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Poly-3-hydroxybutyrate production from carbon-rich hydrolysates of the macroalgae *Gelidium sesquipedale* using halotolerant bacteria

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Preface

This document was created under the supervision of Dr. Maria Teresa Ferreira Cesário Smolders, Dr. Pedro Carlos De Barros Fernandes and Prof. Dr. Maria Manuela Regalo da Fonseca. Every experimental measurement was performed at the Institute of Bioengineering and Biosciences (IBB) at the Instituto Superior Técnico in Lisbon, Portugal.

This master thesis contains a review of the most recent literature, results from experimental assays with discussion and a part dedicated to conclusions and future prospects.

Declaration

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

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(*In Czech/ česky: Děkuji mé rodině, i přes vzdálenost, která nás dělila jste mě nezapomněli denně podporovat, a překonat některé krušné chvíle Doufám, že jednoho dne navštívíme společně tuhle nádhernou zemi, jako je Portugalsko.)*

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Děkuji

Abstract

The *Gelidium sesquipedale* residues after agar extraction proved to be a sustainable source of carbohydrates (44.8 % dw.), mainly originated from structural cellulose and residues of agar. These carbohydrates can be used as a carbon source for the production of the biodegradable polymer, poly-3-hydroxybutyrate by the halotolerant bacteria *Halomonas boliviensis.*

In order to release simple carbohydrates (e.g. glucose) for microbial cultivation, the algal polysaccharides went through a combined hydrolysis process characterised by a chemical pre-treatment at 121 °C for 30 minutes with 0.5 % (w/v) sulfamic acid followed by enzymatic hydrolysis using an enzymatic cocktail with a cellulase complex and β-glucosidase. The combined hydrolysis of 86.4 $gL^{-1}$of lyophilized and blended biomass yielded a glucose concentration of approximately 25 gL^{-1}. The only detected toxic compound resulting from sugar dehydration was 5-hydroxymethylfurfural (HMF). However, the concentration of HMF formed during the pre-treatment did not surpass 0.02 gL^{-1} thus not being toxic towards *Halomonas boliviensis* at these concentrations.

The algal hydrolysates were examined as a carbon source for the production of P3HB by *H. boliviensis* in shake flask and in 2L stirred-tank bioreactor. The best results in shake flask attained a CDW of 8.3 gL^{-1} and $P3HB_{conc.}$ of 3.27 gL^{-1} corresponding to a 39.2 % $P3HB\ cont.(\%)$, a yield of product over substrate ($Y_{P/S}$) of 0.155 ($g_{product}/g_{substrate}$) and a maximum volumetric productivity ($Prod._{max\,P}$) of 0.056 $g\ L^{-1}h^{-1}$.

A fed-batch bioreactor assay using a concentrated algal hydrolysate as feed and a dual limitation of nitrogen and oxygen (2.0% *DO*) yielded a CDW of 22.0 gL^{-1} , $P3HB_{conc.}$ of 5.72 gL^{-1} corresponding to 26.0 % $P3HB\ cont.(\%)$, $Y_{P/S}$ of 0.237 ($g_{product}/g_{substrate}$) and a $Prod._{max\ P}$, of 0.109 $g\ L^{-1}h^{-1}$.

Keywords

Poly-3-hydroxybutyrate (P3HB); Gelidium sesquipedale; Halomonas boliviensis

Resumo

Os resíduos da alga vermelha *Gelidium sesquipedale* após extração do agar provaram ser uma fonte sustentável de carbohidratos (44.8 % dw), constituídos principalmente por celulose e resíduos de agar. Estes polissacáridos podem ser usados como fonte de carbono para a produção do polímero biodegradável poli-3-hidroxibutirato (P3HB) pela bactéria halotolerante *Halomonas boliviensis.*

De forma a produzir açúcares simples (ex: glucose) para poderem ser usados diretamente por microrganismos, os polissacáridos das algas sofreram um processo de hidrólise combinada composta por um pré-tratamento químico a 121ºC durante 30 minutos com uma solução de 0.5 % (w/v) de ácido sulfâmico seguido de um tratamento enzimático usando um cocktail de cellulases e β-glucosidase. A hidrólise combinada de 86.4 gL^{-1} de biomassa previamente liofilizada e moída, originou uma solução de 25 gL^{-1} de glucose. O único composto tóxico detetado proveniente da degradação dos açúcares foi o 5-hidroximethilfurfural (HMF). A concentração de HMF formado durante o pré-tratamento não ultrapassou 0.02 gL^{-1} ficando abaixo do limiar de toxicidade para a bactéria *Halomonas boliviensis.*

Os hidrolisados das algas foram avaliados como fonte de carbono para a produção de P3HB por *H. boliviensis* em frascos agitados e num bioreactor agitado (STR) de 2 litros. Os melhores resultados em balão atingiram uma biomassa (CDW) de 8.3 gL^{-1} e uma concentração de P3HB de 3.27 gL^{-1}, correspondendo a uma acumulação de polímero nas células de 39.2 % (g/g), um rendimento de produto em substrato $Y_{P/S}$ de 0.155 (g/g) e uma produtividade volumétrica máxima ($Prod._{max\ P}$) de 0.056 $g\ L^{-1}h^{-1}$. Um ensaio em bioreactor e em modo fed-batch usando um hidrolisado concentrado de algas como alimentação e uma limitação dupla de azoto e oxigénio (2% oxigénio dissolvido) deu origem a uma concentração de biomassa (CDW) de 22 gL^{-1}, uma concentração de P3HB de 5.72 gL^{-1}, correspondendo a uma acumulação celular de P3HB de 26 % (g/g), um rendimento $Y_{P/S}$ de 0.237 (g /g) e uma $Prod._{max\ P}$ de 0.109 $g\ L^{-1}h^{-1}$.

Palavras Chave

Poli-3-hidroxibutirato (P3HB); Gelidium sesquipedale; Halomonas boliviensis

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Lists of Symbols and Abbreviations

AHG	Anhydro galactose
STR	Stirred-tank reactor
SD	Spray-dryer
RE	Vacuum rotatory evaporator
$P3HB\ cont.\ (\%)$	P3HB content (%)
$P3HB_{conc.}\ (gL^{-1})$	P3HB concentration (gL^{-1})
P3HB	Poly-3-hydroxybutyrate
O.D.	Optical density
MSG	Monosodium glutamate
HPLC	High performance liquid chromatography
HMF	5-hydroxymethylfurfural
GC	Gas chromatography
Fr	Froude number
DW (g)	Dry weight (g)
DO (%)	Dissolved oxygen (%)
CDW (gL^{-1})	Cell dry weight (gL^{-1})
$Y_{P/S}\ (g\ P/\ g\ S_{cons.})$	Yield of product over substrate $(g\ P/\ g\ S_{cons.})$
$Prod_{max\ P}\ (g\ L^{-1}h^{-1})$	Maximal volumetric productivity $(g\ L^{-1}h^{-1})$
$M_W\ (g\ mol^{-1})$	Molecular weight ($g\ mol^{-1}$)

1 Introduction

1.1 Context

Based on the world´s total population which is estimated to reach 7.8 billion in 2020[1], the demand for energy, food and materials will be increasing along to this trend. Therefore, to preserve the environment for next generations, it is vital to re-orient our needs towards more environmentally friendly energy, food and chemical production.

One of the most discussed issue in recent years is the plastic pollution, caused both by elevated high production rates of plastic polymers with non-efficient recycling approaches and by a long-life persistence of the plastic waste which is being accumulated in the marine, aquatic and terrestrial environment [2]. As the merits of plastic materials are indisputable, the solution of banning plastics is not feasible in a short-term, a more promising way being thus the production of biodegradable plastics, such as polyhydroxyalkanoates (PHAs). These polymers are produced intracellularly in granules by a variety of bacterial species [3]. PHAs have a unique property of being biodegradable in every environmental condition and in a short period of time.

Poly-3-hydroxybutyrate (P3HB), is the best described polymer from the polyhydroxyalkanoate family. Also, this polymer proved to be biodegradable in the marine environment, just within 14 days [4]. The reason why the production of polyhydroxyalkanoates has not found a stable position in the market yet, is due to the higher production costs, mainly influenced by the cost of the carbon source (40-50 % of the overall costs) [5-6].

The macroalgal waste material after phycocolloid extraction (e.g. agar, carrageenan, alginate) is a sustainable and yet not fully explored source of carbon. Annually, the agar extracted from red algae overcomes 14 thousand tons, with an enormous waste being generated [7]. Within this work a species of red-algae, *Gelidium sesquipedale*, more specifically the waste biomass after the agar extraction was investigated as a possible carbon-rich platform for the P3HB production by the halotolerant bacteria *Halomonas boliviensis*. The moderately halophilic bacteria *H. boliviensis* is a gram-negative, aerobic, alkalitolerant and psychrophilic bacterial species capable of accumulating P3HB up to 80% (w/w) of the cell dry weight [8-9] under conditions of excess carbon source and depletion of an essential nutrient such as nitrogen, phosphorus or oxygen [10]. A self-sterile saline environment in the cultivation of *H. boliviensis*, could save time and energy by avoiding sterilisation of the cultivation medium and equipment. Moreover water resources could also

be spared, as sea water could be used instead of fresh water.

1.2 Motivation

The main experimental objectives proposed within the framework of this study were:

1. Establish a method for preparation of *G. sesquipedale* residues to attain a homogenized material for further assays.
2. Determine the composition of *G. sesquipedale* in terms of carbohydrates, moisture, ash and total solids contents.
3. Develop a sustainable process to hydrolyse algal polysaccharides to monomers to be more accessible as C-source for *H. boliviensis* cells.
4. Determine the composition of *Gelidium* hydrolysates in terms of sugars and possible toxics from sugar degradation. Examine the inhibition impact of toxics on the growth of *H. boliviensis*.
5. Establish the feasibility of using carbon-rich hydrolysates from *G. sesquipedale* residues for the P3HB production by *H. boliviensis* in shake-flask and bioreactor scales.
6. Determine the best medium composition to attain high P3HB yields and productivities in shake flasks and in 2L stirred-tank bioreactors. Establish the best operational conditions to perform fed-batch cultivations using *Gelidium* hydrolysates.
7. Tackle the issue of algal hydrolysates evaporation to produce carbon-rich concentrates for fed-batch assays.
8. Conduct a fed-batch assay of growth and P3HB production by *H. boliviensis* using the concentrate of algal hydrolysate as feeding.

2 State of the Art

2.1 Plastics - Nowadays and Future Trends

The introduction of plastic materials, that we commonly called plastics, to the world in the 20th century as a new material made a significant impact in our daily lives. Plastics are high molecular mass, synthetic organic polymers mainly manufactured from hydrocarbons obtained from crude oil and natural gas [11]. In contrast to other materials (glass, paper and metals) plastics offer much preferable properties such as mechanical strength, lightness, flexibility, durability, chemical inertia and low production costs. These exact qualities allowed to produce cheaper, lighter and durable products used in our daily purposes. Unfortunately, thanks to the durability of plastics altogether with a massive production and non-efficient recovery techniques resulted in a plastic waste pollution that presents nowadays a considerable threat to the marine, aquatic and terrestrial environment [2].

The mass production of plastics started in 1950s, and the amount of produced plastics reached 265 million tons in 2010 [12] and 300 million tons (million tons, Mt) in 2015 [4]. An estimation proposed by Geyer et al. (2017) describes that by the end of 2050 manufacturers will produce 26,000 Mt of pure polymer resins, 6,000 Mt polyamide and acrylic fibres and 2,000 Mt of additives. In a proportion of how much of those amounts will not pollute the environment is estimated to be only about 62 % - about 12,000 Mt could eventually enter landfills or other environmental areas [13]. This rapidly growing issue got the attention of the science community, public and government in recent years as a challenge to find out a solution to this global concern [14].

Also, another problem closely related to the pollution by macroplastic emerged in recent years, and that is the problem of “microplastics”. Whilst the global attention on macroplastic pollution has been addressed for a decade, microplastics gained their attention since the end of the 20th century. Numerous plastic fragments, fibres and granules with sizes less than 10 mm in diameter are collectively called the “microplastics”. According to the nomenclature, the microplastics are further divided into two groups namely primary and secondary microplastics.

Primary microplastics are manufactured on purpose as “micro-beads” or “micro-exfoliates” for their application in cosmetics, medicine as a drug delivery or as an air-blasting media. Upon discharge these primary microplastics are often contaminated with heavy

metals such as Cd, Cr or Pb.

Secondary microplastics are derived from the natural degradation process of macroplastics. Over a certain period of time, the contribution of physical, biological and chemical processes often with exposure to a sunlight or to UV rays cause a weakening of plastic bonds, leading to a complete breakdown of the chemical bond in the end [15]. Further reduction in the size of microplastics could even end up with particles with nano- dimensions (so-called nanoplastics).

This scenario is especially worrying for the marine environment, as these particles could easily be mistaken by small marine organisms such as mussels, polychaete worms, barnacles, etc. for food. Ingestion of these small-size particles poses not just threat to a small marine organisms, but also for other organisms as we go higher in the food chain, eventually leading to humans [16]. The ingestion of small-size plastic particles presents a great threat to the human health. The health risk originates in the monomeric building blocks (bisphenol A, BPA), additives (e.g. plasticizers) or combination of both.

In order to preserve a sustainable environment for future generations, it is inevitable to restrict the usage of non-degradable plastics, develop advanced technologies that will enhance the recovery of plastics, or even better find an alternative to petroleum-based plastics. In the future, the majority of manufactured plastics should be originated in renewable resources and preferably biodegraded in their end-of-life. Regarding to plastic recovery, future trends will extend the current philosophy of three R´s plastic recovery (Reduce, Reuse and Recycle) with other two R´s (energy Recovery and molecular Redesign) **[17]**.

2.1.1 The plastic recovery

The increase of the plastic solid waste (PSW) volume together with the decreasing area of landfills forced researches to discover new and more efficient methods for plastic recovery **[18]**. Plastic re-usage is an environmentally friendly way to dispose PSW since it saves energy, resources and reduce CO_2, NO_x. and SO_2 emissions **[19]**. Since 1994 the European Union (EU) has started working on objectives for plastic waste recycling and recovery. Through the directive 94/62/EC the EU set up a minimal recycling target of 15 % of PSW, which was later raised up to 22.5 %. Strategies (**Table 1**) being used to access the life-cycle assessment (LCA) of plastics are highlighted in the directive 94/62/EC [20].

Table 1 – Plastic solid waste management strategies reported by the European Union directive 94/62/EC [20].

PSW Management Strategy	Description
Feedstock recycling	*Transformation of PSW into hydrocarbons which can be used for a manufacturing of new plastic polymer*
Mechanical recycling	*Various technologies involving polymer separation, decontamination, size reduction, re-melting and extrusion into pellets*
Incineration	*Incineration of PSW in cement kilns. Throughout this process either electricity or heat could be recovered*
Landfilling	*Using engineered landfills with both leachate and landfill gas extraction*

Because PSW does not comprise a single type of a plastic polymer, for recycling purposes the PSW must be identified and segregated. Neglecting plastic segregation could deteriorate properties of the final recycled plastic material. For this purpose, a branch of segregation techniques could be used such as Laser-induced breakdown spectroscopy (LIBS); Triboelectric separation, X-ray fluorescence, FT-IR and others. Nevertheless, the

whole cycle of plastic recovery is limited up to a certain number of recycling cycles. After several cycles, the plastic material could eventually lose some of its properties like strength, stability etc. [21].

Since, sorting and grinding can recycle only up to 15-20 % of PSW, the chemical method is the most preferable disposal method in which raw materials (necessary to complete plastic recovery) are eventually generated from PSW [22]. Thus, chemical technologies (the thermal and catalyst pyrolysis) are receiving more attention in the challenge of PSW disposal.

Throughout the incineration of plastic polymers, energy-rich components like liquid oil, solid residues (char) and gases are generated. However, thermal pyrolysis as an endothermic process requires high temperatures in the range from 300 up to 900 °C. Due to a high temperature-dependent reaction it was necessary to find out a less energy requiring conditions for the pyrolysis. A possible solution is to conduct a catalytic pyrolysis. For this purpose, several catalysts were proposed such as the Cu-Al_2O_3; $Al(OH)_3$; $Ca(OH)_2$; Zeolite-β and others. The addition of a catalyst significantly could reduce either the temperature and time required for this process [23].

