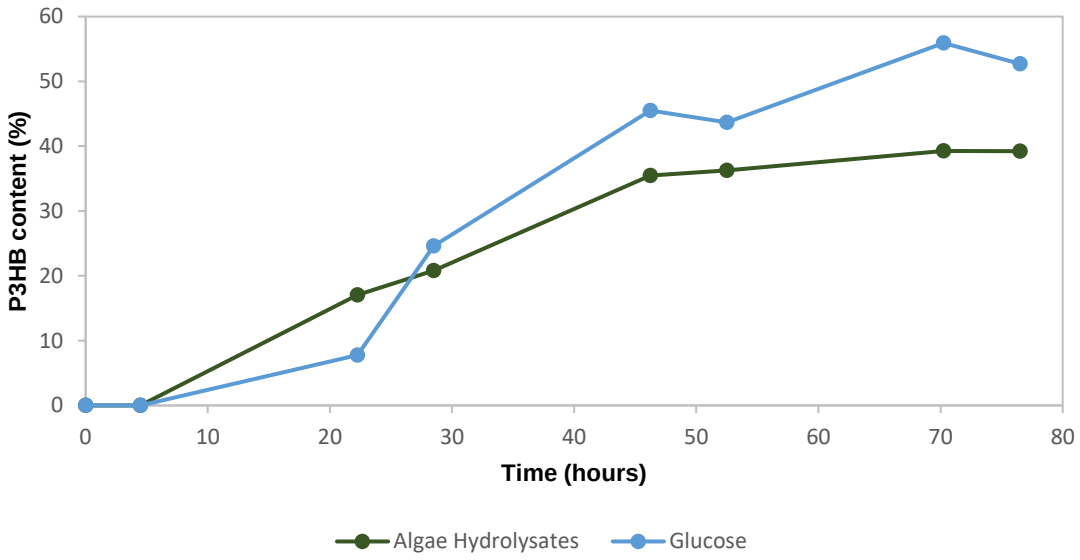



**Figure 33.** P3HB ($gL^{-1}$) content regarding different carbon sources scenarios, glucose (blue) and algae hydrolysates (green). P3HB content (weight percent) was obtained as the percentage of the ratio of P3HB concentration to cell dry weight. Legend: Algae hydrolysate (o); Glucose (o).

Figure 30B displays the uptake of glucose and galactose and the consequent production of gluconic acid. During the first 10 hours, that is, in the batch phase, a preferential assimilation of galactose is observed, which may be due to its higher concentration relative to glucose. With the start of the fed-batch phase, there is a rise in glucose content since the feed used (algae hydrolysate residues), solely comprises glucose as a carbon source. Galactose continued to be consumed until around 28.5 hours

of cultivation (end of log phase) and remained constant around 9 $gL^{-1}$ during the rest of the assay, although with a slight increase. Since the feed lacks galactose, this increase could be attributed to the production of a by-product with a similar retention time, or the inherent uncertainty associated with the HPLC process. With regard to glucose uptake, although pulses have been programmed in order to keep its content constant, an abrupt decrease can be observed. The sudden intake of glucose overrides the exponential production of gluconic acid, corroborating the hypothesis discussed in section 4.2. suggesting that glucose concentrations of bellow 40 $gL^{-1}$ promote the cellular metabolism to produce gluconic acid.

As depicted in the fermentation acquisition graph (Figure 30C), the feed intake remained constant for most of the cultivation, with the exception of the interval between 62h and 70h, during which no feed pulse was scheduled. Despite this, glucose concentration appears to have become negligible after 46 hours, prior to the absence of feed. These findings imply that, after biomass growth stabilized, the programmed feed pulses were insufficient to sustain the required glucose concentration.

In Table 6, the results of the seaweed waste are summarized and compared to the results of the glucose assay. Despite the apparent similarity of XR ($gL^{-1}$) values between the algal assay and glucose, it is important to note that these values do not reflect accurate measurements due to the presence of hydrolysate residues. Therefore, parameters such as CDW ($gL^{-1}$), XR ($gL^{-1}$), and P3HB (%) are prone to errors and cannot be considered as representative of the actual values.

The study findings highlight the hydrolysate's potential for bacterial cultivation. Nonetheless, in order to achieve the intended biomass level prior to entering the limitation phase, a greater supplementation of phosphate and nitrogen is necessary for the hydrolysate utilized in the batch phase. Moreover, a thorough investigation into the sugar consumption rate in the residue hydrolysate utilized as feed is imperative to enhance the feed strategy and prevent sugar depletion, consequently, P3HB production.

