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

Maria Carlota de Albuquerque Mota Mendes de Abreu
Supervisors: Professor Maria Teresa Ferreira Cesário Smolders
Professor Pedro Carlos De Barros Fernandes
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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 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 gluconic acid as by-product, a commodity with wide application in food, pharma, chemical and construction industries.

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

1. INTRODUCTION

Plastic, invented during the Second Industrial Revolution, is derived from various synthetic or semi-synthetic materials from nature such as natural gas, oil, coal, minerals, and plants. The demand for petroleum-based plastics is rapidly increasing due to industrialization and population growth. Unfortunately, the exponential growth of plastic production and inadequate waste management have led to environmental leakage. In the European Union, it is estimated that 80% to 85% of marine litter consists of plastic. To address these concerns, the European Parliament and Council implemented Directive 2019/904, aiming to mitigate the adverse impacts associated with plastic [1].

In recent decades, several possible solutions were found and are being implemented to solve the plastic pollution issue. However, the current solutions are not realistic at the present day. Of all plastic produced in the planet, only 9% is recycled. Therefore, shifting towards more sustainable alternatives, as biodegradable polymers, is mandatory [2]. There are three main types of bio-based polymers produced: agropolymers (starch, cellulose derivatives and natural rubbers), monomers produced by fermentation followed by conventional chemistry polymerization (such as polylactic acid and polybutylene succinate), and lastly, microbial polymers, such as polyhydroxyalkanoates (PHAs) [3]. However, despite these various options and manufacturing methods, not

all bio-based polymers are biodegradable. From the bio-based and biodegradable alternatives, PHAs are attracting much interest due to their environmental benefits and multiple applications, such as packaging, agriculture, pharmaceutical and medical [4]. PHAs are a family of aliphatic biopolyesters, synthesized by numerous microorganisms, as energy reserve materials stored in form of granules. 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.

However, one of the major limitations to continuous market growth is PHA superior production costs when comparing with fossil-based 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 [5], [6]. Microbial contaminations, complexity of sterile operations, energy consumption, expensive substrates and low substrates to product conversion efficiency are some of the drawbacks of current biotechnology. To overcome these drawbacks, it is critical to develop new technologies and strategies. [7]. Contamination is a common problem in fermentation, causing financial damage, especially in industrial-scale processes. Sterilization methods like heating, filtration, irradiation, sonic vibration, and chemical agents are used, with heating being the most common due to its efficiency. However, heating consumes a lot of energy, adding to production costs. Non-sterile fermentation may be a cost-effective technique in the future, but maintaining bioreactor efficiency is a challenge [8].

Halophiles are a unique and diverse group of microorganisms that thrive in high salinity environments. They have the ability to accumulate PHA, making them attractive for bioplastic production. By utilizing high saline media, cross-contamination from non-halophilic microorganisms can be minimized or eliminated, reducing the energy and water consumption associated with sterilization of bioreactors and tubing [9].

Regarding production costs, carbon sources contribute significantly to expenses, representing a major portion of the high production cost of polymers. Traditional PHAs manufacturing relies on expensive raw materials like pure carbohydrates (glucose, sucrose, maltose, starch) and fatty acids. Utilizing cheaper carbon source alternatives, such as industrial waste, is crucial to reduce costs and improve the economic viability of PHA production [10].

The seaweed industry produces approximately 12 million tons annually, with applications in food, industrial specialties, fertilizers, cosmetics, pharmaceuticals, and feed markets. *Gelidium*, a red macroalgae, is commonly used for commercial agar production and its residues contain valuable carbohydrates. Maximizing the utilization of macroalgae waste as a carbon source can support the sustainability of the seaweed industry [11].

2. MATERIAS AND METHODS

2.1. 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, supplemented with 20 gL^{-1} of glucose and incubated at 30ºC in an orbital at 170 rpm for 16h.