In terms of number, the EU achieved in ten years, from 2006 to 2016, an increase of the PSW recycled by almost 80 % (from 4.7 to 8.4 Mt). Within these years, the energy recovered from PSW was enhanced by 79 % (from 7 to 11.3 Mt of processed PSW) and the landfilling decreased by 43 % (12.9 to 7.4 Mt of landfilled PSW). For an illustration, the world plastic production reached up to 350 Mt in 2017, in the EU circa 64 Mt of plastics were produced in that same year. The total PSW collected in 2016 in the EU reached up to 27 Mt.

Countries such as Czech Republic, Germany, Netherlands and Sweden were already close to the 50 % of PSW recycling rate in 2016, which is set to be the new PSW recycling target for 2025. The total EU PSW recycling rate in 2016 was circa 41 % [24]. However, the percentage of plastics recovered in EU makes only 28 % of the recovered plastics worldwide [4].

2.1.2 The biorefinery concept

Up to this day the world´s primary source of energy is petrol. Due to an extreme dependence on oil as energy resource, the worldwide demand for crude oil reached up to 99.3 million barrels per day in 2018 [25] and it is estimated that this number could arise up to 116 million barrels per day by 2030. About 4 % of the worldwide oil resources are used in the chemical industry.

To reduce the worldwide dependence on oil it is necessary to explore an alternative production process. A promising field is sustainable economy based on renewable resources such as plant-based raw materials (biomass). The process that concerts biomass into their building blocks (carbohydrates, proteins and oils) and furtherly converts them into valuable products is called biorefinery. The comparison between petroleum refinery and biorefinery is displayed in **Figure 1**. Replacement of petroleum-based chemicals with those from renewable resources will be essential for future growth of chemical industry. The petrol resources are undepletable and limited in terms of composition, on the other hand the renewable resources are composed from various compounds such as cellulose, hemicellulose, oils, lignin, starch and proteins. These compounds could be transformed into valuable products [26]

Nevertheless, utilization of sugar, starch and vegetable oil from wheat, corn or rapeseed oil as a feedstock in biorefinery competes with food and feed industry, therefore it is preferable to develop strategies which will utilize residues from plant-based biomass. These residues can be divided into four sectors: 1. Agriculture; 2. Forestry; 3. Leftovers from industries and households and 4. Aquaculture [27].

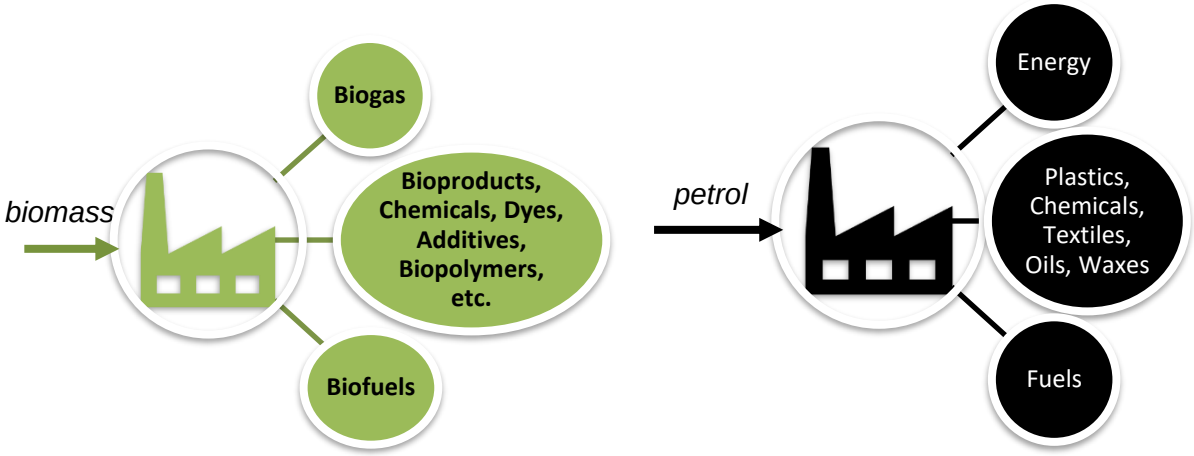


Figure 1 – Comparison of Biorefinery and Petroleum refinery flowcharts [28].

2.1.3 Biodegradable plastics / polymers

Biodegradable polymers or biodegradable plastics are a promising alternative to petroleum-based plastics. The term “biodegradable polymer” is often confused with another term “bio-based polymer”. Biodegradable polymer is defined as a polymer which can be deteriorated with the participation of microorganism(s) in both aerobic or anaerobic

conditions resulting in the change of chemical and physical properties of the polymer eventually leading to the formation of CO_2; H_2O; CH_4 and other low-molecular weight products. An overview of the biodegradability of some biodegradable plastics is displayed in **Table 2**.

Table 2 – An overview of biodegradable plastics with their biodegradability reported by Emadian et al. (2016) [4].

Origin of polymer	Name of polymer	Environment of biodegradation	Conditions of biodegradation	Period of Biodegradation (days)
Renewable resources	*PLA*	*Soil*	*30% moisture*	*98*
		Compost	*58 °C, pH 8.5, 63% humidity*	*58*
		Inoculum from a municipal wastewater treatment plant	*30 °C, aerobic*	*28*
	P3HB	*Soil*	*Real conditions*	*300*
		Soil	*-*	*180*
		Microbial culture from soil	*-*	*18*
		Sea water	*25 °C*	*14*
		River water	*Real condition, ~ 20 °C*	*42*
Cellulose-based	*Cellulose acetate*	*Municipal solid waste mixture*	*-*	*14*
	Sponge doth	*Synthetic material containing compost*	*Aerobic, 58 °C*	*154*
Polyamide-based	*Nylon 4*	*Sea water*	*25 °C*	*21-25*
		Composted soil	*25 °C, 80% humidity, pH 7.5 -7.6*	*120*
Petroleum-based	*PBS*	*Compost*	*Aerobic, pH 7-8, ~60 °C, 50 -55% moisture*	*160*
		Soil	*25 °C, 60% humidity*	*28*
	PCL	*Inoculum from a municipal wastewater treatment plant*	*30 °C, aerobic*	*28*
		Compost	*55 °C*	*6*

On the contrary bio-based polymers are polymers originated from renewable resources. They can be produced by microorganisms, plants or animals, but could be also manufactured *ex-vivo* from bio-based monomers. Thus, some polymers, for instance Polycaprolactone (PCL) or Poly(butylene succinate) (PBS) originated from fossil fuels have a property of biodegradability. On the other hand, some bio-based polymers for example Polyethylene (PE) and Nylon 11 (NY11) lack the quality of biodegradability [29-30]

Biodegradable polymers could be produced either by chemical synthesis or by biosynthesis using biological systems. Up to this day there have been several chemical pathways generating biodegradable polymers, for instance Polyglycolic acid, PLA, PCL, Polyvinyl alcohol. Monomers could be manufactured from renewable feedstocks such as sugar or corn beet, ethanol or crude oil [31]. But even more desirable approach is to use living organisms due to their capability to accumulate biodegradable polymers. Wide range of microorganism could form neutral polysaccharides (Gellan gum; Pullulan; etc.) or polyesters Polyhydroxyalkanoates (PHA) with promising qualities simulating petroleum-based plastics. Microorganism accumulate these polymers intracellularly as storage components mainly in the condition of an excess of a carbon source [31].

The mechanism of biodegradation itself may involve initial steps of photo- or thermal degradation resulting into low-molecular components. But for a polymer to be considered as “biodegradable”, the final degradation steps must be executed by microorganisms. The biodegradation process could be undertaken with industrial composting (aerobic condition) systems in optimized conditions [32]. Referring to european-bioplastics.org, composting of a biodegradable waste could last a period of 6-12 weeks with controlled parameters (temperature, humidity, aeration) under action of microbes, like bacteria or fungi [33].

2.2 Bioplastics

Even though nowadays, only 7 % of the annual oil production is used in plastic production, the potential threat of an oil crisis in the future could have a huge impact on the cost-effectiveness of petroleum-based plastics. Using renewable resources could prevent this situation from happening. But not just a potential shortage of fossil fuels is at stake nowadays. Humanity needs also to minimize CO_2 emission from fossil fuels to avoid potential threat of “global warming”, and therefore there is a need to look for new “green” products [34].The production of bioplastics could be achieve either by a polymerisation of bio-based monomers or through extraction from living organisms.

A renewable polymer and the most abundant biopolymer, cellulose, has been used in traditional applications for decades (wood, cotton, hemp, etc.). Novel technologies, as for example the German automotive company Daimler, developed composites of polypropylene with abaca fibres. Other widespread, naturally occurring biopolymer is lignin. The lignin was used by the German company Tecnaro, which introduced this polymer compound in a mix of natural fibres consisting of thermoplastic lignin, flax, hemp and others. Also, starch, chitosan, proteins, terpenes could be used as renewable polymers. Nowadays, attention is being focused on Polyhydroxyalkanoates (PHA). These bio-polyesters are produced by bacteria as storage compound. Under certain humidity and aeration these polymers could be fully biodegraded to CO_2, water and humus [34].

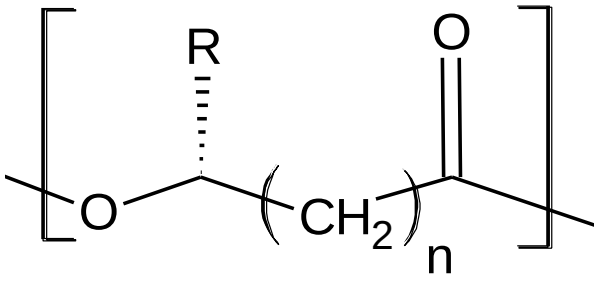
Regarding bioplastic production, is it a more effective approach to purify renewable monomers, instead of purifying natural biopolymers, with a subsequent polymerisation step resulting in a final polymer. Bio-based polymers produced with this approach still have advantages of low carbon footprint, high recycling capability, resources and energy effectiveness over petroleum-based plastics. Bioethanol, butadiene, diols, polyols, diacids, hydroxy-alkanoic acid, amino-alkanoic acid and diamines can undergo melt polycondensation reaction resulting into bio-based polyesters, polyamides, polycarbonates and others [34].

Queiroz and Collares (2009) showed a considerable growth of deposited patents and published articles in the bioplastic area from 1998 up to 2007. The number of these patents and articles doubled or tripled in the last 10 years. Nevertheless, the production of bioplastics is still in an initial phase of development and it will face a difficult task to replace petroleum-based plastics on the market mainly due to high production costs [35].

The biopolymer market generated in 2002 60,000 tonnes of biopolymer products (except of Xanthan Gum) with a cost 4 US $/kg. Growth of the biopolymer market was estimated to be 20 % per year with production cost around 2 US $/kg. Borschiver et al. (2008) stated that the production cost of biopolymers is 50% higher in comparison to petroleum-based plastics [36].

2.2.1 Polyhydroxyalkanoates

Polyhydroxyalkanoates (PHAs) represent a wide class of optically-active poly(oxoesters) with repeating of (*R*)-n-hydroxyalkanoic monomer unit (**Figure 2**). Monomer units could be polymerized by microbes to a M_w ranging from 200,000 up to 3,000,000 Da [37]. A broad family of PHAs can be distinguished by the length of the alkyl groups in the *R*-hydroxyalkanoic acid monomer unit from the small-chain length PHAs with 3-5 carbon units to the long-chain length PHAs with more than 14 carbon units [38]. From 75 different genera up to 300 species both gram-positive and gram-negative bacteria can produce PHAs under an excess of carbon source and nutrient stress caused by the lack of some nutrients such as a nitrogen, phosphorus or oxygen [39].



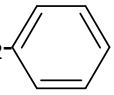
n	R	Polymer
n = 1	-H	poly(3-hydroxypropionate)
	$-CH_3$	poly(3-hydroxybutyrate)
	$-CH_2-CH_3$	poly(3-hydroxyvalerate)
	$-CH_2-CH_2-CH_3$	poly(3-hydroxyhexanoate)
	$-CH_2-$ 	poly(3-hydroxy-5-phenylvalerate)
n = 2	-H	poly(4-hydroxybutyrate)
n = 3	-H	poly(5-hydroxyvalerate)

Figure 2 – General structure of (*R*)-n-hydroxyalkanoic monomer unit.

PHAs can be accumulated up to 90 % of cell-dry weight in intracellular granules (inclusions) located in cytoplasm, where they act as an energy and a carbon source. In the case of recombinant *Escherichia coli* non-storage PHA, poly(3-hydroxybutyrate) (P3HB), participates also in the composition of the cytoplasmatic membrane [40-41].

Size and number of inclusions vary with different species and culture conditions. The composition of these inclusions consist of 98 % of PHAs, almost 2 % of granule-associated proteins (GAPs) and less than 1 % of phospholipids. Different types of GAPs were identified such as PHA synthases, PHA depolymerases, phasins and other proteins like transcriptional regulators, hydrolases, reductases and other enzymes. Amongst GAPs, phasins represent the most frequent protein unit within the inclusions, and they interact with PHAs through the hydrophobic domain and with the cytoplasm through the hydrophilic/amphiphilic domain [38].

2.2.2 Poly-3-hydroxybutyrate

The linear polyester poly-3-hydroxybutyrate (P3HB) is a homopolymer of (*R)*-3-hydroxybutyric acid monomer units. It is also the best well-known and the best described polymer from the PHA family [42]. It was firstly extracted in 1927, by Maurice Lemoigne at the Institute Pasteur, France. Lately, in 1960s the first commercial scale production of a P3HB was explored. The polymer has the promising property of being biodegradable in different environments even in marine waters [42]. However, P3HB itself has drawbacks, as it is brittle, with a high crystallinity and stiffness leading to a low extension to break and with high production costs [43]. A co-polymerisation is a helpful strategy to improve polymer properties. For instance, the copolymer of 3-hydroxybutyrate and 3-hydroxyvalerate with a brand name BIOPOL®, was proposed by the company ICI [44].

A wide range of gram-negative or gram-positive bacteria species accumulate P3HB intracellularly under conditions of excess carbon source and nutrition stress. In a situation of imbalance between carbon and other nutrient in the media, the microorganism assimilates the carbon source and converts it in an energy storage molecule, P3HB. The biosynthesis of P3HB (**Figure 3**) in the cell begins when a 3-ketothiolase (PhaA) provides the combination of two molecules of acetyl-CoA to form an acetoacetyl-CoA. This molecule then goes through reduction by the action of an enzyme Acetoacethyl-CoA reductase (PhaB) and the coenzyme NADH to 3-hydroxybutyryl-CoA. Finally, a PHB synthase (PhaC) initiate the polymerisation step of 3-hydroxybutyryl-CoA monomers into P3HB [39].

Naturally produced P3HB has a semi-crystalline structure with a 100 % *R* configuration which allows an easy degradation.