**Table 6.** Comparison of the cultivation's parameters from the fed-batch P3HB production with *G. corneum* hydrolysates and glucose as the carbon source. CDW ($gL^{-1}$) and XR ($gL^{-1}$) were calculated using the maximum P3HB ($gL^{-1}$).

| Fermentation | Nutrient Limitation | *CDW ($gL^{-1}$)* | *P3HB max ($gL^{-1}$)* | *% P3HB max* | *Xr ($gL^{-1}$)* | *Prod vol max ($gPL^{-1}h^{-1}$)* |
|---|---|---|---|---|---|---|
| **Algal hydrolysate** | Phosphate | 33.17 | 13.02 | 39.26 | 20.14 | 0.185 |
| **Glucose** | Phosphate | 49.33 | 25.86 | 55.92 | 20.79 | 0.468 |

## 4.5. INDUSTRIAL SCALE UP SIMULATION AND ECONOMIC EVALUATION

This chapter will focus on the economic comparison between a P3HB production plant operating under total sterility versus one where equipment and processes for sterilization are not implemented. This analysis takes into account the efficacy of using hypersaline media to prevent contamination of foreign microorganisms, as demonstrated in section 4.3, thereby reducing the need for maintaining strict

sterilization protocols. The simulation without sterilization mechanisms was designed to evaluate the cost savings associated with using halophilic bacteria, which can grow in saline media and are less prone to contamination. This approach is expected to reduce equipment and energy costs, making P3HB production more economically feasible.

However, it is important to note that this study only evaluated the upstream portion of the P3HB production process, and therefore the results do not reflect the actual costs of operating a full-scale production plant. Thus, while the study does not provide a comprehensive analysis of the total costs associated with P3HB production, it does provide valuable insights into the economic trade-offs between implementing strict sterilization protocols and potentially reducing costs by using hypersaline media in the upstream process.

SuperPro Designer, a process simulation software, was used to model the P3HB production process under both sterile and non-sterile conditions. The software allowed for the comparison of key economic parameters such as operating costs of equipment, energy, and water.

### 4.5.1. Flowsheet development and description

The production flowsheet was developed to scale up the experimental laboratory work described in chapter 3.4. The production process utilized fed-batch cultivation with glucose as the carbon source and phosphate as the limiting nutrient to produce P3HB, under identical conditions to those in the experimental laboratory work. To execute this process, two seed reactors and a fermenter were employed, with a scale-up factor of 10x between each reactor. In the batch process, the P3HB production was set to attain, approximately 70Kg in both scenarios. For simplicity reasons, the stoichiometric equations for biomass growth and P3HB production used were described in (Joseph & Renjanadevi, 2021), with the conversion of glucose to P3HB based on experimental data obtained and described in section 4.1.

For a fed-batch process, scheduling information is required, and stream flows are displayed on a per-batch basis. Each operation was scheduled to start at the end of the previous operation (transfer out of the previous operation master slaved with the posterior operation). The size of each equipment was set based on the rated throughput that flows through the equipment.

#### *4.5.1.1. "Full-Sterility" plant design*

In the laboratory setting described in 3.4, glucose, magnesium, sodium chloride, and mineral components were sterilized individually to prevent precipitation and degradation of these compounds. However, due to a software limitation of only allowing 25 pieces of equipment, only sodium chloride could be sterilized separately from the other components.

The seed medium (without NaCl) for this fermentation is prepared in a blending tank (V-101). The operations in the blending tank are charging of water, charging of the mineral components, charging of glucose, agitation and transferred out. The output is then heat-sterilized (ST-101) and transferred to a storage blending tank (V-106), that splits two bulk flow streams into the two seed reactors (SR-101 and SR-102). The seed reactor (SR-101), with a volume of 100 L is employed to generate the pre-inoculum,

then the output inoculum is transferred to the second bioreactor (SR-102), with a volume of 1000 L. The operations of both seed reactors are transfer in of seed medium, transfer in of NaCl solution, agitation, fermentation and transfer out. In SR-101, the bacteria were charge into to the reactor, while in SR-102 the inoculum is transferred from the first seed reactor.