2.2. Raw Material

Crude whole red algae *Gelidium corneum* and its industrial waste products after agar-agar extraction, were supplied by Iberagar SA-Sociedade Luso-Espanhola de Colóides Marinhos (Coina, Portugal). Prior to use, the algae was oven dried at 40 ºC to obtain a moisture content inferior to 10%, and grounded to particles < 3 mm. Crude *Gelidium* underwent an acid hydrolysis (1.5% H_2SO_4, 150 ºC, 10 min and 10% (w/v) solid loading in total volume of 500 mL) to convert the polysaccharides of the algae into simple sugars. Hydrolysates of crude red algae *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}.

Pretreated industrial *G. corneum* residues (remaining solid fraction after agar extraction) underwent a hydrothermal hydrolysis (170 ºC, 20 min and a 20% (w/v) solid loading in distilled water) and 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.

Both hydrolysates were prepared by the partner, University of Minho, prior to the commencement of this study.

2.3. 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 was used as C-source when assessing the effect of sterility conditions on polymer productivity and *G. corneum* hydrolysates were used when testing the use of algae biomass as alternative carbon sources for P3HB production.

2.3.1. 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} $MgSO_4.7H_2O$, 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} $FeSO_4$. $7H_2O$.The pH of the medium was adjusted to 7.5 using concentrated HCl 37% (w/w) and sterilized in the autoclave at 121 ºC, 1 bar for 20 min.

The inoculum for the bioreactor was prepared by transferring the contents of 2 cryovials (4 mL) in two 500ml 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 accessed in an orbital incubator 30 °C and an agitation speed 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.

2.3.2. Bioreactor production medium: Glucose as the 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} $MgSO_4.7\ H_2O$; 1.5 gL^{-1} K_2HPO_4, 6 gL^{-1} NH_4Cl, 0.005 gL^{-1} $FeSO_4.7\ H_2O$, 20 gL^{-1} MSG and 25 gL^{-1} glucose. After 16 h 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} $MgSO_4.7\ H_2O$, 600 gL^{-1} glucose and 0.125 gL^{-1} $FeSO_4$. $7H_2O$.

2.3.3. 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 concentrations of NH_4Cl, K_2HPO_4 to be added were calculated considering the total N-, P- content of the hydrolysate.

The added medium had the following composition: 45 gL^{-1} NaCl, 5 gL^{-1} $MgSO_4.7\ H_2O$; 0.9 gL^{-1} K_2HPO_4, 0.98 gL^{-1} NH_4Cl, 0.005 gL^{-1} $FeSO_{4.7\ H}2O$, 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 (10M) 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: 100 mL of the production medium (without NaCl, $MgSO_4.7H_2O$ and $FeSO_4$. $7H_2O$), 195mL of a NaCl solution (300 gL^{-1}), 65 mL of a $MgSO_4.7H_2O$ solution (100 gL^{-1}), and 130 µL $FeSO_4$. $7H_2O$ (50 gL^{-1}). The initial volume of the fed-batch cultivations was 1.3 L, including all medium components and inoculum. The $FeSO_4$. 7H2O (50 gL^{-1}) solution was prepared by dissolving first in H_2SO_4 (10M) 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 75mL of a NaCl solution (300 gL^{-1}). The composition of both *Gelidium* are described above, in section 2.2.

2.3.4. Culture 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 analyse 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-aseptic 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.

2.4. Analytical methods

Bacterial growth was monitored by measuring optical density (O.D.) at 600 nm in a double beam spectrophotometer (Hitachi U-2000), 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. 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.

To quantify sugar and organic acid concentration, and therefore, monitor the evolution of fermentation, a High-Performance Liquid Chromatography (HPLC) apparatus (Hitachi LaChrom Elite) was used equipped with a Rezex ROA.Organic acid H+ 8% (30 mm x 7.8 mm) column, an autosampler (Hitachi LaChrom Elite L-2200), an HPLC pump (Hitachi LaChrom Elite L-2130) and a Hitachi L-2490 refraction index detector. A column heater for long columns (Croco-CIL 100-040-220P, 40 cm x 8 cm x 8 cm, 30-99ºC) was connected externally to the HPLC system. The injection volume was 20 µL and elution was achieved using 5mM H_2SO_4 solution as mobile phase. The column was kept at 65 ºC under a pressure of 26 bar, and the pump operated at a flow rate of 0.5 mL/min. 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. 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.