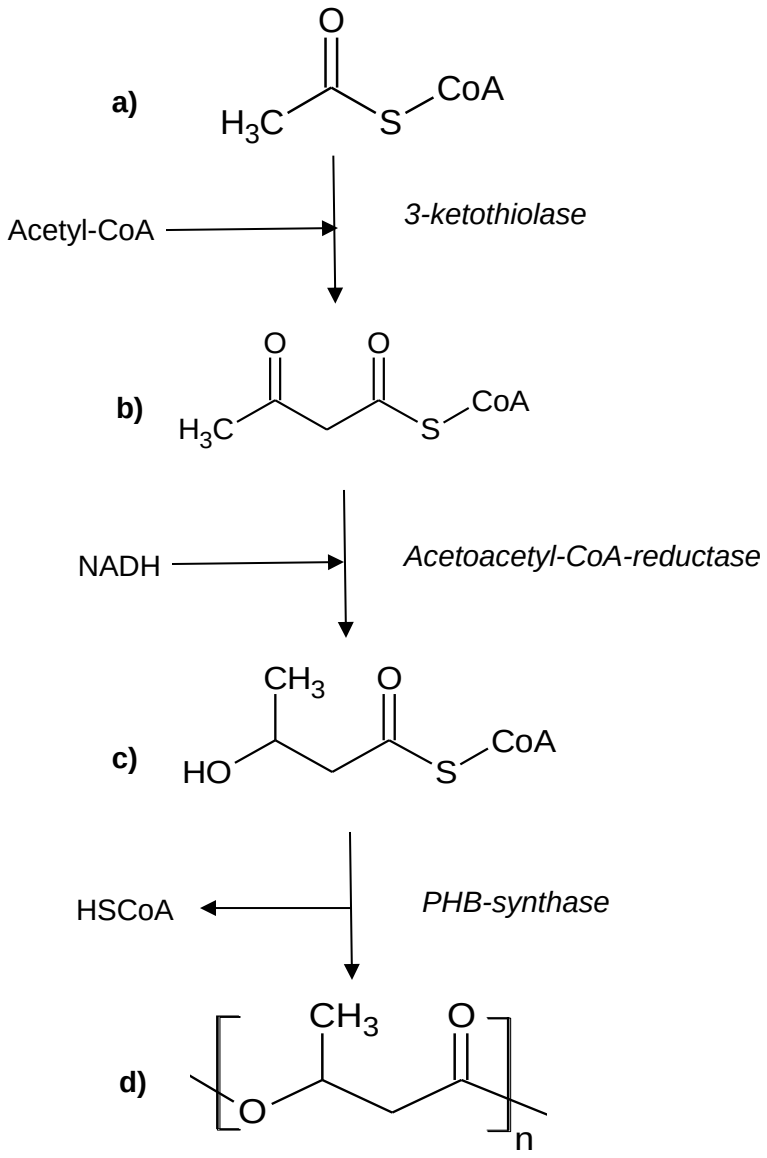


Figure 3 – Biosynthesis of poly-3-hydroxybutyrate; on the left: a) Acetyl-CoA, b) Acetoacetyl-CoA, c) 3-hydroxybutyryl-CoA, d) poly-3-hydroxybutyrate.

The biodegradation of P3HB is achieved by extracellular hydrolases called PHA depolymerases (PhaZs) produced by a variety of different microorganisms. After a hydrolysis step, monomers and oligomers are obtained from P3HB. Under aerobic conditions, the microorganisms could process the P3HB degradation products into energy, CO_2 and water or in the case of anaerobic condition energy, CH_4 and water could be

generated. The degradation rate depends on several factors: concentration of enzymes and polymer, environmental conditions and polymer itself. In the study case done by Martínez-Tobón et al. (2018), the authors tested the rate of P3HB degradation with a variety of bacterial strains namely *Comamonas testosteroni; Cupriavidus sp.; Paucimonas lemoignei; Pseudomonas stutzeri and Ralstonia sp*. Degradation was achieved with all bacterial strains. The highest mass loss of P3HB of (90%) was attained after just 1 week and was observed by the strain *C.testosteroni* [3].

2.2.3 Production of P3HB

Although, some bacterial species like *Alcaligenes latus* and *Azotobacter vinelandii* do not require nutrient limitation to initiate the P3HB accumulation, higher yields of P3HB are obtained in the case of nutrient limitation [10]. The industrial scale production of P3HB is still limited by high production costs mainly influenced by the costs of the carbon source (40-50% of overall costs) and the recovery of P3HB [5-6]. The gap in the production costs between PHAs and synthetic plastics is preventing a full replacement of synthetic plastic so far. Estimated production costs of PHAs can be more than 2 times higher in a comparison to the production of petroleum-based plastics. The production cost of PHAs produced in a fed-batch can reached up to 3.5 €/kg, whilst synthetic plastics like polypropylene or polyethylene can be produced with the production cost circa 1.4 €/kg [45]. One way to reduce the production costs of P3HB is to avoid the use of pure substrates and optimize a production with different industrial by-products as carbon sources (cheese whey permeate, wood hydrolysate, sugarcane molasses, waste glycerol, corn steep liquor, etc.) [46-47].

2.2.4 Different incoming materials

Liu et al. (2011) aimed for the optimization of P3HB accumulation in a batch configuration using a residual activated sludge with a microbial community found in a sewage sludge. Results showed that the absence of nitrogen and/or phosphorus and the presence of sodium acetate as a carbon source, led to a microbial growth (3.7 gL^{-1} CDW) and P3HB accumulation (67% $P3HB\ cont.$) by sewage sludge microbial community [5]. Shen et al. (2015) investigated a fed-batch production of a copolymer poly(3-hydroxybutyrate-co-3-hydroxyhexanoate) (PHBHHx) using a residual activated sludge. The fermentation has been accessed by a mixed bacterial community found in a sewage sludge with a continuous aeration, nitrogen and phosphorus limitation and with the feeding of sodium laureate as a carbon source with a final content of copolymer (more than 23% wt. of PHBHHx) [48].

During biodiesel production crude glycerol is generated through the transesterification of vegetable and animal fats or oils. The crude glycerol has a low market value due to impurities. Since, purification steps from the crude glycerol towards a pure glycerol are costly especially for medium and small sized plants, the crude glycerol became a significant waste by-product in the biodiesel production. Thus, it was necessary to find out an environmentally friendly way to dispose it. A fed-batch fermentation by the microorganism *Cupriavidus necator* for the production of P3HB using the crude glycerol as a carbon source has been investigated. Results showed that the crude glycerol as a primary carbon source could be implemented for the microbial growth of *C.necator* DSM 545 (almost 69 g/L of CDW) and the accumulation of P3HB (38% of P3HB) [49]. A scale up feasibility of P3HB using *Burkholderia cepacia* and crude glycerol from lab-scale to plant-scale was reported in the study done by authors Zhu et al. (2010). They successfully conducted a scale-up of P3HB production up to 200 L with yields above 23 g/L of CDW and slightly more than 7 g/L of P3HB accumulation [47]. The crude glycerol from biodiesel production could be also used as a carbon source by *C. necator* for the production of copolymers such as poly(3-hydroxybutyrate-co-4-hydroxybutyrate) and poly(3-hydroxybutyrate-4-hydroxybutyrate-3-hydroxyvalerate) under limitation of nitrogen [50].

Whey is a by-product in cheese or casein production. The amount of whey generated in the dairy industry is about 80-90% of the whole volume of milk transformed during cheese or casein production. The whey contains about 4.5% (wt/vol) of lactose, 0.8% (wt/vol) of protein, 1.0% (wt/vol) of salts and less than 1.0% (wt/vol) of lactic acid. Since, the whey is produced in very large quantities there is a need to employ it in further applications. Several authors report that whey-based medium could be used as a carbon source by recombinant *Escherichia coli*, *Azohydromonas lata, Bacillus megaterium, C.necator and Pseudomonas oleovorans* aiming at P3HB production. Ahn et al. (2000) reported the accumulation of P3HB in a fed-batch system by recombinant *E.coli* with whey-based medium gaining approximately 120 g/L of cell concertation, 96 g/L of P3HB concentration and attaining a 2 g/L/h P3HB productivity [51]. Das et al. (2018) used whey as a carbon source by *B.megaterium* for P3HB production. The authors achieved almost 76% of P3HB accumulation of its dry weight [52].

Sugar-rich agricultural raw materials such as wheat bran, corn steep liquor, rapeseed cake, molasses and wastewater from starch production plant were implemented in the production of bioplastics as well. Different microorganisms were employed due their

capacity to accumulate P3HB and the ability to grow on these substrates namely *Pseudomonas fluorescens* (sugarcane molasses), *Azotobacter chroococcum* (beet molasses),*Burkholderia cepacia* and *Burkholderia sacchari* (starch). Sugarcane molasses is a promising feedstock with a low price and a sugar-rich composition including sucrose, glucose and fructose. Akaraonye et al. (2011) chose *Bacillus cereus* for P3HB accumulation using sugarcane molasses as carbon source. The cultivation was conducted in 2L and 1L shake flasks with a yield of P3HB accumulation above 51% of CDW in the 2L shake flasks and 61% of CDW in the 1L shake flasks [53]. The potential of corn syrup as carbon source for P3HB accumulation by *C. necator* was explored by Daneshi et al. (2010). Under nitrogen limitation, the highest P3HB productivity and biomass yield obtained were 0.224 g/L/h and 0.57 g/g respectively [54].

2.3 Macroalgae as a Carbon Source

Marine algae, including micro- and macroalgae, as a renewable resource promises a lot of interesting properties in comparison to other industrial by-products or municipal waste materials. Algae represents a widely diverse group of photosynthetic organisms living in the aquatic environment that covers 72 % of the earth´s surface. Besides of a potential application in industry, autotrophic algae could be used to reduce CO_2 emissions due to their high photosynthetic efficiency and fast growth rate. Carbohydrates and lipids from algae biomass, synthetized through the reduction of inorganic carbon. may be used in industrial applications as well [55]. These compounds can be further converted into bioethanol, other chemicals and fuels through chemical and biochemical processes. Production yields of marine algae are much higher comparing to terrestrial plants. Also, algae do not require arable land and could grow even using wastewater. Another advantage is that marine algae do not possess lignin which is essential for the mechanical properties of terrestrial plants. Depolymerisation of marine algae biomass is thus much easier, as it requires less harmful hydrolysis conditions, in contrast to lignocellulosic biomass [56].

Macroalgae can be cultivated offshore, onshore or in integrated seaweed cultivation systems. The offshore cultivation is a preferable choice since it allows better control over growth conditions and harvesting of macroalgae biomass. To access higher growth yield of macroalgae it is essential to provide a set of conditions (**Table 3**). The harvesting of macroalgae is carried out three times a year. Excessive harvest could endanger marine ecosystems.

Some marine areas with proliferated seaweed populations even require removal of seaweed plants to restore the availability of nutrients for other marine organisms [55].

Table 3 – Environmental conditions for the growth of macroalgae reported by Sudhakar et al. (2018) [55].

Factor	Description
Location	Tropical regions
Water quality	Coral reefs, rocky and sandy bottom,
Salinity	28-34 ppt, relatively salty, purely marine
Depth	At least 30 metres in depth
Temperature	27-30 °C
Current	5-10 m per minute
Fauna	Presence of other vegetation such as seagrass or sargassum

2.3.1 Characterization of *Gelidium sesquipedale*

The *Gelidium sesquipedale* is a red algae specie from the *Gelidium* genus and represents the class "Florideophyceae" from the division "Rhodophyta" (red algae) (**Figure 4**). Red algae typically feature water-soluble pigments such as phycobiliproteins (allophycocyanin, phycocyanin and phycoerythrin) located in phycobilisomes, luthein and zeaxanthin. Final colour (bright red, gray-green, blue-green) of macroalgae in general depends on the ratio of those pigments. Populations of *Gelidium sesquipedale* could be found alongside shores of the Northern Atlantic stretched from the Cornwall to Mauritania, where they have a significant economic importance mainly in countries like Spain, Portugal or Morocco [57-58].

Phylum	• **Rhodophyta**
Class	• Florideophyceae
Order	• Gelidiales
Family	• Gelidiaceae
Genus	• *Gelidium*
Species	• *Gelidium sesquipedale*

Figure 4 – The image of Gelidium sesquipedale (on the left) [59] and taxonomic classification of Gelidium sesquipedale (on the right).

Red algae species can accumulate a special type of storage polysaccharide called "floridean starch" based on D-glucose monomers with a (α-1,4-glycosid linkage) and occasionally with a (α-1,6-glycosidic linkage) [60]. Cell wall of red algae consists of microfibrillar cellulose and colloidal polysaccharides agar and carrageenan [61]. Agar fulfils similar functions as lignin for terrestrial plants, but for algae it provides more flexibility and structural resistance to water current and waves motion.

Agar is a colloidal polysaccharide consisting of two different types of polysaccharides agarose (70-80%) and agaropectin (30-20%). The agarose is composed of (α-1,3-linked) D-galactose and (β-1,4-linked) 3,6-anhydro-L-galactose (AHG) monomers [62]. These disaccharides may be esterified to form sulphate esters and methoxyl residues as well. Addition of agar to a water solution forms a slightly viscous solution. After heating and cooling down this viscous solution becomes a thermoreversible gel [63]. Carrageenan is mainly synthetized by red algae of *Gigartinales* order, it is derived by repetition of a α- and β-galactopyranose. Esterification by sulphuric acid is occurring for carrageenan as well as in the case of agar but with a higher degree of esterification [64]. An overview of chemical composition of some species within red algae division is displayed in **Table 4**.

Table 4 - Chemical composition of some red algae species.

Component	***Gracilaria verrucosa***	***Gelidium amansii***	***Gelidium elegans***	***Gracilaria gracilis***
Ash	7.4 [a)]	n.r. [b)]	30.4 [c)]	19-35 [d)]
Carbohydrates	67 [a)]	n.r. [b)]	n.r. [c)]	n.r. [d)]
Agar Yield	n.r. [a)]	n.r. [b)]	n.r. [c)]	9.9-14.9 [d)]
Cellulose	n.r. [a)]	17 * [b)]	17.2 [c)]	n.r. [d)]
Starch	n.r. [a)]	0 [b)]	n.r. [c)]	n.r. [d)]
Hemicellulose	n.r. [a)]	59 [b)]	29.5 [c)]	n.r. [d)]
Dry matter	n.r. [a)]	n.r. [b)]	n.r. [c)]	14.9-22.7 [d)]
Lipids	0.65 [a)]	n.r. [b)]	n.r. [c)]	1.4-3.6 [d)]
Moisture	7.8 [a)]	n.r. [b)]	n.r. [c)]	n.r. [d)]
Proteins	9.5 [a)]	n.r. [b)]	n.r. [c)]	3-5.8 [d)]

[a)] Kwon et al. (2016a) – reported as % dw. for *Gracilaria verrucosa* [65].

[b)] Oh et al. (2015) – reported as % dw. for *Gelidium amansii* [66].

[c)] Chen et al. (2016) – reported as % dw. for *Gelidium elegans* [67].

[d)] Ben Said et al. (2018) – reported as % dw. for *Gracilaria gracilis*, ranges are reflecting variability of a chemical composition due to the different growing conditions of algae such as depth and fragment weights [68].

* *α-cellulose; n.r.: non-reported*

2.3.2 Agar waste as a feedstock

Agar is the most valuable phycocolloid in food industry, biotechnology and cosmetics where it is used as a gelling agent. The agar is extracted mainly from two red seaweed genera *Gelidium* and *Gracilaria*, the so-called agarophytes. The industrial extraction of agar is firstly carried out with an alkaline treatment which provides the desulfation of agar polymer and with the subsequent solubilisation of agar with hot water under pressure [69]. Dissolved agar must be next filtered to remove the residual algal biomass. Phycocolloids extraction has developed rapidly in recent years, resulting in a production of diverse products from seaweed phycocolloids with a total revenue about 1000 million US$ in 2015. However, after the filtration step an enormous amount of algae waste is left untapped, about 70 % of the raw material is discarded. This non-valuable residual biomass could have a negative impact on the environment; thus, it has to be properly disposed. Interestingly, these residues still contain a non-negligible amount of polysaccharides like cellulose, hemicellulose and a small amount of floridean starch [70] Thus, this biomass could be implemented in a biorefinery as feedstock for further production of valuable bio-based products [7].

Some authors reported the application of algal residues after agar extraction in the polymer and biofuels fields. Madera-Santana et al. (2015) studied the possible implementation of polysaccharides from agar residues in the manufacture of biocomposites with poly-lactic acid (PLA). The enrichment of PLA improve mechanical and thermal properties of the biocomposite [70]. Pei et al. (2013) explored the valorisation of seaweed residues in papermaking industry. Authors suggested that the integration of agar waste biomass as a fibre source and filler in the papermaking industry could solve the worrying issue of deforestation. These sheet composites also exhibited better qualities such as higher antimicrobial activity and lower permeability [71]. Meinita et al. (2017) used agar waste from *Gracliaria verrucosa* and *Gelidium latifolium* as a raw material for a bioethanol production. Authors obtained after 12 hours of the fermentation of *G. verrucosa* hydrolysates a maximum concentration of ethanol around 5.5 g/L corresponding to 57% of the theoretical yield while during the fermentation of *G. latifolium* hydrolysates the highest ethanol concentration obtained was circa 10.8 g/L, responding to 64% of the theoretical yield [7].