A concentrated NaCl solution was prepared separately, to avoid precipitation, in a batch blending tank (V-103), with the operations: charge NaCl solution, agitation and transfer out. The output is then heat-sterilized (ST-103) and transferred to a storage blending tank (V- 107), that splits four bulk flow streams into the seed reactors (SR-101 and SR-102), the fermenter (FR-101) and the feed blending tank (V-102), to achieve a final 45 $gL^{-1}$ NaCl in all the vessels. As the composition of the medium for the seed reactors and the fermenter differ, an additional blending tank (V-104) is needed for the production medium preparation (without NaCl). The operations in the blending tank are charging of water, charging of mineral components, agitation and transferred out. The output is then heat-sterilized (ST-104) and transferred directly to the fermenter. The fermentation is carried out with aeration rate of 1.0 v.v.m and at a temperature of 30℃. The operations in the stoichiometric fermenter (FR-101) are transfer in of the production medium, transfer in of NaCl solution, transfer in of inoculum, agitation, and fermentation. The feed (without NaCl) for the fed-batch process is prepared in a blending tank (V-105). The operations in the blending tank are charging of water, charging of mineral components, charging of glucose, agitation and transfer out. The output is heat-sterilized (ST-105) and then transferred to another blending tank (V-102), which is connected to the fermenter (FR-101). Three compressors (G-101, G-102 and G-103) are used to supply required air in the respective vessels (SR-101, SR-102 and FR-101). Additionally, air filters are also employed in both air inlet and exhaust gas outlet. Chilled water is used to remove the heat produced by the exothermic process and maintain a constant temperature in both seed reactors and fermenter.

Clean-in-place (CIP) and steam-in-place (SIP) operations were implemented in all the equipment, following heat sterilization operations, apart from the compressors and the air/exhaust gas filters. The CIP procedure was composed by two steps of water and one with NaOH (0,5M) with a total process duration of 50 minutes. For sterilizing the equipment, SIP procedures were configured with vapor at 152ºC. The “Full-Sterility” flowsheet is shown in Figure 34.