In order to perform the 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. Acidic methanolysis of the cells in pellet was carried out, to extract the polymers. The cells were re-suspended on 1mL of chloroform and transferred to Pyrex hermetic test tube with Teflon cases and 1mL 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 Na2CO3 (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 G C vials and kept at -20°C until GC analysis was performed.

A Gas Chromatography (GC) apparatus was used for offline determination of the P3HB concentration. The GC used was (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.

2.5. Microbial identification

Various methods of microbiological identification were employed to assess possible contaminations, including phenotypic and genomic approaches. For phenotypic identification, samples were aseptically collected from each fermentation condition and plated on different solid media, such as *H. boliviensis* production medium, marine agar, TSA, and LB agar. The plated samples were incubated at 30 ºC until sufficient colony growth was observed and then stored at 4 ºC.

For genotypic identification, the previously collected samples, stored at -80 ºC with 17% glycerol, were subjected to DNA purification using the GeneJET Genomic DNA Purification Kit. Two different protocols were employed for gram-negative and gram-positive bacteria, involving lysis buffers and silica-based membrane spin columns. The purified DNA was quantified using a NanoDrop UV-Vis Spectrophotometer and stored at -20 ºC for further analysis.

2.6. 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 [12]. 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.

3. 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 were 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 an up-scale simulation and an economic evaluation of the two scenarios, one with "Full-Sterility" and the other with "Non-Sterility" conditions. Lastly, the final section focuses of other approaches to improve the economic viability of P3HB production, namely the use of a algae waste stream as the carbon platform for the production of P3HB.

3.1. Fed-batch bioreactor cultivations: Sterility assay

Fed-batch experiments were carried out aiming to understand the growth and P3HB production by *H. boliviensis* under different levels of sterility: “Full-Sterility”, “Semi-Sterility”, and “Non-Sterility”. 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 phosphate limitation. The outputs of the fed-batch cultivations under the different levels of sterility are shown in **Figure 1.** The results take into account four cultivations replicas on “Full-Sterility”, two on “Semi-Sterility”, and two on “Non-Sterility” with the respective mean with standard deviation displayed. A first look on the results obtained from the fed-batch cultivation of *H. boliviensis* suggests similar bacterial behavior and P3HB production under the different conditions. Regarding biomass growth, dry weights and absorbance were monitored during the course of each cultivation.

As presented in **Figure 1**, both, “Full-Sterility” and “Semi-Sterility” conditions, showed remarkably equivalent growth rates, attaining 49.6 gL^{-1} ± 2.9 and 50.3 gL^{-1} ± 9.2 of dry weight after 76,5 hours of cultivation. In contrast, the complete non-sterility exhibited a mild decrease of the biomass growth, achieving around 40.2 gL^{-1} ± 4.7.

Concerning the P3HB production, the highest P3HB concentration of 30 gL^{-1} ± 6.3 was achieved on the “Semi-Sterility” assay, next, 25.9 gL^{-1} ± 3.5 on the control assay. The unsterile assays had the lowest productivities of 22.4 gL^{-1} ± 7.5. Additionally, in all type of assays, the P3HB content were higher than 50%, the highest content, 59% ± 1.75, was achieved by the “Semi-Sterility” tests and the lowest of 52% ± 6.11 was attained by the control assays.

The slightly less promising results in biomass growth, observed in non-sterility assays, could be attributed to the effect of heat sterilization on the compounds present in the medium. It is possible that the sterilization process may have led to favourable changes, such as the breakdown of complex structures of compounds into simpler and easily usable forms by the organisms present in the assay [13].

In addition, 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 also explain the different productivities between the three conditions, as the P3HB synthesis is directly influenced by the carbon source concentration.