2.3.3 Carbon-rich hydrolysates from macroalgae

The conversion step of macroalgae biomass to carbon-rich hydrolysates is a crucial part for the further production of bioproducts and biofuels. Pre-treatment of biomass should provide an efficient hydrolysis of polysaccharides into mono- ; di- or oligosaccharides However, macroalgae biomass possess a structural rigidity and complexity, therefore it is essential to develop and optimize this step in order to obtain high fermentable sugar yields with the lowest costs and minimal formation of inhibitory products [66]. A wide range of different pre-treatment techniques were explored during last decades (**Table 5**).

Table 5 – Overview of the pre-treatment methods reported by Alvira et al. [72] (2010)

Pre-treatment	Mechanism of Pre-treatment	Methods
Biological	Employment of microorganisms for their hydrolytic potential accessed by hydrolytic enzymes – mainly for a lignocellulosic biomass	• Hydrolysis by white-rot fungi *Phanerochaete chrysosporium; Ceriporia lacerata;* etc. • Hydrolysis by *Actinomyces* [66] • Enzymatic hydrolysis
Physical	Implementation of mechanical forces in order to reduce the particle size and crystallinity of the polymer, resulting of an increase of a surface area	• Milling techniques • Chipping • Grinding • Extrusion

Chemical	Digestion by chemical agent(s)	• Alkali (NaOH; $Ca(OH)_2$)
		• Acids (H_2SO_4; HCl)
		  ○ Concentrated
		  ○ Diluted
		• Ozonolysis
		• Organic solvents (methanol; ethanol; acetone; etc)
		• Ionic liquids

Physico-chemical	Combination of physical pre-treatments together with chemical agent(s)	• Ammonia-fibre explosion • Steam explosion

2.3.3.1 Chemical pre-treatment

Aiming at pre-treatment, different catalysts may be used such as acids, alkalis and organic solvents. In general, chemical pre-treatment usually requires high temperatures ranging from 140 – 210 °C for a shorter period of time. For this reason, these methods are classified also as thermochemical pre-treatment methods [66]. A widely explored pre-treatment technique is the digestion by acids. Pre-treatment with concentrated acids is less favourable approach in comparison to a digestion with diluted acids due to a higher corrosivity and an increased rate of monosaccharide degradation [72].

The saccharification of agar (or agarose) could be obtained by a mild acid pre-hydrolysis with a subsequent enzymatic hydrolysis step. It is worth mentioning that a weak acid prefers to cleave the α-1,3 linkages of agarose resulting in the release of agarooligosaccharides [62]. Under certain conditions, during the chemical hydrolysis of agar, a D-galactose could be released from galactosyl residues. However, the anhydrogalactose compounds (AHG) are prone to further dehydration leading to 5-hydroxymethylfurfural (HMF) and eventually to levulinic acid [73].

Abd-Rahim et al. (2013) optimized the chemical hydrolysis of the red seaweed *Kappaphycus alvarezii*. In order to release the most reducing sugars, the authors alternated several parameters such as temperature, hydrolysis time, acid and substrate concentration. Using 8% (w/v) biomass load, a temperature of 110 °C and 90 minutes hydrolysis time they achieved just above 34 g/L of the total reducing sugars with 0.2M H_2SO_4 and slightly over 35 g/L with 0.2M HCl [74]. Kim et al. (2015) optimized the acid hydrolysis process for *G. verrucosa.* The highest concentration of the total reducing sugars released was circa 7.5 g/L under conditions of 0.1N H_2SO_4; 2% (w/v),121°C for 15 minutes [75]. The use of phosphoric acid for thermo-chemical hydrolysis of *G.verrucosa* was reported by Kwon et al. (2016b). The authors reported almost 33% yield of total reducing sugars release under conditions of 1.5% concentration of acid; 10% (w/v) biomass load; 140 °C and with 60 minutes of reaction time [76]. Another promising catalyst is sulfamic acid reported by Park et al. (2018). Sulfamic acid has a dual-catalytic property with a Brønstead and Lewis acid sites. Optimized pre-treatment with 100 mM sulfamic (7.5% (w/v) biomass concentration; 130°C; 90 minutes) yielded almost 40% release of total reducing sugars [77].

2.3.3.2 Enzymatic hydrolysis

The enzymatic hydrolysis of biomass or biological hydrolysis managed by microorganisms requires less harmful conditions compared to thermochemical methods [7]. The enzymatic hydrolysis has advantages of less energy-dependent conditions and eco-friendly impact on the environment over chemical pre-treatment methods. The lower energy input of enzymatic hydrolysis causes less formation of inhibitory compounds. On the other side, the enzymatic hydrolysis requires a prolonged time of hydrolysis, during that time the risk of contamination is significant [66].

An enzymatic treatment is frequently used as a subsequent treatment step after chemical pre-treatment in the so called "combined hydrolysis" to enhance sugars release. The study aimed for saccharification of brown seaweed *Sargassum sp*. conducted by Azizi et al. (2016) demonstrated that employing enzymatic hydrolysis after mild acid hydrolysis (0.15N H_2SO_4; 10% biomass concentration (w/v); heating at 121 °C for 30 minutes) the amount of released sugars increased from 12 g/L up to 20 g/L. The authors used for enzymatic hydrolysis the enzymes cellulase (Celluclast ® 1.5L) and cellobiase (Novozyme 188) [78]. Kwon et al. (2016a) reported in their study the combined hydrolysis of *G. verrucosa* with the chemical pre-treatment using citric acid followed by the enzymatic hydrolysis with enzyme mixtures Viscozyme ®, Cellic ® CTec2 and HTec2. After the chemical pre-treatment with 0.1M citric acid, 10% (w/v) biomass load at 150 °C for 60 minutes, the authors obtained circa 51 % of the yield of total reducing sugars. Following the subsequent enzymatic hydrolysis that yield increased up to 58 % [65].

2.3.4 Bacterial growth inhibitors

The chemical pre-treatment of biomass has one consideration. During thermal acid hydrolysis certain dehydration products originated from pentoses and hexoses are generated (**Figure 5**). Three main groups of dehydration by-products are furans, organic acids and phenols [79]. Furans, such as 5-hydroxymethylfurfural (HMF) from hexoses and 2-furaldehyde (furfural) from pentoses, could above certain concentration inhibit microbial growth. It was reported that these toxic compounds can inhibit the bacterial growth by reducing the specific growth rate (**Table 6**), productivity, protein and RNA synthesis and may as well participate in DNA breakdown [80].

Table 6 – Overview of the inhibitory effect of different dehydration by-products on growth rate of microorganisms reported by van der Pol et al. (2014) [79].

Inhibitory product	Concentration (g/L)	Relative growth rate (%)	Microorganism
Furfural	0.5	53	*S.cerevisiae*
	1	19	
	4	11	
5-HMF	1	35	
	3	17	
	4	29	
Acetic acid	3.5 (pH 3.5)	66	
	5 (pH 5.6)	79	
	15 (pH 5.6)	56	
Formic acid	1.67 (pH 6)	84	*Z.mobilis*
	3.91 (pH 5.8)	50	
	11.04 (pH 5.8)	0	

Also, the enzymatic hydrolysis of biomass by cellulytic enzymes can be retarded by inhibitory compounds [66]. It has been reported that phenolic compounds may interact through hydrophobic interaction between aromatic compounds and cellulolytic enzymes. However, even products of the enzymatic hydrolysis by cellulolytic enzymes such as monosaccharides (e.g. glucose), disaccharides (e.g. cellobiose) or oligosaccharides may inhibit the activity of cellulolytic enzymes as well [81] .Under very harsh conditions (longer hydrolysis time, higher temperature and acid concentration) HMF is further degraded to levulinic and formic acid [81].

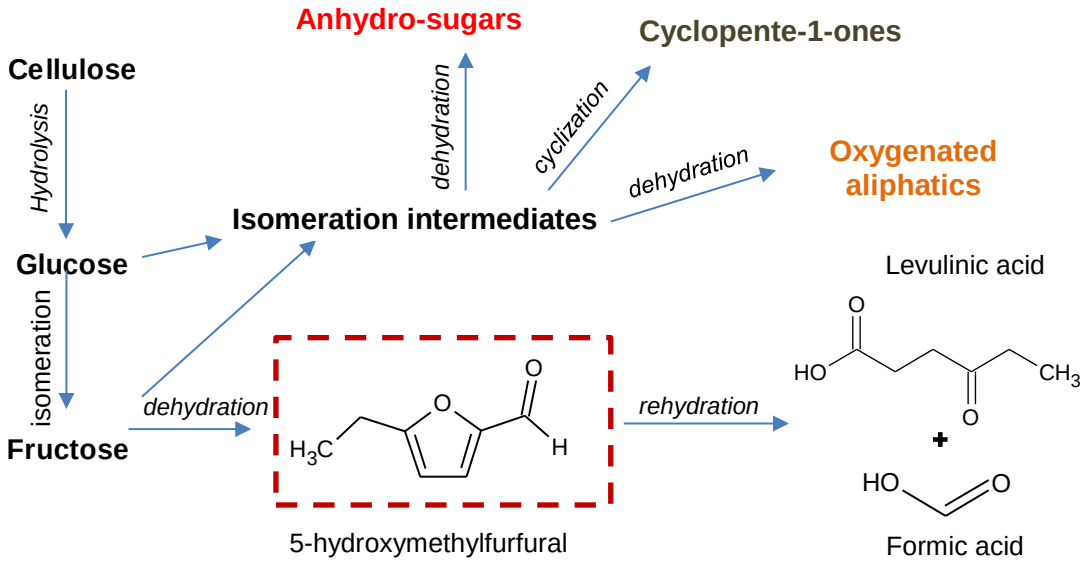


Figure 5 – Conversion of carbohydrates to dehydration by-products [82].

Several approaches to reduce the formation of inhibitory compounds have been explored up to this day. One of these methods is the pre-treatment with diluted acid which increases the enzymes accessibility for subsequent hydrolysis. This methodology was described in the previous chapter. However, even if low acid concentrations are used, still a little concentration of inhibitory compounds can be formed. For instance, in the article by Kim et al. (2013) hydrolysis of *Gelidium amansii* by diluted acetic acid 1-5% (w/v) generated 0.1-2.3% (w/v) of HMF; furfural was not detected [83].

Very promising results were obtained by a unique treatment called "Ammonia Fibre Explosion (AFEX)". Biomass with anhydrous ammonia is placed in the pressure vessel with exposure to moderate temperatures (60-100 °C) and high pressures (250-300 psi) for 5 minutes. Then by the action of a quick decompression in the vessel with a further enzymatic hydrolysis step it is possible to obtain yields of released sugars above 80% with little or no degradation. In addition, AFEX is a dry process meaning that the treated biomass is preserved for a long time and further enzymatic hydrolysis can be fed at very high solids loadings [84].

Another established pre-treatment technique is wet oxidation (WO). For this reaction elevated temperatures (100-320 °C), high pressures (0.5-20 MPa) of pure oxygen (or air) and the presence of a catalyst such as a sodium hydroxide, hydrogen peroxide or sodium carbonate are required [85].The WO has a capability to easily solubilize hemicellulose and to make it more accessible for the further enzymatic conversion towards monosaccharides without an elevated rate of carbohydrate dehydration [79].

Also, few techniques may detoxify sugar-rich hydrolysate from growth inhibitors. Feldman et al. (2015) focused on the transformation of HMF by a white-rot fungus *Pleurotus ostreatus*. Results showed that *P.ostreatus* is capable of metabolizing and detoxifying HMF to a 2,5-bis-hydroxymethlyfuran (HMF alcohol) and to a 2,5-furandicarboxylic acid (FDCA). Extracellularly, HMF is oxidized to FDCA in two-step oxidation reaction catalysed by aryl-alcohol oxidase enzymes generating H_2O_2. Intracellularly, HMF could be reduced to HMF alcohol by aryl-alcohol dehydrogenase enzymes and the cofactor NADHP. Since, a FDCA is not occurring in media it's plausible that FDCA is subsequently metabolized by further metabolic pathways, whereas HMF alcohol was identified in the media [86].

It is even possible to detoxify hydrolysates from inhibitory compounds by sorption on activated carbon. However, the addition of a sorption operation unit to a upstream processing will increase the overall product costs, as it will add yet another step in solid-liquid separation in order to separate activated carbon from hydrolysates [87].

2.3.5 Concentration of carbon-rich hydrolysates

Concentration of the biomass-derived hydrolysates is usually needed when these sugar-rich solutions are used as feeding solution in fed-batch assays. During the concentration process a large volume of solvent (water) is evaporated, leading to an increase of carbohydrate concentration in the hydrolysates. However, due to the vulnerability of the carbohydrates to degradation at high temperatures , the heat impact on carbon-rich hydrolysates needs to be taken in the consideration. A brief review of some less-harmful evaporation techniques is presented in this chapter.

Techniques such as heating, vacuum evaporation and membrane separation have been reported to be successful for the concentration of the hydrolysates, but a high energy input makes these methods undesirable in a large scale. Pressure-driven membrane processes applicable in this area such as: nanofiltration (*NF*) and ultrafiltration (*UF*) have attracted a lot more attention in recent years, due to a low energy demand and unique separation ability. A possible detoxification is also possible by *NF* as reported by Weng et al. (2009). These authors have separated acetic acid from xylose in biomass hydrolysates. A combined setting using a *UF* and a *NF* unit could also be used to separate and reuse the enzymes used in the enzymatic hydrolysis. The *UF* separates the enzymes and the permeate (small molecules, e.g. glucose) is further treated with *NF*, where small molecules are retained and water will pass as permeate [88]. Nevertheless, several technical issues need to be taken into consideration during the membrane processes. During the concentration of carbon-rich hydrolysates by *NF* an increase of the carbohydrate concentration is responsible for the build-up of a Concentration Polarization (CP) layer, as the solids are deposited on a membrane surface, retarding the separation process. This layer could be disrupted by a higher applied pressure ΔP [89]. Another issue that needs to be overcome is the osmotic pressure$\Delta\pi$, which is especially significant for compounds with low M_W at high concentrations. To attain higher concentration factors C_F, enough pressure to surpass the osmotic pressure $\Delta\pi$ needs to be applied [90].

Spray-drying is a widely used technique in the food and pharmaceutical industries. This method converts a liquid/ slurry material to a dry powder. The dry powder offers a lot of advantages as it will prolong shelf-life significantly and reduces costs for storage and transportation. The final dry-powder can be distinguished in two main groups: sticky and non-sticky. If the material tends to stick / attach to walls of the drying chamber or forms agglomerates, it will lead to loss of products and thus lower product yields. Unfortunately, sugars (glucose, fructose, etc.) and organic acids are compounds that have sticky properties. The stickiness of sugars is due to their thermoplastic properties Above the temperature of sticky-point, which is circa 10-20 °C higher than a glass transition temperature (T_g), the sugars will change from glassy to a liquid-rubbery state. For the low molecular weight compounds like sugars the T_g is low and as the content of sugars is increasing during the spray-drying process, the T_g decreases even below the temperature of the outlet air. To minimize this phenomenon, the difference between the inlet and the outlet temperatures of the drying air should diminish [91]. The issue of stickiness can also be avoided by several steps such as: 1) addition of drying agent with higher molecular weight (e.g. maltodextrin, gum Arabic, starch); 2) low humidity and temperature of air and 3) cooling walls of drying chamber [92].

2.4 Halotolerant Bacteria for P3HB Production

For the P3HB production, using carbon-rich hydrolysates from *G. sesquipedale* as feedstock, the halotolerant bacteria *Halomonas boliviensis* (**Figure 6**) has been drafted as a promising P3HB producer. *H. boliviensis* is a Gram negative, aerobic and rod-shaped microorganism with a cell length and of 2.25 and a width of 0.5 µm. Mobility is accessed by lophotrichous flagella. *H.boliviensis* is a moderately halophilic, alkalitolerant and psychrophilic microorganism and high yields of P3HB accumulation (50-80 %) have been reported [8-9]. The polymer production by *H. boliviensis* is accessed mainly during late exponential and stationary phase [93].