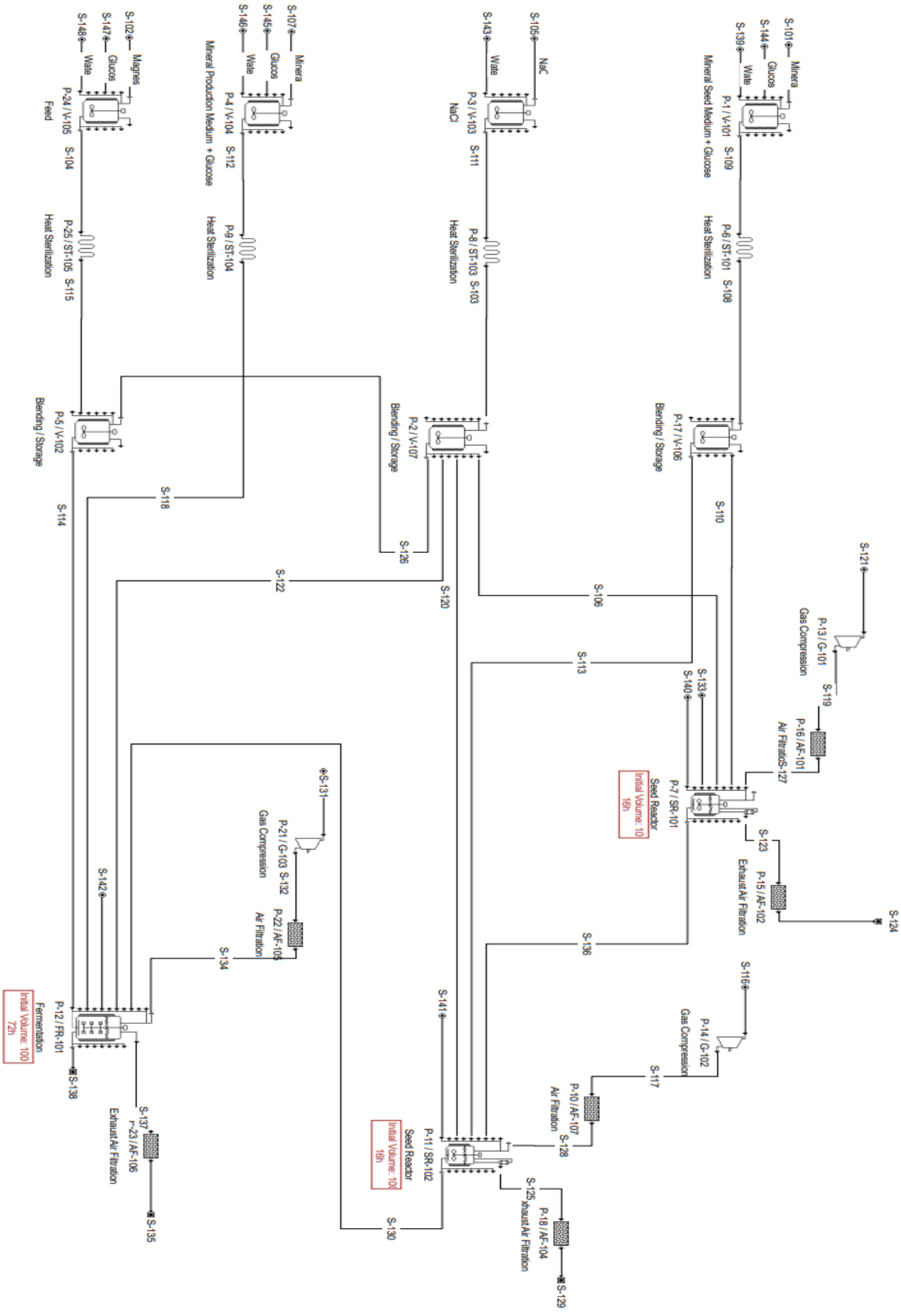



**Figure 34.** “Full-Sterility” flowsheet of P3HB production created with SuperPro Designer v.12 software.

#### *4.5.1.2. "Non-Sterility" plant design*

In the "Non-Sterility" condition the process design, as shown in Figure 35, is simplified as there's no need for heat sterilization, air filters for the inlet air, and SIP (Sterilize-In-Place) operations in the equipment. The seed medium (with all the components) is prepared in a blending tank (V-101). The operations in the blending tank are charging of water, charging of the seed mineral medium components, charging of glucose, charging of NaCl, agitation and transferred out. The output splits two bulk flow streams into the two seed reactors (SR-101 and SR-102). A second blending tank (V-104) is needed for the production medium preparation (with all components). The operations in the blending tank are charging of water, charging of the mineral production medium components, charging of glucose, charging NaCl, agitation and transferred out. The output is then transferred directly to the fermenter. Finally, in a third blending tank (V-105), which is directly connected to the fermenter, the feed is prepared: charging of water, charging of mineral feed components, charging of glucose, agitation and transfer out. In this condition, the NaCl solution isn't prepared separately.