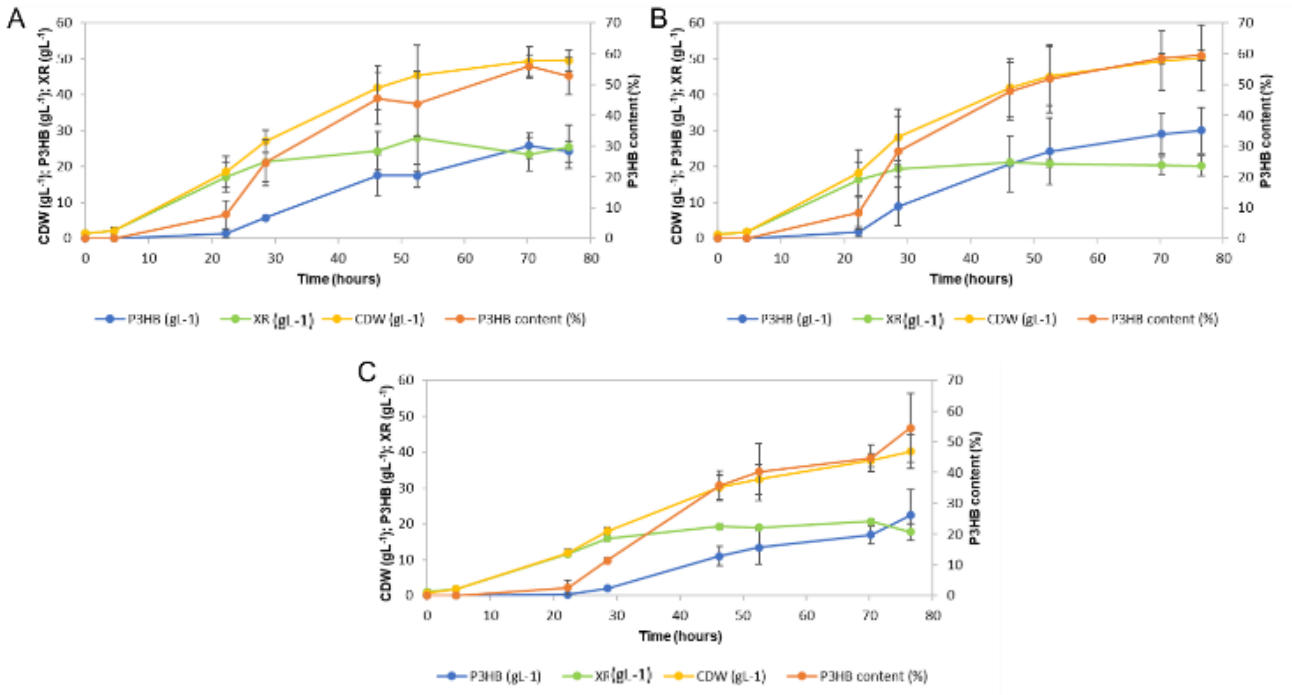

Figure 1. Graphic representation of *H. boliviensis* growth and P3HB accumulation in "Semi-Sterility" conditions. All values are Mean with SD (Standard Deviation); n=2; Legend: P3HB gL^{-1}(o); XR gL^{-1}(o); CDW gL^{-1} (o); P3HB Content (w/w) % (o). Legend: Full-Sterility (A); Semi-Sterility (B); Non-Sterility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

3.2. 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. HPLC restrictions led to varying glucose concentrations and different gluconic acid productions in fermentations. Glucose concentrations below 40 gL^{-1} induced gluconic acid production, while concentrations around and above 50 gL^{-1} and 60 gl^{-1} inhibited the glucose oxidation pathway, as depicted in **Figure 2**. To maximize P3HB synthesis, understanding the primary metabolic pathways for glucose intake is crucial. Nutrient concentration and the presence of stressors affect glucose utilization via the pentose phosphate pathway or glycolysis [14].