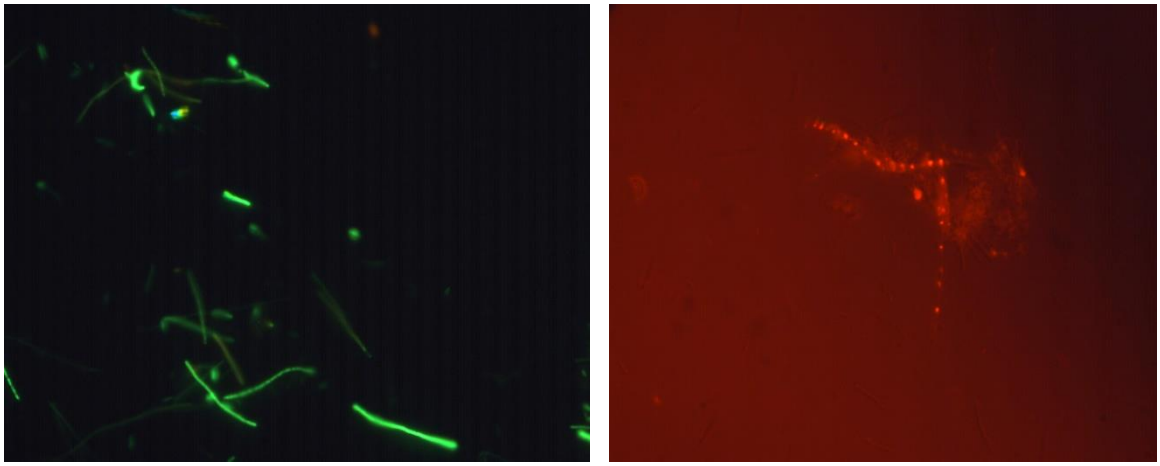
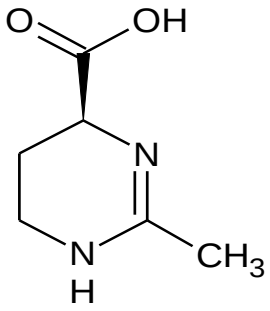


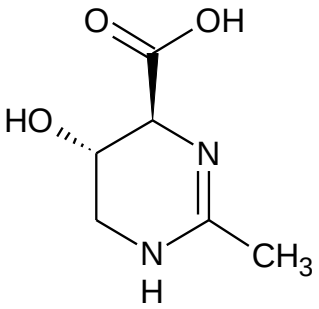
Figure 6 – Microscopic images of *H. boliviensis* strain; on the left (green) viable cells stained with SYTO ® 9 + propidium iodide; on the right image (red) inclusions bodies containing polymer stained with Nile red.

To withstand osmotic stress caused by an elevated NaCl concentration bacterial species within the *Halomonadaceae* family developed the synthesis of ectoines and hydroxyectoines, collectively known as ectoines. These solutes are very soluble, with a low M_w and mostly uncharged or may have zwitterionic properties. Ectoines are derivatives from heterocyclic amino acids or from partially hydrogenated pyrimidine (**Figure 7**). Accumulation of ectoines is promoted by an increased environmental osmolarity. The increase of osmotic stress in the environment is counteracted by the accumulation of ectoines until optimal conditions for cell growth are obtained. Then the content of ectoines inside cells decreases by the release of osmolytes via specific efflux systems, in order to restore the osmotic balance [94]. Ectoines also protect cells against a potential damage caused by heat, freeze or

desiccation and have protective qualities on biological compounds such as enzymes, DNA, antibodies, etc. The protection ability of ectoines has a bright future in fields related to the molecular biology, food processing, biotechnology, etc. [95].



Ectoine



Hydroxyectoine

Figure 7 – Chemical structures of ectoines.

Both P3HB and ectoines are intracellular products, therefore yields of these products are in direct relation with microbial growth. Conditions in which each product is favoured are however not the same. In an environment with high salt concentrations, above optimal 4.5% (w/v), cells prioritize to utilize carbon source for the synthesis of ectoines over cell growth and P3HB accumulation. The increase of P3HB content is apparent at lower salt concentrations, triggered by lack of nutrient in culture medium [96].

2.4.1 Taxonomy of microorganism

Two strains LC1^T and LC2 within the genus *Halomonas* were successfully isolated by Quillaguamán et al. (2004) from soil samples around hypersaline lakes of the south-western region of Bolivia, receiving the name *Halomonas boliviensis*. The majority of this region lies more than 4000 m above sea level therefore the survival in such conditions is not an easy task. Many of these local lakes contain salt concentration up to 100 g/L due to the evaporation and presence of intravolcanic basins [9]. A complete phylogenetic classification is described in the **Table 7** based on the classification by Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures [97].

Table 7 – Taxonomic classification of *Halomonas boliviensis* species.

Domain	• **Bacteria**
Phylum	• Proteobacteria
Class	• γ-proteobacteria
Order	• Oceanospirillales
Family	• Halomonadaceae
Genus	• *Halomonas*
Species	• *Halomonas boliviensis*

2.4.2 P3HB production by *H. boliviensis*

Several studies have been conducted in order to produce P3HB by *H. boliviensis*. The research article by Rivera-Terceros et al. (2015) aimed for the accumulation of P3HB by *H.boliviensis* using combinations of glucose, xylose and maltose as carbon sources and monosodium glutamate (MSG) as nitrogen source. The culture medium composition was designed to promote P3HB production upon nitrogen limitation. The growth of *H. boliviensis* on a starch hydrolysate as a carbon source was also followed. While maximum P3HB content of 60% was obtained in shake flasks scale using starch hydrolysate or a mixture of glucose and xylose as a carbon sources, the maximum P3HB accumulation in an air-lift reactor was above 40% [6].

Truly a pioneering work in the field of P3HB production by *H. boliviensis* was done by the group led by Quillaguamán. These authors describe the production of P3HB by *H. boliviensis* using different carbon sources in shake flasks and in bioreactors (Quillaguamán et al,2016). Under controlled cultivation conditions such as pH 8 and non-limiting DO (%), with a 4.5% (w/v) concentration of NaCl and combination of butyric acid and sodium acetate as carbon source, the authors improved the polymer production up to 88% of P3HB content. Other carbon sources such as glucose and sucrose also induced the P3HB production,

resulting in the highest accumulation of P3HB about 55% within 33 hours [93].

The study conducted by Quillaguamán et al. (2007) optimized batch culture conditions in order to obtain higher yields of P3HB and to increase microbial cell growth. Using 1.5% (w/v) of yeast extract as a nitrogen source 2.5% (w/v), sucrose as a carbon source and oxygen limitation resulted in 54% of P3HB accumulation, almost 8 g/L of P3HB concentration and 14 g/L of CDW [98]. It is notable that yeast extract as nitrogen source enhanced the cell growth, whilst P3HB accumulation was suppressed. This could be explained by the fact that yeast extract contains a wide spectrum of nitrogen components which could enhance the cell growth, and only after depletion of such nutrient, the P3HB accumulation may be triggered.

Therefore, Quillaguamán et al. (2008) suggested a study to determine essential nitrogen nutrients to access a better control of nutrient supply and to optimize the fed-batch strategy. Culture broth with glucose as a carbon source was initially supplemented by 10 mL of 25% (w/v) MSG during the first 9 h. of fed-batch cultivation to maintain a constant the concentration of MSG at 20% (w/v). Also, 0.4% (w/v) of NH_4Cl and 0.22% (w/v) of a KH_2PO_4 were added at 3rd (4 mL), 6th (1 mL), 9th (1mL) and 12th (3 mL) hour of the cultivation. Initial supplementation helps to avoid an early nutrient depletion and increase a cell density together with a volumetric productivity. The P3HB production was triggered when both NH_4^+ and *MSG* were being depleted in the medium, PO_4^{3-} decreased slightly during the first 6h of cultivation. Authors obtained in this assay 81% wt. of P3HB content; 44 g/L of CDW; more than 35 g/L of P3HB concentration and 1.1 g/L/h of P3HB productivity [10].

Recently, a study published by García-Torreiro et al. (2016) describes the metabolic behaviour of *H. boliviensis* under different nutrient limitations. The *H. boliviensis* growth and P3HB production was examined by single restriction of N and O_2 and combined restriction of both N and O_2 in fed-batch cultivation. The highest P3HB accumulation was accomplished under conditions of N limitation and low O_2 supply during the fed-batch cultivation. After 24 h. of fermentation the nitrogen source MSG was depleted, yielding a N/C ratio 0.02, at the same time the DO was below 20%. Combination of both conditions lead to 35 g/L of P3HB concentration, 48 g/L of CDW at the same time of cultivation (60 h.), 73% of P3HB content and 1.32 g/L/h of P3HB productivity [99].

3 Materials and Methods

3.1 Raw Materials

Gelidium sesquipedale residues after agar extraction were supplied by Iberagar SA, Portugal. The algal biomass was kept in sealed bags at -18 °C in the freezer.

To prepare a powder from lyophilized *G. sesquipedale* residues, the biomass was first cleaned with tap water to remove any impurities such as rocks, sand, shelves, etc. Polished biomass was then dried at room temperature for 2-3 hours to remove excess water. The biomass was then brought overnight to -80 °C. Deeply frozen biomass was then lyophilized for 48 hours and grinded with a coffee blender to a lyophilized powder (**Figure 8**). This lyophilized powder was stored in a desiccator until use. Every assay was performed using the lyophilized algal powder.

Figure 8 – Lyophilized powder from the *Gelidium sesquipedale* residues after an agar extraction.

3.2 Enzymes

The enzymes used within this work were cellulase complex (NS 22086), β-glucosidase (NS 22118) and glucoamylase (NS 22035) with enzyme activity 14.8 FPU/g, 32.0 NPGU/g and 750 AGU/g, respectively [100] the first two were purchased from Novozymes A/S while α-amylase from *Bacillus amyloliquefaciens* (≥ 250 KNU/g) was purchased from Sigma-Aldrich [101]. The protein concentration, determined by Bradford method using BSA as protein standard, was 120 $mg\ mL^{-1}$ for (NS 22086) and 40 $mg\ mL^{-1}$ for (NS 22118) [102].

3.3 Microorganism

The halotolerant bacteria *Halomonas boliviensis* DSM 15516, a strain able to accumulate P3HB, was used within the framework of this work.

3.3.1 Strain storage and preparation

Cultures of *H. boliviensis* were stored at -80 °C in 2 mL cryovials. The stock cultures were prepared by transferring 1.5 mL of an inoculum in the late exponential growth phase, to sterile cryovials containing 300 µL of pure sterilized glycerol. The preparation of stock cultures was performed under aseptic conditions in a laminar flow chamber (BioAir Instruments aura 2000 MAC 4 NF, Italy). The flow chamber was sterilized with a UV light for 15 minutes.

3.3.2 Culture media

3.3.2.1 Inoculation medium

The inoculation medium for *H. boliviensis* contained: 45 gL^{-1} of NaCl; 25 mL/L of 100 gL^{-1} $MgSO_4 \times 7\ H_2O$; 0.55 gL^{-1} K_2HPO_4; 2.3 gL^{-1} NH_4Cl; 15 gL^{-1} Tris; 3 gL^{-1} monosodium glutamate (MSG), 0.005 gL^{-1} $FeSO_4 \times 7\ H_2O$. Solutions of NaCl (300 gL^{-1}) and $MgSO_4 \times 7\ H_2O$ (100 gL^{-1}) were prepared separately to avoid precipitation during the sterilization in the autoclave. After all salts were dissolved the pH of inoculation medium was adjusted to 7.5 with 37% (w/w) HCl. The inoculation medium altogether with solutions of NaCl and $MgSO_4 \times 7\ H_2O$ were then sterilized in the autoclave at 121 °C, 1 bar for 20 minutes.

3.3.2.2 Production medium used in the shake flask assays

The medium for P3HB production in shake flasks assays contained: 45 gL^{-1} NaCl; 50 mL/L of 100 gL^{-1} $MgSO_4 \times 7\ H_2O$; 2.2 gL^{-1} K_2HPO_4; 0.005 gL^{-1} $FeSO_4 \times 7\ H_2O$; 1.0 gL^{-1} NH_4Cl; 15 gL^{-1} Tris and 20 gL^{-1} of MSG. Solutions of NaCl (300 gL^{-1}) and $MgSO_4 \times 7\ H_2O$ (100 gL^{-1}) were prepared separately to avoid a precipitation during the sterilization in the autoclave. In a particular study to determine the influence of the nitrogen content on the P3HB production different concentrations of NH_4Cl (0 or 1 gL^{-1}) and MSG (0; 5; 10 gL^{-1}) were tested. After all salts were dissolved the pH was adjusted to 7.5 with 37% (w/w) HCl. The P3HB production medium altogether with solutions of NaCl and $MgSO_4 \times 7\ H_2O$ were then sterilized in the autoclave at 121 °C, 1 bar for 20 minutes.

For the growth on *G. sesquipedale* hydrolysates a 20-fold concentrated production medium was prepared. The pH of this concentrated medium was not adjusted as previously. The concentrated NaCl and $MgSO_4 \times 7\ H_2O$ were also prepared and sterilized separately.

3.3.2.3 Production medium used in the bioreactor assays

In the bioreactor assays, the medium used to trigger polymer production by imposing N limitation had the following composition: 45 gL^{-1} NaCl; 50 mL of 100 gL^{-1} $MgSO_4$ x 7 H_2O; 15 gL^{-1} K_2HPO_4; 0.005 gL^{-1} $FeSO_4$ x 7 H_2O, 10 gL^{-1} MSG and 20 gL^{-1} glucose. The pH of production medium was adjusted to 7.5 with 37% (w/w) HCl. The production medium was sterilized inside the bioreactor at 121 °C, 1 bar for 20 minutes. Concentrated solutions of $MgSO_4$ x 7 H_2O (100 gL^{-1}), NaCl (300 gL^{-1}) and glucose (500 gL^{-1}) were prepared and sterilized separately to avoid precipitation during the sterilization process. These solutions were later added aseptically to attain the desired concentrations. The initial volume in the bioreactor was 1.3 L.

The medium composition to trigger P3HB by P limitation contained: 45 gL^{-1} NaCl; 50 mL of 100 gL^{-1} $MgSO_4$ x 7 H_2O; 5 gL^{-1} K_2HPO_4; 0.005 gL^{-1} $FeSO_4$ x 7 H_2O, 10 gL^{-1} MSG and 25 gL^{-1} glucose. After all salts were dissolved, the pH was adjusted to 7.5 as previously. The medium was sterilized inside the bioreactor at 121 °C, 1 bar for 20 minutes.

3.3.3 Shake flask assays

3.3.3.1 Preparation of inoculum

The inoculum of *H. boliviensis* was prepared in 500 mL using: 76.5 mL of inoculation medium; 15 mL of 300 gL^{-1} NaCl; 2.5 mL of 100 gL^{-1} $MgSO_4 \times 7\ H_2O$; 4 mL of 500 gL^{-1} glucose to attain a total volume of 100 mL. A 2 mL cryovial prepared, as described in section **3.3.1**, was added.

Growth of the inoculum was accessed in an orbital shaker Aralab ® Agitorb 200 at 30 °C and an agitation speed of 170 rpm during 24 h until an exponential phase was reached. This corresponded to an optical density at 600 nm $O.D._{.600nm}$ of 5. For the production of P3HB production assays a 5 % (v/v) of inoculum was used.

3.3.3.2 *Shake flasks cultivation*

The shake flask assays were carried out to follow growth and P3HB production of *H. boliviensis* in different conditions. These assays were performed in 500 mL shake flasks containing production medium supplemented with 20 gL^{-1} glucose or *G. sesquipedale* hydrolysate to a 100 mL final working volume. A volume of previously prepared inoculum was added to attain 5 % (v/v) concentration. Samples from shake flasks were periodically taken for cell growth determination by $O.D._{.600nm}$ and cell dry weight CDW, sugar consumption and P3HB production. The assays were performed in duplicate and the average of the results considered.

3.3.4 Fed-batch assays

3.3.4.1 *Fed-batch cultivation*

The inoculum for Fed-batch assay was prepared as It was described in section (*3.3.3.1*), in this case to a total volume of 65 mL corresponding to 5% (v/v) of the bioreactor initial volume.