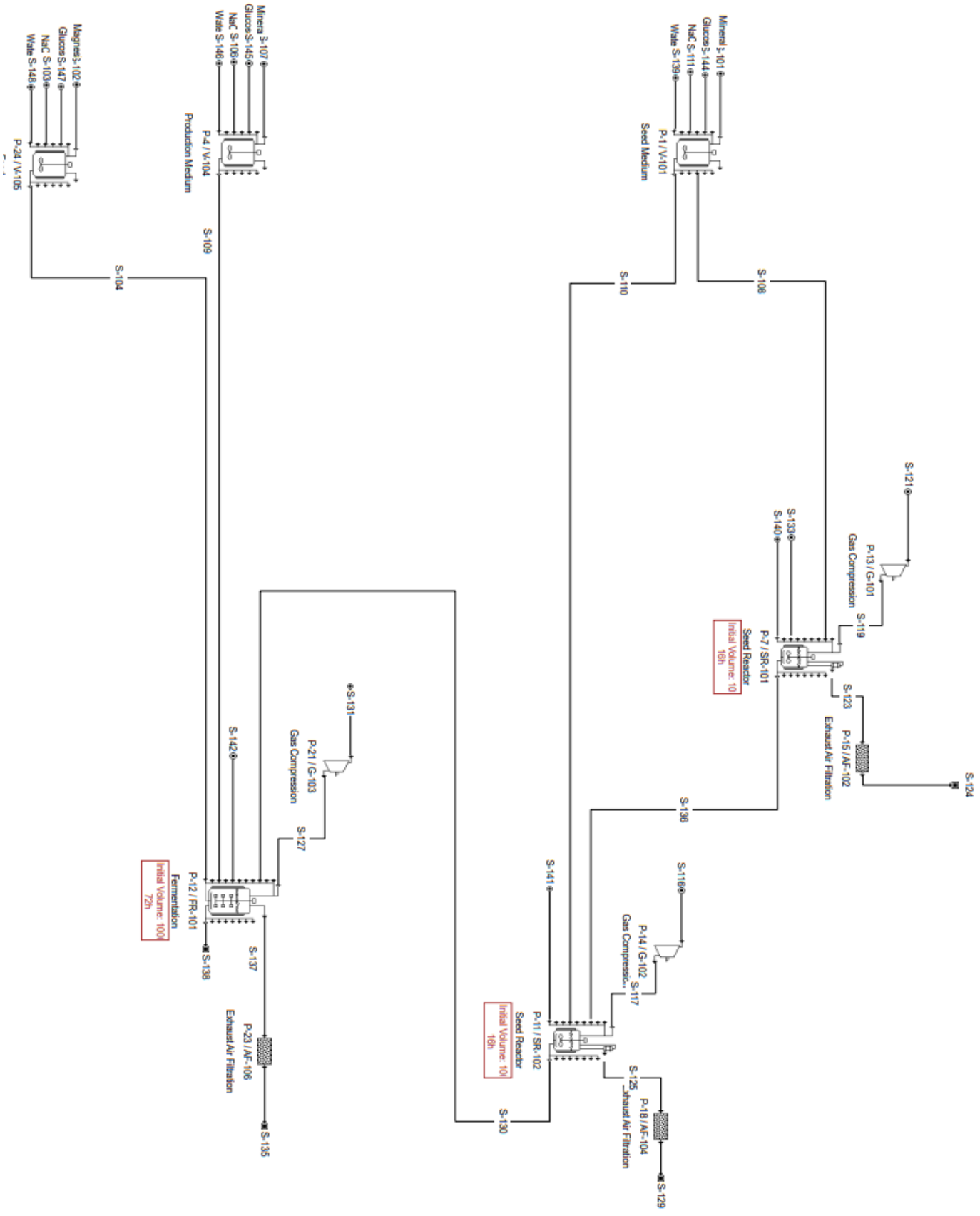



**Figure 35.** “Non-Sterility” flowsheet of P3HB production created with SuperPro Designer v.12 software.

### 4.5.2. Economic evaluation

This study focuses exclusively on operational costs, including energy and water usage, as well as equipment expenses. Other factors that may affect the production process, such as productivity per batch and raw materials, were held constant across both simulations, not being relevant for the purposes of this analysis.

SuperPro Designer performs thorough cost analysis, estimating capital costs as well as operating costs, and generating the following two pertinent reports: the Economic Evaluation Report (EER), and the Itemized Cost Report (ICR). Figure 36 displays the relevant information of EER reports for both simulations.

**Economic Evaluation Report**
*for 1. All sterile*

**1. EXECUTIVE SUMMARY (2022 prices)**

| | |
|---|---|
| Total Capital Investment | 35181000 € |
| Capital Investment Charged to This Project | 35181000 € |
| Operating Cost | 8887000 €/yr |

**3. FIXED CAPITAL ESTIMATE SUMMARY (2022 prices in €)**

| | |
|---|---|
| **3A. Total Plant Direct Cost (TPDC) (physical cost)** | |
| 1. Equipment Purchase Cost | 5491000 |
| 2. Installation | 2285000 |
| 3. Process Piping | 1922000 |
| 4. Instrumentation | 2197000 |
| 5. Insulation | 165000 |
| 6. Electrical | 549000 |
| 7. Buildings | 2471000 |
| 8. Yard Improvement | 824000 |
| 9. Auxiliary Facilities | 2197000 |
| TPDC | 18100000 |
| **3B. Total Plant Indirect Cost (TPIC)** | |
| 10. Engineering | 4525000 |
| 11. Construction | 6335000 |
| TPIC | 10860000 |
| **3C. Total Plant Cost (TPC = TPDC+TPIC)** | |
| TPC | 28960000 |
| **3D. Contractor's Fee & Contingency (CFC)** | |
| 12. Contractor's Fee | 1448000 |
| 13. Contingency | 2896000 |
| CFC = 12+13 | 4344000 |
| **3E. Direct Fixed Capital Cost (DFC = TPC+CFC)** | |
| DFC | 33304000 |

**Economic Evaluation Report**
*for 2. Non Sterility*

**1. EXECUTIVE SUMMARY (2022 prices)**

| | |
|---|---|
| Total Capital Investment | 22315000 € |
| Capital Investment Charged to This Project | 22315000 € |
| Operating Cost | 6387000 €/yr |

**3. FIXED CAPITAL ESTIMATE SUMMARY (2022 prices in €)**

| | |
|---|---|
| **3A. Total Plant Direct Cost (TPDC) (physical cost)** | |
| 1. Equipment Purchase Cost | 3479000 |
| 2. Installation | 1431000 |
| 3. Process Piping | 1218000 |
| 4. Instrumentation | 1392000 |
| 5. Insulation | 104000 |
| 6. Electrical | 348000 |
| 7. Buildings | 1565000 |
| 8. Yard Improvement | 522000 |
| 9. Auxiliary Facilities | 1392000 |
| TPDC | 11450000 |
| **3B. Total Plant Indirect Cost (TPIC)** | |
| 10. Engineering | 2862000 |
| 11. Construction | 4007000 |
| TPIC | 6870000 |
| **3C. Total Plant Cost (TPC = TPDC+TPIC)** | |
| TPC | 18320000 |
| **3D. Contractor's Fee & Contingency (CFC)** | |
| 12. Contractor's Fee | 916000 |
| 13. Contingency | 1832000 |
| CFC = 12+13 | 2748000 |
| **3E. Direct Fixed Capital Cost (DFC = TPC+CFC)** | |
| DFC | 21068000 |