Co-production of intracellular P3HB with extracellular gluconic acid could enhance the economic viability of the process. Previous research shows that co-production of P3HB with other chemicals reduces production costs and enables economically viable downstream processing [15]. Gluconic acid, a noncorrosive and nontoxic organic acid, finds applications in the food, metal, leather, and pharmaceutical industries.

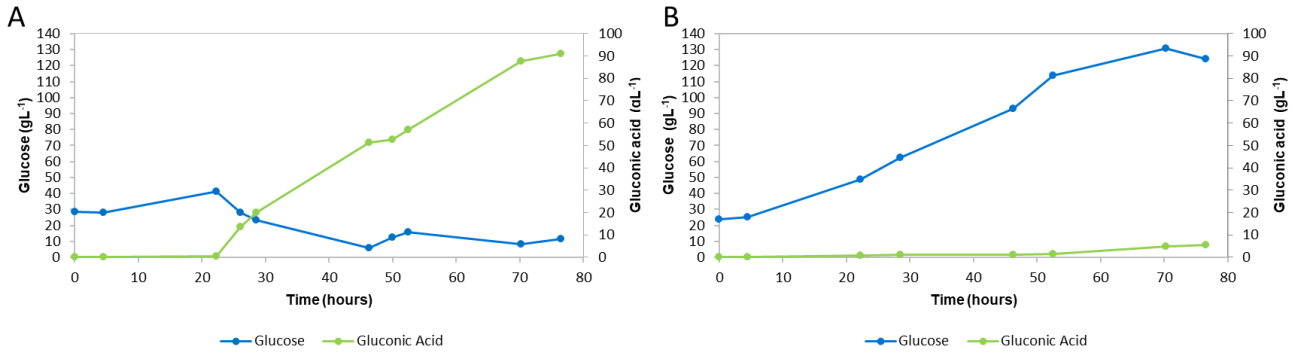

Figure 2. *H. boliviensis* glucose uptake and gluconic acid accumulation throughout 76.5 hours of cultivation. Both glucose and gluconic acid concentrations were monitored using off-line analysis by high performance liquid chromatography. Legend: Gluconic acid gL^{-1} (o); Glucose gL^{-1} (o).

3.3. Study of the microbial population dynamics- Microbial identification

3.3.1. Phenotypic Method: Morphology

To analyse microbial population dynamics in different sterility scenarios, samples from each cultivation type were plated on various solid media. Phenotypic morphology of colonies varied across the different media, indicating distinct responses induced by the media [16]. Samples from all three conditions were plated on *H. boliviensis* medium, which mirrored the bioreactor composition, providing a more realistic depiction of microbial dynamics during the experiment. Additionally, generic media plates were used to assess the presence of dormant contamination due to nutrient restrictions or high salt concentrations. Comparing the plates from all three sterile assays, colonies exhibited similar surface appearance and colour across the different solid media. Although colony sizes differed slightly, the variations were not significant enough to suggest contamination.

Data regarding this section is not presented in this extended abstract.

3.3.2. Genotypic Method: 16S amplicon sequencing

To confirm the findings, samples were subjected to 16S amplicon sequencing analysis. DNA extraction and purification were carried out using two protocols: Gram-positive and Gram-negative, to preserve DNA integrity. The DNA samples were then sequenced using the Illumina MiSeq platform by StabVida. The analysis revealed three different taxa, as shown in **Figure 3**, but the relative frequencies of the two unexpected taxa were below 0.1%, making them negligible. The results aligned with the phenotypic analysis conducted on the plates, confirming the presence of only the genus *Halomonas*, with the absence of contamination.

Despite the lack of evidence for contamination in the phenotypic analysis, it is worth mentioning that the genomic 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 favourable 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.

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 [17]. 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.