Fed-batch cultivations were carried out in 2L stirred-tank bioreactors (New Brunswick Bioflo 115) using the BioCommand Batch Control software, which enabled control, monitoring and data acquisition. The pH was controlled at 7.5 with 10% NH_4OH or 2.5 M NaOH, depending on the type of nutrient limitation used to trigger P3HB production, namely phosphorus or nitrogen, respectively. The gas flow used was 2.0 L/min, unless stated otherwise. The dissolved oxygen ($DO\ (\%)$) set point was set at 20% saturation, unless stated otherwise. The agitation speed (max. 1200 rpm) was set in cascade with the $DO\ (\%)$. The initial volume of cultivation was 1.3L including all medium components and inoculum.

One litre (1L) of a concentrated glucose solution (500 gL^{-1}) was prepared as feed for the fed-batch assays. This solution was sterilized previously in the autoclave at 121 °C, 1 bar for 20 minutes. The feeding was carried automatically using the DO-stat strategy.

In the assay carried out using P limitation as trigger for the P3HB production , two pulses of 9 mL of 250 g/L MSG were added during the batch phase. This was carried out aseptically with a syringe. The 250 gL^{-1} solution of *MSG* was prepared separately from the 500 gL^{-1} glucose solution, to avoid a Maillard reaction during the sterilization in the autoclave. The $DO\ (\%)$ was maintained at 20% with 2.0 $L\ min^{-1}$ of gas flow.

3.3.5 Cultivation parameters

The yield of product over substrate $Y_{P/S}$ in shake flasks was calculated according to the **Eq. 1**. The production yield was calculated by dividing the maximal concentration of product (P3HB polymer) $[P_{max}]$ with the consumed substrate (glucose) $[S_{cons.}]$ at the corresponding time. Variations in sample volumes were neglected in shake flask assays.

$$Y_{P/S}\ (\mathrm{g\ P/\ g}\ S_{cons.}) = \frac{[P_{max}]}{[S_0] - [S_{max}]} = \frac{[P_{max}]}{[S_{cons.}]} \quad (1)$$

In the bioreactor assays, product yields were computed by the **Eq. 2**. In this case a mass balance was performed for each sample retrieved and therefore for each mass of product obtained. The production yield in the bioreactor was determined by dividing a sum of product mass in each sample plus final (maximal) concentration of product $\sum m_{P_S} + m_{P_{max}}$ with the mass of consumed substrate (glucose) $m_{S_{cons.}}$ consisting of initial mass of substrate m_{S_i}, the mass of substrate in feed $m_{S_{feed}}$ subtracted by amount of substrate at time appropriate to cultivation time of $m_{P_{max}}$.

$$Y_{P/S}\ (\mathrm{g\ P/\ g}\ S_{cons.}) = \frac{\sum m_{P_S} + m_{P_{max}}}{m_{S_i} + m_{S_{feed}} - m_{S_{max}}} = \frac{m_{P_{sum.}}}{m_{S_{cons.}}} \quad (2)$$

Also, a maximum productivity for both shake flasks and bioreactor were determined according to **Eq. 3.** The maximal productivity was computed by dividing the maximal concentration of product $[P_{max}]$ by correspond time of sample $t_{s\,max}$.

$$Prod_{max\,P}\ (g\ L^{-1}H^{-1}) = \frac{[P_{max}]}{t_{s\,max}} \quad (3)$$

A polymer content, $P3HB\ cont.(\%)$ and residual cell dry weight, $CDW_{residual}\ (gL^{-1})$ were acquired by **Eq. 4** and **5**.

$$P3HB\ cont.(\%) = \frac{P3HB_{conc.}\ (gL^{-1})}{CDW\ (gL^{-1})} \quad (4)$$

$$CDW_{residual}\ (gL^{-1}) = CDW\ (gL^{-1}) - P3HB\ (gL^{-1}) \quad (5)$$

3.4 Analytical Assays

3.4.1 Carbohydrate quantification

The quantification of glucose, galactose, HMF and phosphate was determined with a High-Performance Liquid Chromatography (HPLC) equipment (Hitachi LaChrom Elite), with a column (Rezex ROA. Organic acid H+ 8% 30 mm x 7.8 mm, an autosampler (Hitachi LaChrom Elite L-2200), an HPLC pump (Hitachi LaChrom Elite L-2130) and with a Hitachi L-2490 RI detector and a Hitachi L-2420 UV/VIS detector. A column heater was connected externally. The temperature of column was set to 65 °C. The 5 mM H_2SO_4 eluent was used with 0.5 $mL\ min^{-1}$ flow rate in HPLC measurements.

Samples of culture medium were withdrawn from the bioreactor or shake flask and centrifuged (1-15P microcentrifuge, Sigma) for 5 minutes at 10 000 rpm. An aliquot of the supernatant (200 µL) was mixed with vortex with the same volume (200 µL) of 50 mM H_2SO_4 centrifuged again at 10 000 rpm for 5 minutes. HPLC samples were prepared in HPLC vials by diluting 100 µL of supernatant from the second centrifugation cycle with 900 µL of 50 mM H_2SO_4 . After vortexing, the samples were ready for the carbohydrate quantification.

A linear regression equation were obtained from calibration curves for glucose, galactose, HMF and phosphate. Calibration data are attached in the **Appendix**. The detection of monosaccharides and phosphate was done by the RI while the HMF was quantified using the UV/VIS detector.

3.4.2 Total carbohydrates

The determination of total carbohydrates in algal biomass was done according to the National Renewable Energy Laboratory (NREL) protocol called: “Determination of Total Carbohydrates in Algal Biomass” [103].

This assay was initiated by the hydrolysis of 25 mg weighted biomass with 250 µL 72% (w/w) sulfuric acid in glass tubes (3 samples) at 30 °C in a water bath. After an hour the hydrolysate was diluted to a final concentration of 4% (w/w) sulfuric acid with 7 mL of Mili-Q ® water. Samples were then placed in an autoclave for one hour at 121 °C. Once the samples were cooled down to room temperature, an aliquot was taken for neutralisation to pH 6-8 using calcium carbonate. To separate the solids after the neutralisation, a centrifugation step was applied at 10 000 rpm for 5 minutes. Samples were then prepared for the HPLC quantification of carbohydrates.

The total carbohydrate content was then computed according to **Eq. 6** and **7**, where the term *DW* in **Eq. 7** represents a dry weight of sample.

$$Total\ Monosaccharides\ (\mathrm{mg}) = \mathrm{c_{monosaccarides}}(\mathrm{mg/mL}) \times 7.25\ (\mathrm{mL}) \quad (6)$$

$$Total\ Carbohydrates\ (\%) = \frac{Total\ Monosaccharides}{DW_{sample}} \times 100 \quad (7)$$

3.4.3 Ash, moisture and total solids

To determine ash, moisture and total solids in algal biomass the protocol reported by NREL "Determination of Total Solids and Ash in Algal Biomass" was followed [104].

Pre-conditioned crucibles (575 °C in a muffle furnace overnight) were weighted (3 samples), and to these pre-weighted crucibles an amount of 100 mg of biomass was added. The samples were then placed in a convection drying oven at 60 °C for at least 18 hours until constant weight was achieved. Results from this assay were used for the total solids and moisture determination of algal biomass, according to **Eq. 8** and **9**, respectively.

To determine the ash content of algal biomass, cooled crucibles containing the dried sample from previously described procedure were subsequently put in a muffle furnace at 575 °C for at least 24 hours. Cooled samples were weighted and the ash content of algal biomass was calculated (**Eq. 10**).

$$Total\ Solids\ (\%) = \frac{\mathrm{m_{dried\ sample+crucible}} - \mathrm{m_{crucible}}}{\mathrm{m_{sample}}} \times 100 \quad (8)$$

$$Moisture\ (\%) = 100 - Total\ Solids\ (\%) \quad (9)$$

$$Ash\ (\%) = \frac{m_{ashed\ sample} - m_{crucible}}{DW_{sample}} \times 100 \quad (10)$$

3.4.4 Starch content

To verify the starch presence in the algal biomass the procedure "Total Starch Assay Procedure" reported by Megazyme [105] was followed with few alterations.

To three glass tubes an amount of 100 mg of algal biomass was added to each flask. To each tube a volume of 0.2 mL of 80 % v/v aqueous ethanol was added to wet the samples, after the addition of ethanol, the glass tubes were vortexed carefully. After vortexing an amount of 3 mL of α-amylase (NS 22035) solution was added to each flask. The solution of α-amylase was prepared by dilution of the enzyme with acetate buffer (100 mM, pH 5.5 + calcium chloride 5 mM) in a ratio of 1:30. After the addition of the α-amylase solution the glass tubes were placed in a water bath and incubated for 6 minutes at 90 °C. The samples were stirred after 2,4 and 6 minutes. After the digestion of starch by α-amylase, 1 mL of amyloglucosidase (Sigma-Aldrich), pre-diluted with deionized water in a ratio of 1:10, was added to each glass tube. The samples were then put in an incubator for 30 minutes at 65 °C. After the starch hydrolysis each flask was transferred to a 50 mL volumetric flask. The final volume of 50 mL was adjusted using Mili-Q ® water.

An aliquot of 500 µL from each flask was taken, centrifuged at 10 000 rpm for 5 minutes. Samples were then prepared for the HPLC quantification of carbohydrates. The total starch content (% dw.) was calculated according to **Eq. 11**.

$$Starch\ (\%\ dw.)\ = \frac{c_{\text{glucose}} \times V_{final}}{DW_{\text{sample}}} \times 0.9 \times 100 \qquad (11)$$

The 0.9 factor in **Eq. 11** was introduced to take into account the conversion of starch to glucose and the introduction of water molecule during the hydrolysis [106].

3.4.5 Optical density

To measure the $O.D._{600nm}$, a sample volume of 600 µL was transferred to 1.5 mL Eppendorf tube. The O.D. of culture was measured with a Hitachi U-200 spectrophotometer at the wavelength of 600 nm. The samples were diluted to a ratio of 1:10 or 1:100 with Mili-Q ® water and measured against the Mili-Q ® water, which represented a blank sample. In the case of culture growth on *G. sesquipedale* hydrolysates the culture medium before addition of the inoculum represented the blank.

3.4.6 Cell dry weight

In order to determine the CDW, a sample volume of 1.2 mL was transferred to a pre-weighted dried 1.5 mL Eppendorf tube. This sample was centrifuged at 10 000 rpm for

5 minutes, and after the centrifugation the supernatant was separated. The pellet was then resuspended with 1 mL of distilled water, and after resuspension the sample was centrifuged for the second time at 10 000 rpm for 5 minutes. After the second centrifugation step the supernatant was discarded. The cleaned pellet was then dried in an oven Memmert (model 200) at 60 °C for at least 48 hours. The CDW was then calculated as the mass difference between the weight of the Eppendorf tube containing the dried sample and the empty pre-dried Eppendorf tube divided by the total volume of the sample.

3.4.7 P3HB quantification

3.4.7.1 Acid methanolysis of P3HB

To determine the concentration of P3HB produced and accumulated by *H. boliviensis*, the polymer was converted into stable and volatile hydroxycarboxylic acid methyl esters, through the acid methanolysis reaction and the esters were further analysed by gas chromatography (GC).

The reaction started by adding 1 mL of chloroform to the cell pellet in the Eppendorf after the centrifugation of culture broth. After, the chloroform addition, the cells were resuspended and then transferred to a glass tube. In order to initiate the acid methanolysis, 1 mL of "solution A" containing 97 mL methanol, 3 mL of 96% H_2SO_4 and 330 µL hexanoic acid, was added to each glass tube. These tubes were then vortexed and placed in an oven Memmert (model 200) for 5 hours at 100 °C. The samples were vortexed once during the reaction time. After 5 hours the reaction was stopped by adding 1 mL of 60 gL^{-1} Na_2CO_3 solution to each tube. Each glass tube was vortexed and subsequently centrifuged for 5 mins at 5 000 rpm in a Heraeus Labofuge 200 from Thermo Scientific. After the centrifugation 200 µL from the bottom phase (organic phase) was transferred from the glass tube to a GC vial. The vials were stored at -18 °C until analysis of GC.

3.4.7.2 Quantification of P3HB by gas chromatography

The GC vials with the organic phase were analysed by using gas chromatograph (Agilent Technologies 5890 series II) equipped with a FID detector and a 7683B injector. The oven, injector and detector temperatures were set to 60, 120 and 150 °C. The GC column used within this study was a HP-5 from Agilent J&W Scientific with a 30 metres in length and 0.32 mm of internal diameter. The data acquisition and integration were performed by the Shimadzu CBM-102 communication Bus Module and Shimadzu GC

solution software (Version 2.3).

Peak identification was achieved by using an internal standard of 3-methyl hydroxybutyrate from Sigma-Aldrich. For the quantification, a calibration curve of 3-methyl hydroxybutyrate was carried out in a concentration range from 0 to 8 gL^{-1}. The calibration curve for P3HB quantification is enclosed in **Appendix**.

3.4.8 Ammonium quantification

The concentration of ammonium ion present in the fermentation broth was determined using the phenate method, described in the manual, Q. A.,1999 (Standard Methods for the Examination of Water and Wastewater Part 4000 inorganic non-metallic constituents) [107]. This off-line method requires measurement of the optical density (OD) of samples at 640 nm in a double beam spectrophotometer (Hitachi U-2000) and, because of technical issues, also in a T70 UV/Vis Spectrometer (PG Instruments Ltd), always using 1 mL plastic cuvettes with an optical path length of 1 cm. For the OD determination, samples were diluted with deionized water to obtain an absorbance value lower than the threshold (ca. 0.5-0.6). The reference used was the mineral medium described above.

Supernatant aliquots were used to prepare samples for the quantification. The supernatant was diluted 1:2500 with deionized water to a final volume of 5 mL, followed by the addition of 200 µL of phenol solution, 200 µL of sodium nitroprusside and 500 µL of oxidizing solution. For colour to develop, a dark environment is required for 1 hour at room temperature. The samples were analysed in the next 24 h, as the colour keeps stable during this period. Phenol solution, sodium nitroprusside (0.5%w/v) and the oxidizing solution were prepared according to the reference mentioned before [107]

The calibration curve for ammonium determination was obtained for working ranges of 0.1 to 0.5 mgL^{-1}. The calibration curve for the ammonium quantification is enclosed in **Appendix**.

3.5 Preparation of Carbon-rich Hydrolysates

3.5.1 Chemical pre-treatment

The chemical pre-treatment assays of algal biomass were performed by optimizing the reaction parameters such as type of catalyst, concentration of biomass and reaction time. All chemical pre-treatments with acids were conducted in an autoclave at 121 °C, the

chemical pre-treatment using sodium hydroxide was performed at -20 °C in the freezer. An overview of the chemical agents used, and the hydrolysis conditions as well is displayed in **Table 8**.

Table 8 – Overview of the conditions of chemical pre-treatment of algal biomass

Catalyst	Catalyst Concentration % (w/v)	Time	T (°C)
Sulfuric acid	0.25; 0.5 and 0.1	15; 30 and 60 minutes	121
Phosphoric acid	0.5; 1.0 and 1.5	15; 30 and 60 minutes	
Sulfamic acid	0.5; 1.0; 1.5 and 2.0	30; 60 and 90 minutes	
Sodium hydroxide	0.25; 0.5 and 0.1	24; 42; 72 and 168 hours	-20

The algal biomass was weighted to three 50 mL glass flasks and 10 mL of acid or alkali was added in each glass flask to attain biomass concentration of 21.6; 43.2 and 86.4 gL^{-1}. These flasks were then sealed with a rubber cover and an aluminium cap and placed in the autoclave or in the freezer. After each chemical hydrolysis assay, aliquots from the three flasks were centrifuged (10 000 rpm for 5 minutes) and prepared for sugar quantification.

3.5.2 Enzymatic hydrolysis

The enzymatic hydrolysis of algal biomass by cellulolytic enzymes was carried out in three 50 mL glass flasks containing 10 mL of Mili-Q ® water or phosphate buffer 0.135 M. An amount of biomass was added to a final biomass concentration of 43 gL^{-1}. These flasks were sealed with a rubber cover and with an aluminium cap. Samples were first sterilized in an autoclave at 121 °C for 20 minutes to avoid microbial contamination during the enzymatic hydrolysis assay. The pH of the samples was then adjusted to 4.8 with NaOH 2 M/ HCl 1 M before addition of the enzymes.