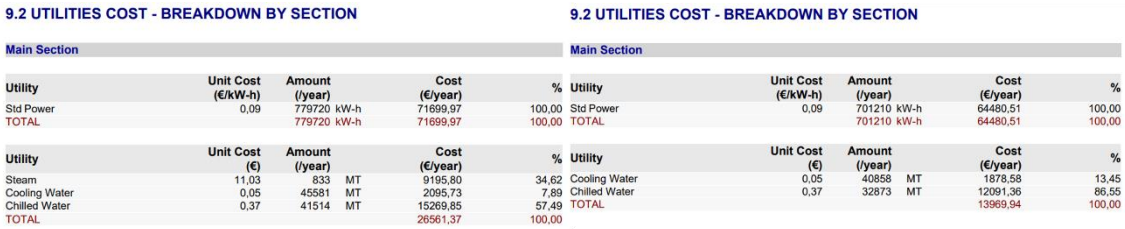
**Figure 36.** Executive summary and fixed capital estimate summary, extracted from the Economic Evaluation Report from “Full-Sterility” (left) and “Non-Sterility”( right) simulations.

The total capital investment for the “Full-Sterility” facility is approximately 35 million euros, contrasting with the 22 million euros investment for the “Non-Sterility” facility. This reflects a decrease in investment of approximately 13 million euros. This includes equipment purchase and installation costs; other costs related to plant construction; startup and validation costs; and the working capital required for this project. Regarding the operations costs, the implementation without sterilization process reflected in a 2.5 million decrease.

The study found that the total cost of the plant (as shown in Figure 36.3C) was approximately 4 million euros lower without the use of sterilization mechanisms, representing a significant 37% decrease. This reduction in cost was due to the elimination of heat sterilization machines, air filters, and the additional blending tanks required for sterilization.

Energy consumption (Std power), shown in Figure 37, was also lower in the absence of sterilization mechanisms and SIP operations, resulting in a 10% decrease in energy costs due to the reduced need

for heat sterilization. Water consumption was also significantly affected with, approximately, a 47% decrease, mainly due to the steam employed in the “Full-Sterility” scenario.



**9.2 UTILITIES COST - BREAKDOWN BY SECTION**

**Main Section**

| Utility | Unit Cost (€/kW-h) | Amount (/year) | | Cost (€/year) | % |
|---|---|---|---|---|---|
| Std Power | 0,09 | 779720 | kW-h | 71699,97 | 100,00 |
| TOTAL | | 779720 | kW-h | 71699,97 | 100,00 |

| Utility | Unit Cost (€) | Amount (/year) | | Cost (€/year) | % |
|---|---|---|---|---|---|
| Steam | 11,03 | 833 | MT | 9195,80 | 34,62 |
| Cooling Water | 0,05 | 45581 | MT | 2095,73 | 7,89 |
| Chilled Water | 0,37 | 41514 | MT | 15269,85 | 57,49 |
| TOTAL | | | | 26561,37 | 100,00 |

**9.2 UTILITIES COST - BREAKDOWN BY SECTION**

**Main Section**

| Utility | Unit Cost (€/kW-h) | Amount (/year) | | Cost (€/year) | % |
|---|---|---|---|---|---|
| Std Power | 0,09 | 701210 | kW-h | 64480,51 | 100,00 |
| TOTAL | | 701210 | kW-h | 64480,51 | 100,00 |

| Utility | Unit Cost (€) | Amount (/year) | | Cost (€/year) | % |
|---|---|---|---|---|---|
| Cooling Water | 0,05 | 40858 | MT | 1878,58 | 13,45 |
| Chilled Water | 0,37 | 32873 | MT | 12091,36 | 86,55 |
| TOTAL | | | | 13969,94 | 100,00 |

**Figure 37.** Utilities cost analysis, extracted from the Itemized Cost Report from “Full-Sterility” (left) and “Non-Sterility” (right) simulations.

Overall, this study has demonstrated that utilizing halophilic bacteria in upstream P3HB production can result in significant cost savings, which have important implications for the development of sustainable and economically viable bioplastic production processes. It should be noted, however, that this type of analysis is dependent on a number of assumptions, including market demand and the selling price of the polymer, as well as the costs of key raw materials, fermentation yields of biomass and P3HB, and potential losses in the primary recovery and purification steps. Therefore, the actual economic outcomes of such an investment may differ significantly from the current projection.