	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 3. 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);

3.4. Industrial scale up simulation and economic evaluation

This section compares the economics of a P3HB production plant with and without sterilization. Hypersaline media are considered for contamination prevention, reducing the need for strict sterilization protocols. The study focuses only on the upstream process of P3HB production and uses SuperPro Designer to simulate and compare economic parameters. The "Full-Sterility" facility required a capital investment of 35 M€, while the "Non-Sterility" facility had a lower investment of 22 M€, resulting in a reduction of approximately 13 M€. Operational costs decreased by 2.5 M€ without sterilization, and the total plant cost was around 4 M€ lower, with a 37% decrease. Eliminating sterilization equipment reduced energy consumption by 10% and water usage by approximately 47%. Data pertaining to this section is not presented in this extended abstract.

3.5. Fed-batch bioreactor cultivations: *Gelidium corneum* hydrolysate assay

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. **Figure 4** 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**).

After 30 hours of cultivation , the growth reached a stable state with a dry weight of 33 gL^{-1}. Examining the growth phases, it can be inferred that during the batch phase (up to 10 hours of cultivation), encompassing the lag phase and part of the log phase, the bacteria exhibited similar behavior as in the glucose substrate assay. This suggests efficient assimilation of sugars, particularly of galactose, found in the hydrolysates of the macroalgae *G. corneum*. This finding supports previous research indicating that galactose, like glucose, serves as an effective substrate for P3HB production by *H. boliviensis* [18]. However, the short duration of the log phase indicates 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 earlier, hindering biomass growth and promoting P3HB accumulation. As a result of the reduced biomass growth, the polymer production also declined, yielding approximately 13.02 gL^{-1}, corresponding to 39.26% P3HB accumulation in cells. Furthermore, the programmed feeds designed to sustain glucose concentration, in the fed-batch phase, were insufficient, resulting in its decline to negligible levels after 46 hours. This factor likely played a significant role in the reduced production of P3HB.

The study findings emphasize the potential of the hydrolysate for bacterial cultivation. However, to reach the desired biomass level before entering the limitation phase, increased supplementation of phosphate and nitrogen is required for the hydrolysate used in the batch phase. Additionally, it is crucial to investigate the rate of sugar consumption using the residual hydrolysate as feed to improve the feeding strategy and prevent sugar depletion, thereby enhancing P3HB production.