Enzymes were firstly pre-diluted 10 times with deionized water or with 0.135M phosphate buffer to access better addition of originally viscous solutions of the enzymes. The enzymatic cocktail used for the enzymatic hydrolysis contained: 1.2 mg of cellulase complex (NS 22086) and 0.3 mg of β-glucosidase (NS 22118), corresponding to an enzyme

load 2.8 mg cellulase complex/ g biomass (0.04 FPU/g) and 0.7 mg β-glucosidase/ g biomass (0.022 NPGU/g). The enzymatic hydrolysis was executed in an incubator at 50 °C for 48 hours with an agitation speed of 660 rpm. Samples were taken from each flask at different times during the hydrolysis, centrifuged at 10 000 rpm for 5 minutes and subsequently prepared for the sugar quantification.

3.5.3 Combined hydrolysis

Combined hydrolysis of algal biomass was performed by chemical pre-treatment followed by enzymatic hydrolysis. After chemical pre-treatment (under the most promising conditions), the pH of samples was adjusted to 4.8 with NaOH 2M/ HCl 1M. Afterwards, the enzyme load (mg enzyme/g biomass) which was described in the previous procedure "Enzymatic Hydrolysis" was added. The enzymatic hydrolysis was carried in the incubator out for optimal period at 50 °C with an agitation speed of 660 rpm. Samples were taken when the combined hydrolysis assay was completed, centrifuged at 10 000 rpm for 5 minutes and prepared for the carbohydrate quantification. The assay was performed in triplicate.

3.5.4 Scale-up of hydrolysis

An assay to determine the feasibility of scaling-up of the hydrolysis was carried out in 50, 100 mL glass flasks and 2 L Erlenmeyer flasks. The scale-up was accomplished by increasing of a total working volume while maintaining constant the biomass concentration and a ratio of biomass/load of enzymes. In order to maintain flow characteristics, the Froude number *Fr* (**Eq. 12**) was kept constant.

$$Fr = \left(\frac{2(\pi N)^2 d}{g}\right) \quad (12)$$

Where (**Eq. 12**) *N* is a rotational speed (rpm); *d* is the characteristic length scale – corresponding to the flask diameter. If *d* increased during the scale-up, *N* was appropriately decreased to maintain *Fr* constant.

3.5.5 Storage of hydrolysates

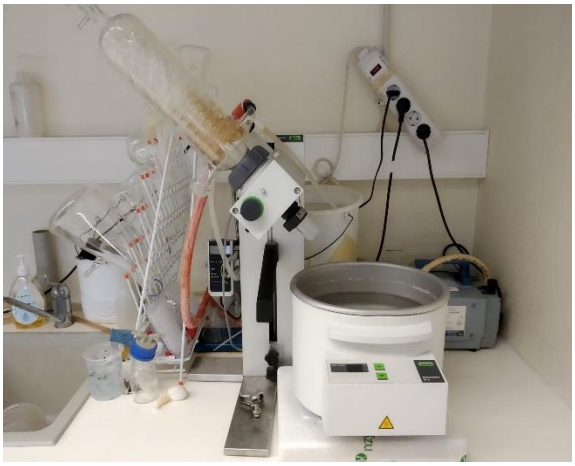
After hydrolysis the hydrolysate was sterilized in an autoclave at 121 °C, 1 bar for 20 minutes. After the sterilization the sterile hydrolysate was centrifuged in aseptic 500 mL centrifugation tubes by Thermo Scientific Sorvall RC6 ^{TM} centrifuge with the 500 mL Tube

Rotor type SLC-3000 no.:35 at 4 000 rpm, 20 °C for 45 minutes. After the first centrifugation step the supernatant was poured to a sterile 500 mL Schott flask ®. The centrifuged hydrolysate was then stored at 4 °C overnight. The next day, another centrifugation step was carried out to remove any precipitate that had formed in the hydrolysate. The second centrifugation was conducted at 4 000 rpm, 4 °C for 45 minutes in aseptic 500 ML centrifugation tubes. After the second centrifugation the hydrolysate was poured to another sterile 500 mL Schott flask ® and stored at 4 °C until further usage.

3.5.6 Concentration of hydrolysates

To produce a carbon-rich feed to be used in fed-batch cultivations the produced *G. sesquipedale* hydrolysate was concentrated. Two possible concentration techniques were carried out, namely: vacuum rotatory evaporator (BUCHI Rotavapor R-3) and Spray dryer (BUCHI Mini Spray Dryer B-290). The working conditions are described in **Table 9**.

Table 9 – The working conditions of two evaporation techniques, namely Vacuum rotatory evaporator (BUCHI Rotavapor R-3) on the left and Spray dryer (Buchi Mini Spray Dryer B-290).



Vacuum rotatory evaporator

Spray Dryer

Process parameter	Value (Unit)	Process parameter	Value (Unit)
Pressure	50 ± 5 (mbar)	T_{inlet}	150 (°C)
$T_{Water\ Bath}$	50 ± 5 (°C)	T_{out}	93 ± 2 (°C)

4 Results and Discussion

4.1 Composition Analysis of the *G. sesquipedale* Residues

The determination of the chemical composition of *G. sesquipedale* residues after agar extraction was a fundamental step towards the preparation of algal hydrolysates. The results of the composition analysis are displayed in **Table 10.**

Table 10 – The chemical composition of the *G. sesquipedale* residues.

Parameter	Value	Unit
Dry-Weight	93.90 ± 0.83	(%)
Moisture	6.10 ± 0.83	(%)
Ash	16.39 ± 1.15	(% dw.)
Carbohydrate Content	44.80 ± 1.90	(% dw.)
• Glucose • Galactose • 3,6-Anhydro-L-Galactose	37.50 ± 0.02 3.217 ± 0.001 4.086 ± 0.002	
Agar	7.30	(% dw.)
Cellulose	36.70 ± 1.72	(% dw.)
Starch	0.80 ± 0.33	(% dw.)

One of the agar constituents 3,6-anhydro-L-galactose (L-AHG) was calculated regarding the molar ratio (1:1,27) [108] between galactose and L-AHG. A possible quantification of L-AHG by HPLC is unfeasible due to an inaccessibility of the L-AHG chemical standard . The amount of cellulose in the residues (% dw.) was quantified by subtracting the glucose released in the hydrolysis of starch (**Eq.11** in the section **3.4.4**) from the total amount of glucose determined during the acid hydrolysis for the carbohydrate determination (section **3.4.2**). The amount of starch was calculated according to **Eq. 11** in section (**3.4.4**). By subtracting the total carbohydrate content by the cellulose and starch content, the agar amount was determined.

The results showed that *G. sesquipedale* residues still contain a nonnegligible content of carbohydrates (44.80% dw.) originated from the polysaccharidic fraction. The

carbohydrate present in larger amounts was cellulose (37.50% dw.), followed by agar (7.30 % dw.). The presence of agar in residues indicates that the extraction process was not complete ant thus the waste biomass still contains a significant portion of this polysaccharide.

4.2 Preparation of Carbon-Rich Hydrolysates

In order to obtain high yields of released carbohydrates with few or no degradation products, an optimization of the hydrolysis process consisted of a chemical pre-treatment followed by enzymatic hydrolysis of *G. sesquipedale* residues was carried out.

4.2.1 Chemical pre-treatment of algal residues

Acid-catalysed hydrothermal hydrolysis of *Gelidium* residues was carried out using the conditions stated in **3.5.1**. The results were already presented in a previous report [109] and are not depicted in this thesis. The three most promising conditions are summarized in (**Figure 9**).

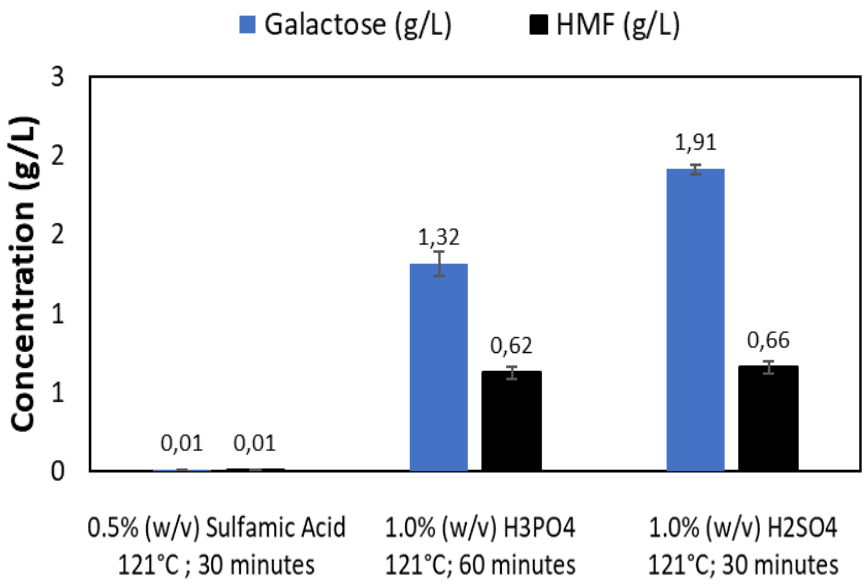


Figure 9 – Chemical pre-treatment of *G. sesquipedale* residues. The concentration of biomass used for this assay was 43.2 gL^{-1}.

These are the conditions that have yielded a higher sugar concentration and the least amount of inhibitors. During the chemical pre-treatment, merely galactose was released, showing that the mild acid conditions used are not strong enough to hydrolyse

cellulose into glucose. Nonetheless, even in mild conditions HMF is released, and this is caused by the AHG fragility even in the mild acidic environment [110]. Besides HMF, no other degradation products originated from the dehydration of carbohydrates, was detected in each condition. The hydrolysis with sulfuric and phosphoric acid was able to release 64.0% and 44.2% of the total galactose, which is given by carbohydrate determination (section 3.4.2). However, the dehydration of the agar monomers to HMF was significantly higher using these acid catalysts comparing to sulfamic acid.

Although the condition with sulfamic acid was unable to release galactose from agar it was further chosen in the pre-treatment step, due to the low amount of HMF produced. This is a reasonable decision for processing this type of biomass because the amount of agar in the residues and thus of released galactose is quite low and thus it might be neglected for further valorisation to P3HB.

4.2.1.1 Inhibition effect of HMF on H. boliviensis growth

Regarding the results from the chemical pre-treatment, it was vital for further studies on *H. boliviensis* growth on algal hydrolysates to understand the magnitude of the HMF inhibitory effect on microbial growth. Therefore, a shake flask assay of *H. boliviensis* growth on P3HB production medium (section *3.3.2.2*) with different concentrations of HMF and 20 gL^{-1} of glucose.

Results obtained from the HMF inhibitory study (**Figure 10**) suggest that above HMF 0.1 gL^{-1} the inhibitory effect on *H. boliviensis* growth is significant. At 0.1 gL^{-1} HMF the relative growth rate decreased approximately 92.8 % of the maximum attained in the control, i.e. same cultivation medium supplemented with 20 gL^{-1} of glucose and no HMF. Therefore, the chemical pre-treatment condition with 0.5% (w/v) sulfamic acid at 121 °C for 30 minutes in an autoclave was chosen for the further production of hydrolysates from *G. sesquipedale* residues, as under this condition not more than 0.01 gL^{-1} of HMF was released.

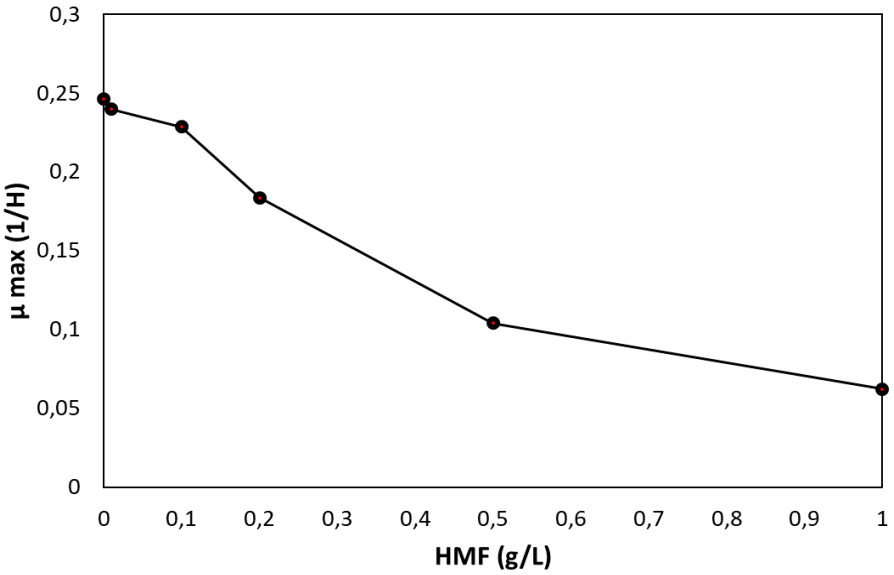


Figure 10 – The inhibitory effect of HMF on *H. boliviensis* growth. The y-axis is represented by the maximum specific growth rate $\mu_{max}(1/H)$.

4.2.2 Enzymatic hydrolysis of algal residues

To extend the yield of released sugars, the enzymatic hydrolysis of algal residues was carried out (**Figure 11**). A mixture of cellulolytic enzymes were used to hydrolyse cellulose to its monomeric form, i.e. glucose. The conditions used in this study were described in section (**3.5.2**).

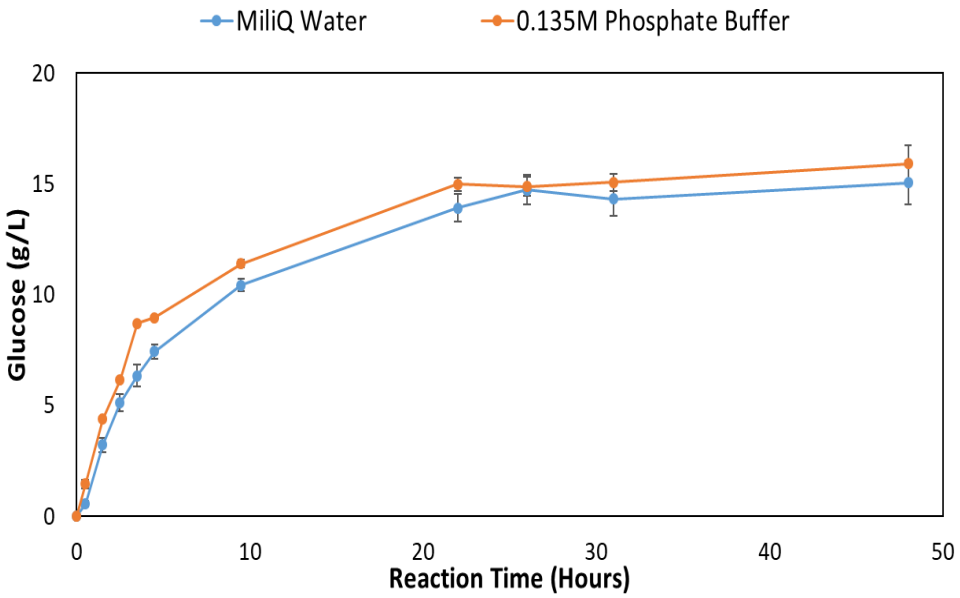


Figure 11 – Enzymatic hydrolysis of *G. sesquipedale* residues, the concentration of biomass used for this assay was 43.2 gL^{-1}.

After 30 hours of enzymatic hydrolysis the concentration of glucose released was not increasing anymore. This reaction time was thus chosen in further assays. Since, no significant improvement of released glucose was observed using the 0.135M phosphate buffer, this condition was left out for further assays.

4.2.3 Combined hydrolysis of algal residues

Knowing the best conditions for chemical pre-treatment and enzymatic hydrolysis, the so-called "combined hydrolysis" of biomass was performed. To increase the amount of sugars released during the combined hydrolysis, an assay with different biomass concentrations was explored (**Figure 12**). Lastly, for the production of hydrolysates in large quantities, a scale-up of this process was conducted (**Figure 13**).