## 5. CONCLUSION AND FUTURE WORK

As the harmful effects of conventional synthetic plastics become more widely recognized, the production of biopolymers, such as P3HB, is gaining popularity around the world. P3HB is a short-chain length PHA that has similar physical and thermal properties to polyethylene, making it useful in a range of industries. However, the cost of industrial manufacturing is currently high, making it unsustainable and uncompetitive when compared to petrochemical plastics. To reduce production costs, improvements must be made to the fermentation process.

The primary objective of this work, to demonstrate the effectiveness of using halophilic bacteria to prevent cross-contamination in open fermentations without reducing P3HB productivity, was achieved. The genomic analysis conducted successfully identified only the presence of the genus *Halomonas* in all three conditions, providing compelling evidence to support the initial hypothesis of this study. This finding strongly suggests that only halophilic bacteria are capable of flourishing in environments characterized by high salinity concentrations.

The production of P3HB and growth under different levels of sterility were analyzed, and the results showed that the three tested conditions had similar P3HB production levels, with the “Semi-Sterility” regime achieving the highest concentration of P3HB. Moreover, the P3HB content was consistently above 50% in all assays, indicating that *H. boliviensis* has the potential to produce P3HB under different levels of sterility. In the non-sterile assays, growth and productivity were slightly lower, which could be attributed to several factors, such as variations in feeding strategies and effect of the heat sterilization process on the compounds present in medium.

Furthermore, to compare the cost-effectiveness of each strategy, the data obtained from the experiments were used to design two production plants using SuperPro designer software: one with "Full-Sterility" and the other with "Non-Sterility" conditions. The results indicated that using non-sterilized processes led to a significant reduction in capital investment and operational costs, including energy and water usage. In fact, the total cost of the plant was approximately 37% lower without sterilization mechanisms, with energy costs reduced by approximately 10% and water consumption by 47%. The development of these simulations highlights the practical applications of the experimental findings and their potential significance in industrial production processes. Future studies, such as "what-if" analyses using the SuperPro software, could provide additional insights into the potential risks and benefits of implementing the strategies described above.

However, the study also identified a significant challenge in P3HB production - the accumulation of gluconic acid by *H. boliviensis*, which competes for glucose as a substrate. To maximize P3HB synthesis, understanding the primary metabolic pathways involved in glucose intake is crucial. Further research is necessary to determine which pathways are triggered or suppressed under specific conditions. Additionally, co-production of intracellular P3HB combined with gluconic acid, an extracellular product, could provide an alternative to optimizing economic viability.

Finally, the study demonstrated that *G. corneum* hydrolysates have the potential to reduce the manufacturing costs of P3HB production. Galactose and glucose, present in the hydrolysates, were an

effective substrate for P3HB production. However, the low nutrient availability, such as phosphate and nitrogen, in the hydrolysate may have impeded biomass growth and favored P3HB accumulation. Further investigation into the sugar consumption rate when the hydrolysate produced from the industrial *Gelidium* residues is utilized as feed is necessary to optimize the feed strategy and prevent sugar depletion. Nonetheless, these findings highlight the potential of seaweed waste hydrolysate for bacterial cultivation and provide insights into the cultivation parameters for the efficient production of P3HB.

Overall, this study provides valuable insights into the production of P3HB by *H. boliviensis* and contributes to the growing body of knowledge on bioplastics production using microbial fermentation. The results obtained in this study will help pave the way for further research in this field, with the ultimate goal of developing sustainable and eco-friendly alternatives to traditional plastic materials.

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## APPENDIX

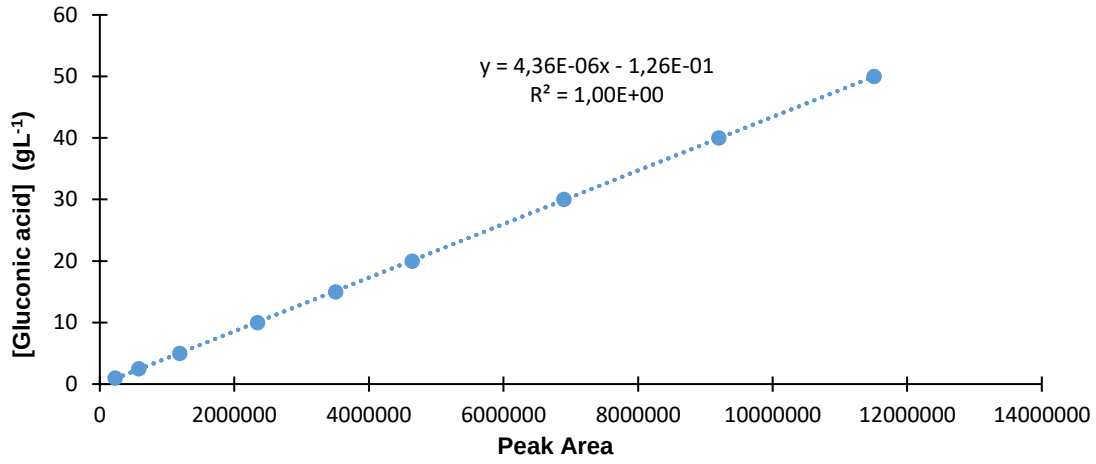


**Figure 38.** Calibration slope for gluconic acid concentration ($gL^{-1}$) correlating peak area accessed by HPLC UV-VIS detector under a standard solution with a retention time of 12.7 min.