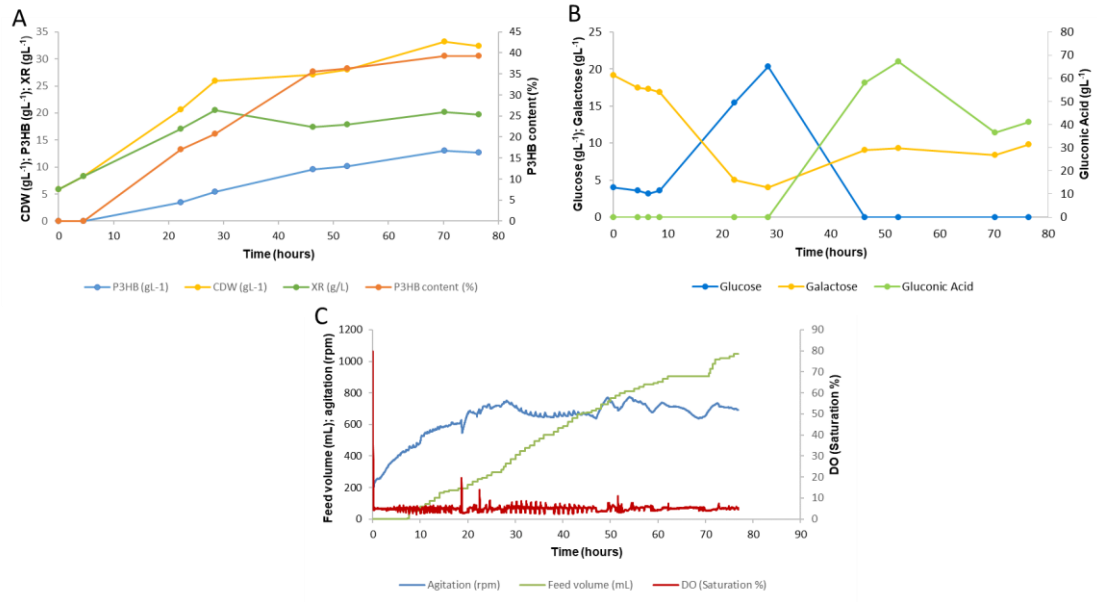

Figure 4. (**A**) Graphic representation of *H. boliviensis* growth and P3HB accumulation with crude *G. corneum* hydrolysate (Batch phase) and its industrial residues hydrolysate (Fed-batch phase) as a carbon source and under nitrogen limiting conditions; Legend: P3HB gL^{-1}(o); XR gL^{-1}(o); CDW 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).

4. CONCLUSION AND FUTURE WORK

The popularity of biopolymers like P3HB is rising due to growing awareness of the harmful effects of conventional plastics. P3HB shares properties with polyethylene and is used in various industries. However, high manufacturing costs hinder its competitiveness against petrochemical plastics. To address this, improvements in the fermentation process are necessary.

This study successfully demonstrated the effectiveness of using halophilic bacteria to prevent cross-contamination in open fermentations while maintaining P3HB productivity. Different levels of sterility were analysed, with the "Semi-Sterility" regime achieving the highest P3HB concentration. *H. boliviensis* consistently produced P3HB with a content above 50% under various sterility levels. Non-sterile assays showed slightly lower growth and productivity, likely due to variations in feeding strategies or even the effect of the heat sterilization process on the compounds present in medium.

Two production plants were designed using SuperPro designer software to compare cost-effectiveness: one with "Full-Sterility" and the other with "Non-Sterility" conditions. The non-sterilized process significantly reduced capital investment and operational costs, including energy and water usage. The total plant cost was approximately 37% lower without sterilization mechanisms, with energy costs reduced by around 10% and water consumption by 47%. These simulations demonstrate the practical applications of the experimental findings in industrial production processes.

One challenge identified in P3HB production was the accumulation of gluconic acid by *H. boliviensis*, which seems to compete with glucose as a substrate. Understanding the primary metabolic pathways involved in glucose intake is crucial to maximize P3HB synthesis. Co-production of intracellular P3HB and extracellular gluconic acid could also optimize economic viability and requires further research. Additionally, *G. corneum* hydrolysates showed potential in reducing manufacturing costs. Galactose and glucose in the hydrolysates were effective substrates for P3HB production. However, low nutrient availability, such as phosphate and nitrogen, may have impeded biomass growth and consequently lower P3HB productivities. Investigating the sugar consumption rate in the residue hydrolysate as feed is necessary to optimize the feed strategy and prevent sugar depletion. These findings underscore the potential of seaweed waste hydrolysate for bacterial

cultivation and provide insights into efficient P3HB production.

Overall, this study contributes valuable insights into P3HB production by *H. boliviensis* and bioplastics production via microbial fermentation. The results will guide further research towards sustainable and eco-friendly alternatives to traditional plastic materials.

REFERENCES

[1] "The New Coal: Plastics & Climate Change." https://www.beyondplastics.org/plastics-and-climate (accessed Jan. 11, 2022).

[2] C. Schmidt, T. Krauth, and S. Wagner, "Export of Plastic Debris by Rivers into the Sea," 2017, doi: 10.1021/acs.est.7b02368.

[3] R. P. Babu, K. O'Connor, and R. Seeram, "Current progress on bio-based polymers and their future trends," *Prog Biomater*, vol. 2, no. 1, p. 8, 2013, doi: 10.1186/2194-0517-2-8.

[4] V. Sharma, R. Sehgal, and R. Gupta, "Polyhydroxyalkanoate (PHA): Properties and Modifications," *Polymer (Guildf)*, vol. 212, Jan. 2021, doi: 10.1016/j.polymer.2020.123161.

[5] C. Kourmentza *et al.*, "Recent Advances and Challenges towards Sustainable Polyhydroxyalkanoate (PHA) Production," *Bioengineering*, vol. 4, no. 2, Jun. 2017, doi: 10.3390/BIOENGINEERING4020055.