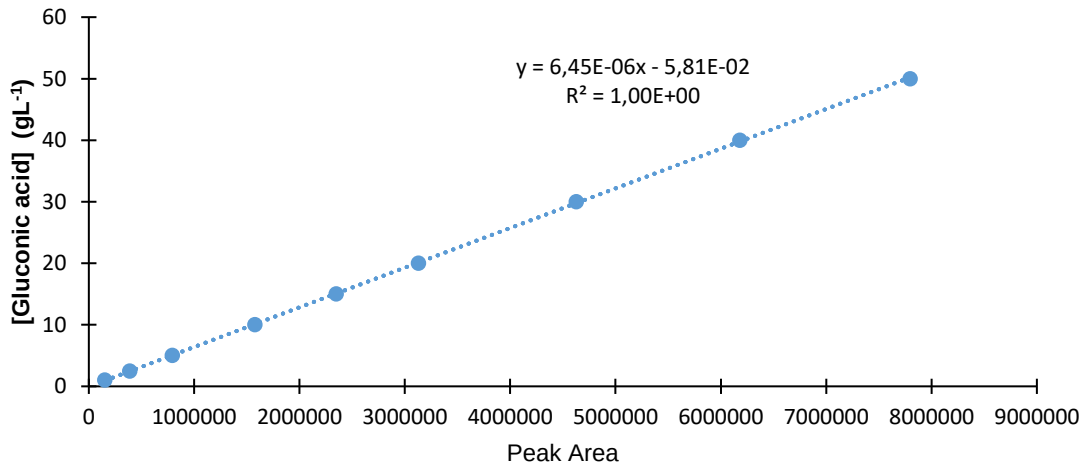


**Figure 39.** Calibration slope for gluconic acid concentration ($gL^{-1}$) correlating peak area accessed by HPLC RI detector under a standard solution with a retention time of 13.4 min.

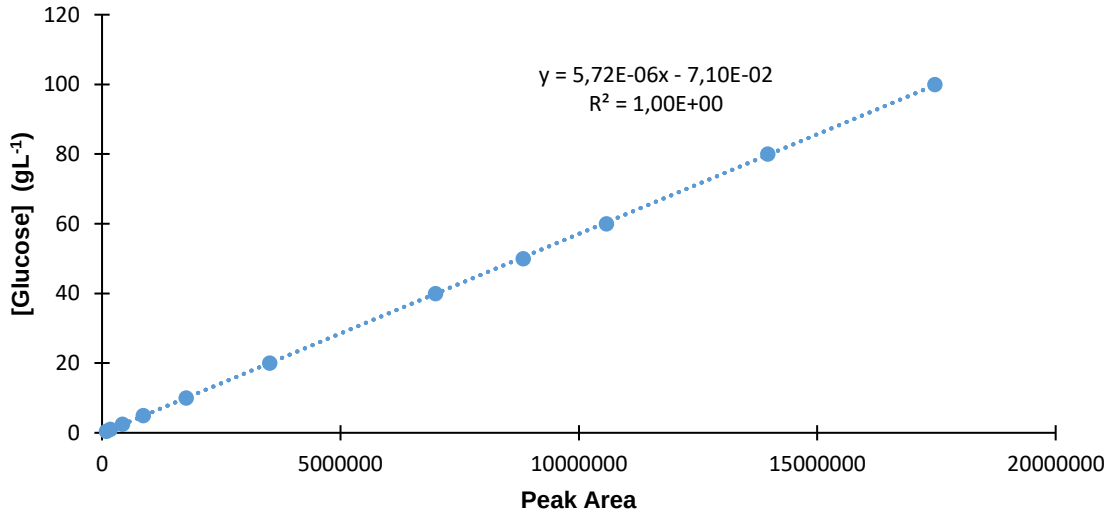


**Figure 40.** Calibration slope for glucose concentration ($gL^{-1}$) correlating pick area accessed by HPLC RI detector under a standard solution with a retention time of 13.4 min.