[6] "Polyhydroxyalkanoate (PHA) Market Share, Size | 2022 - 2027 | MarketsandMarkets." https://www.marketsandmarkets.com/Market-Reports/pha-market-395.html (accessed Nov. 20, 2022).

[7] G. Q. Chen and X. Liu, "On the future fermentation," *Microb Biotechnol*, vol. 14, no. 1, pp. 18–21, Jan. 2021, doi: 10.1111/1751-7915.13674.

[8] Z. Chen and C. Wan, "Non-sterile fermentations for the economical biochemical conversion of renewable feedstocks," *Biotechnol Lett*, vol. 39, no. 12, pp. 1765–1777, Dec. 2017, doi: 10.1007/S10529-017-2429-8/TABLES/3.

[9] M. F. Edbeib, R. A. Wahab, and F. Huyop, "Halophiles: biology, adaptation, and their role in decontamination of hypersaline environments," *World J Microbiol Biotechnol*, vol. 32, no. 8, Aug. 2016, doi: 10.1007/S11274-016-2081-9.

[10] K. Khosravi-Darani, Z. B. Mokhtari, T. Amai, and K. Tanaka, "Microbial production of poly(hydroxybutyrate) from C1 carbon sources," *Applied Microbiology and Biotechnology 2013 97:4*, vol. 97, no. 4, pp. 1407–1424, Jan. 2013, doi: 10.1007/S00253-012-4649-0.

[11] J. S. Gomes-Dias, A. Romaní, J. A. Teixeira, and C. M. R. Rocha, "Valorization of seaweed carbohydrates: Autohydrolysis as a selective and sustainable pretreatment," *ACS Sustain Chem Eng*, vol. 8, no. 46, pp. 17143–17153, Nov. 2020, doi: 10.1021/acssuschemeng.0c05396.

[12] D. Petrides and R. G. Harrison, "Bioprocess Design and Economics Bioseparations Science and Engineering," 2003, Accessed: Nov. 22, 2022. [Online]. Available: http://www.intelligen.comhttp://www.oup-usa.org/isbn/0195123409.html

[13] K. Pontes Eliodório, C. Pennacchi, G. Caetano de Góis e Cunha, A. de Araújo Morandim-Giannetti, R. Giudici, and T. Olitta Basso, "Effects of caramelization and Maillard reaction products on the physiology of Saccharomyces cerevisiae," *Fungal Biol*, 2023, doi: https://doi.org/10.1016/j.funbio.2023.06.009.

[14] J. F. Kornecki *et al.*, "Enzyme production of d -gluconic acid and glucose oxidase: successful tales of cascade reactions," *Catal Sci Technol*, vol. 10, no. 17, pp. 5740–5771, Sep. 2020, doi: 10.1039/D0CY00819B.

[15] T. Li, D. Elhadi, and G. Q. Chen, "Co-production of microbial polyhydroxyalkanoates with other chemicals," *Metab Eng*, vol. 43, pp. 29–36, Sep. 2017, doi: 10.1016/J.YMBEN.2017.07.007.

[16] M. C. F. van Teeseling, M. A. de Pedro, and F. Cava, "Determinants of Bacterial Morphology: From Fundamentals to Possibilities for Antimicrobial Targeting," *Front Microbiol*, vol. 8, no. JUL, Jul. 2017, doi: 10.3389/FMICB.2017.01264.

[17] A. Chatterjee and J. Abraham, "Microbial Contamination, Prevention, and Early Detection in Food Industry," *Microbial Contamination and Food Degradation*, pp. 21–47, Jan. 2018, doi: 10.1016/B978-0-12-811515-2.00002-0.

[18] M. Mesquita Marques, "From Macroalgae to Bioplastic - Seaweed Hydrolysates for Polyhydroxyalkanoate Production by Marine Bacteria," Instituto Superior Técnico - Universidade de Lisboa, 2017.