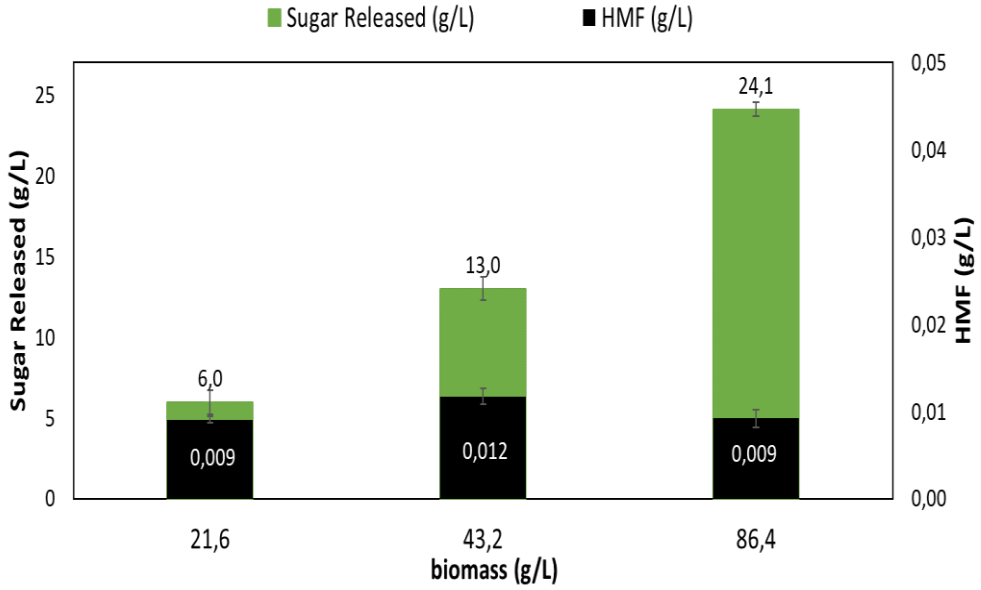


Figure 12 – Combined hydrolysis of *G. sesquipedale* (0.5% (w/v) sulfamic acid with cellulolytic hydrolysis) using different biomass concentrations.

From **Figure 12** an increase of the total amount of sugars released with higher biomass concentration is observed. The hydrolysis of 86.4 gL^{-1} biomass yields a hydrolysate with 24.1 gL^{-1} of total sugars and concentration of HMF below 0.01 gL^{-1}. Two monosaccharides, glucose and galactose, were released during the combined hydrolysis of *G. sesquipedale* residues using 0.5% (w/v) sulfamic acid. However, glucose was predominantly released in all conditions in **Figure 12**. A small concentration of 0.3 gL^{-1} of

galactose was released in the hydrolysis of 21.6 gL^{-1} biomass concentration. This could be explained by the higher volume/biomass ratio and thus higher accessibility of the acid to the biomass cells, which may cause a partial hydrolysis of agar, eventually leading to the release of galactose.

The results from the scale-up of the combined hydrolysis assay (**Figure 13**) show that it is feasible to increase the working volume from 10 to 700 mL in tested conditions and attain similar results. To maintain a constant *Fr* number (section **3.5.4**) and thus similar hydrolysis yields, the rotation speed *N* (rpm) decreased as the processed volume was increased (**Eq. 13**). In **Table 11** changes in fluid dynamics are showed in order to obtain a constant *Fr* through the scale-up assay.

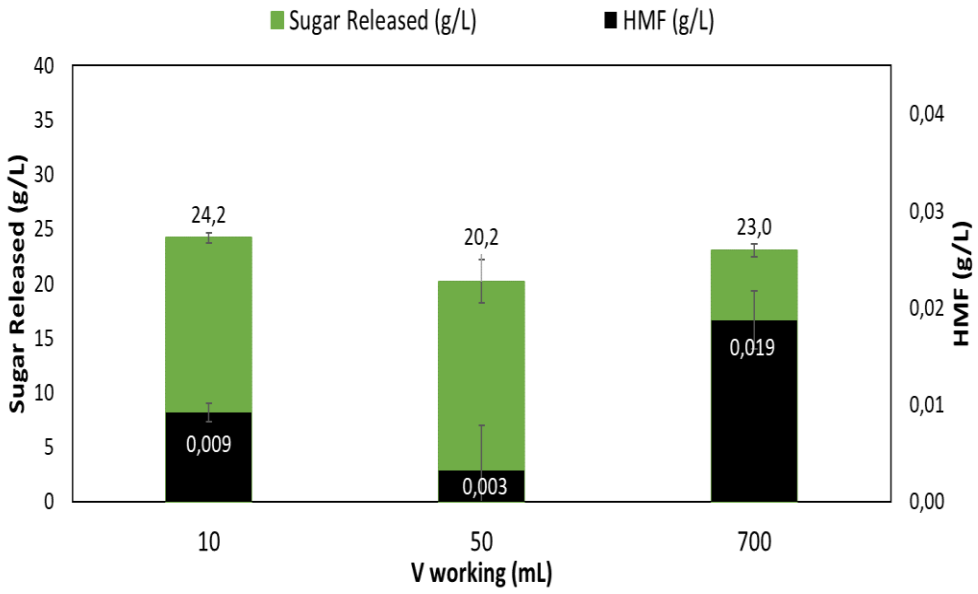


Figure 13 – The scale up of combined hydrolysis of *G. sesquipedale* residues by 0.5% (w/v) sulfamic acid treatment and subsequent enzymatic hydrolysis, with 86.4 $gL^{-1}$of biomass concentration used for this assay.

$$N_2(rpm) = N_1 \sqrt{\left(\frac{d_1}{d_2}\right)} \qquad (13)$$

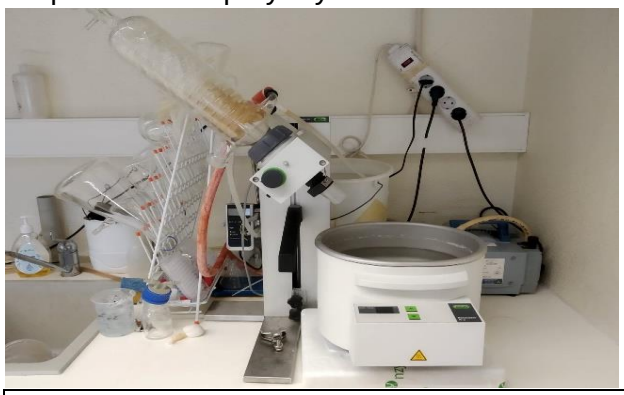
Table 11 – Changes in the fluid dynamics to access constant *Fr*.

V working (mL)	d – Flask diameter (cm)	N_{SP}- Rotational speed (rpm) - Setpoint	N_{AC}- Rotational speed (rpm) - Actual
10	4.4	660	660
50	7.8	495	490
700	15	357	150

4.2.4 Concentration of hydrolysates from algal residues

To prepare a concentrate from the algal hydrolysates, two evaporation techniques were explored, namely the vacuum rotatory evaporator (RE) and the spray dryer (SD). The results obtained by both methods are mentioned in **Table 12.**

Table 12 – Comparison in two evaporation techniques, namely vacuum rotatory evaporator and spray dryer.



Vacuum rotatory evaporator		**Spray-dryer**	
Process Conditions			
Process Parameter	**Value (Unit)**	**Process parameter**	**Value (Unit)**
Pressure	50 ± 5 (mbar)	T_{inlet}	150 (°C)
$T_{Water\ Bath}$	50 ± 5 (°C)	T_{out}	93 ± 2 (°C)
Results			
Parameter	**Value (Unit)**	**Parameter**	**Value (Unit)**
$V_{initial}$	45 mL	$V_{initial}$	420 mL
Concentration Factor; C_F	3.461	Yield of Product; Y_P	62.56 %
$c\ glucose_{initial}$	26.3 gL^{-1}	$c\ glucose_{initial}$	24.8 gL^{-1}
Duration of Process	3 h 50 min	Duration of Process	2 h 54 min
Final State of Concentrate	Viscous liquid	Final State of Concentrate	Dry powder
Further Degradation	Mild	Further Degradation	Harsher

Concerning **Table 12**, both evaporation techniques managed the task of concentration of the algal hydrolysate. However, the concentration of glucose-containing hydrolysates by evaporation poses the risk of further degradation reactions, resulting in an elevated concentration of the inhibitory products in concentrates. Evaporation by RE seems to have a milder impact on the formation of degradation products in comparison to SD (**Table 13**).The concentration of HMF was even lower than the predicted one, regarding the concentration factor C_F. The concentrate obtained by RE contained other dehydration products that were identified (not quantified) as acetic and formic acid. This suggests that the evaporation by RE causes further degradation of HMF to acetic acid and eventually to formic acid. In the case of SD, after resuspension of the dry powder in distilled water, the concentration of HMF was almost three times higher than the predicted value.

Table 13 – Comparison between two evaporation techniques on the further formation of inhibitory products.

Vacuum rotatory evaporator		**Spray-dryer**	
$V_{initial}$	45 mL	$V_{initial}$	420 mL
$c_{initial}$ HMF	0.012 gL^{-1}	$c_{initial}$ HMF	0.03 gL^{-1}
V_{final}	13 mL	$V_{final} - resuspended$	40 mL
c_{final} HMF	0.025 gL^{-1}	c_{final} HMF	0.90 gL^{-1}

There are a few limitations to reach higher concentrations or higher concentration factors C_F . Above, C_F 8 a deposition of poorly soluble precipitates was observed in the evaporation by *RE*, therefore the loss of products is inevitable. Another disadvantage of *RE* is a very high energy demand, which is significantly higher than in the case of *SD*. On the other hand, the evaporation of algal hydrolysate by *SD* is faster, with lower energy input, and it lead to a dry powder, which has a substantial advantage of long-shelf life without absence of humidity and reduced costs for storage and potential transportation. However, due to the high adhesiveness of glucose, a small fraction of solids remains attached to parts of the equipment during the drying process resulting in a considerably low product yield of 62.56 % Y_P [92].

4.3 Shake Flasks Assays

Shake flasks experiments were carried out aiming to understand the growth and P3HB production by *H. boliviensis G. sesquipedale* residues hydrolysate.

The algal hydrolysates tested as feedstock were prepared using the combined hydrolysis with 0.5% (w/v) sulfamic acid followed by enzymatic hydrolysis with the enzymatic cocktail described before. The sugar composition varied slightly from 24 to 26 gL^{-1} of glucose and HMF concentration was always below 0.02 gL^{-1}.

4.3.1 Growth of *H. boliviensis* on glucose

Since, merely glucose is present in the produced hydrolysates, the growth rate of *H.* boliviensis based solely on glucose was determined (**Figure 14**).

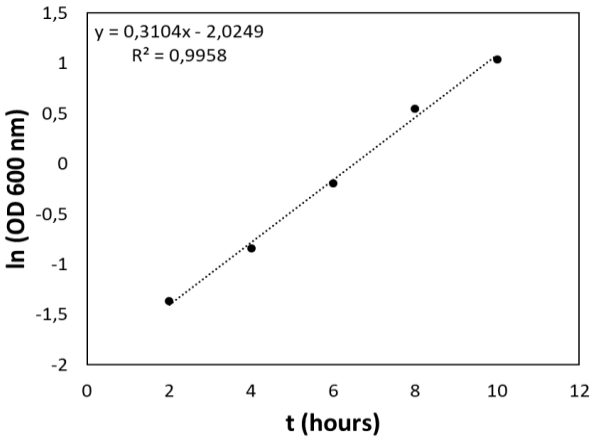


Figure 14 – Determination of *H. boliviensis* growth rate on production medium containing 20 gL^{-1} *MSG* and 1 gL^{-1} NH_4Cl supplemented with 20 gL^{-1} glucose.

Regarding the results of *H. boliviensis* growth on polymer production medium supplemented with 20 g/L (**Figure 14**), the maximum specific growth rate μ_{max} $0.31\ h^{-1}$ was determined as the slope of the equation ($y = 0.3104x - 2{,}0249$) derived from the linear regression of the function $ln\ (O.D._{600nm}) = f(t)$.

4.3.2 Production of P3HB by *H. boliviensis*

To access the growth and P3HB production of *H. boliviensis* on the produced algal hydrolysate a 20-fold concentrated production medium "A" was used. The composition of this medium was already mentioned in section (*3.3.3.2*) and is repeated here in **Table 14** to ease the comparison with other medium compositions tested. The solutions of $NaCl$,

magnesium salt and glucose were added separately (*3.3.3.*2). The results of the *H. boliviensis* growth and P3HB production on algal hydrolysates and the corresponding simulate assay with the same glucose concentrations are reported in **Figure 15** and **16**.

Table 14 -Composition of polymer production medium "A".

Component	K_2HPO_4	$FeSO_4 \times 7H_2O$	NH_4Cl	*Tris*	*MSG*
Concentration (g/L)	2.2	0.005	1.0	15	20

From **Figures 15** and **16** and the results summarized in **Table 15**, it is observed that a similar CDW of 9 g/L is attained in both conditions. Concerning polymer production, the highest accumulated polymer content in *H. boliviensis* cells grown on algal hydrolysate was only 21% (w/w), while this value attained 34% (w/w) on glucose. Such a low accumulation could be explained by an excess of nitrogen in medium "A", and this nitrogen content might be even higher in the algal hydrolysates. Hence, to optimize the P3HB accumulation in *H. boliviensis* it was important to study the influence of N content on *H. boliviensis* growth and P3HB accumulation.

Table 15 - Cultivation parameters from the *H. boliviensis* growth and P3HB production on production medium "A" supplemented with 18 gL^{-1} glucose or algal hydrolysate with the same glucose concentration.

Cultivation Parameter (Unit)	$Y_{P/S}$ $(g\ P/\ g\ S_{cons.})$	$Prod_{max\ P}$ $(g\ L^{-1}h^{-1})$	CDW (gL^{-1})	P3HB cont. (%)	$P3HB_{conc.}$ (gL^{-1})
Algal Hydrolysate	0.124	0.028	8.63	21.3	2.06
Glucose	0.163	0.042	9.00	33.9	3.03

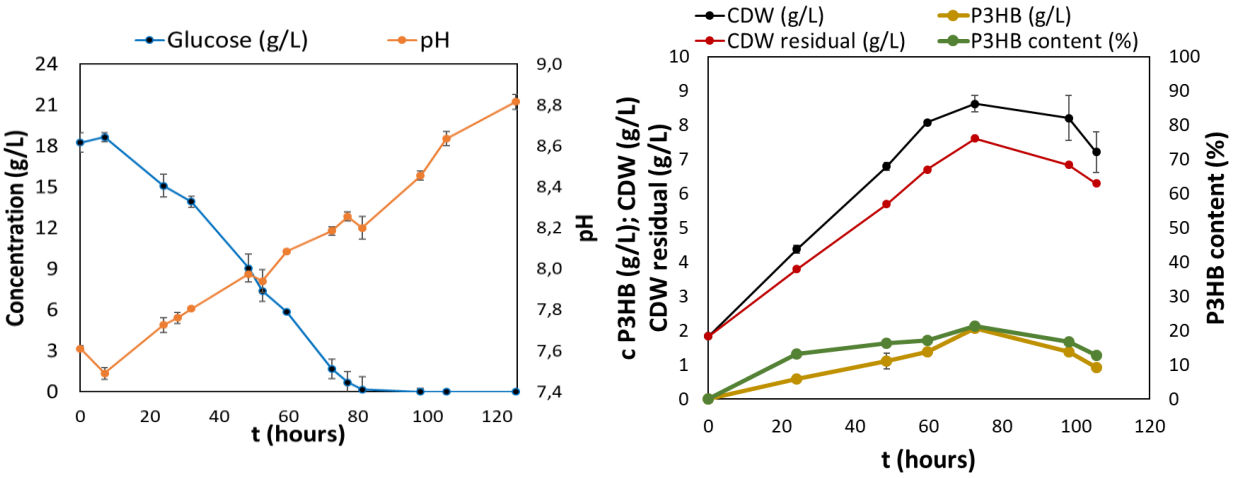


Figure 15 – *H. boliviensis* growth and P3HB accumulation on the production medium "A" with the algal hydrolysate as feedstock.

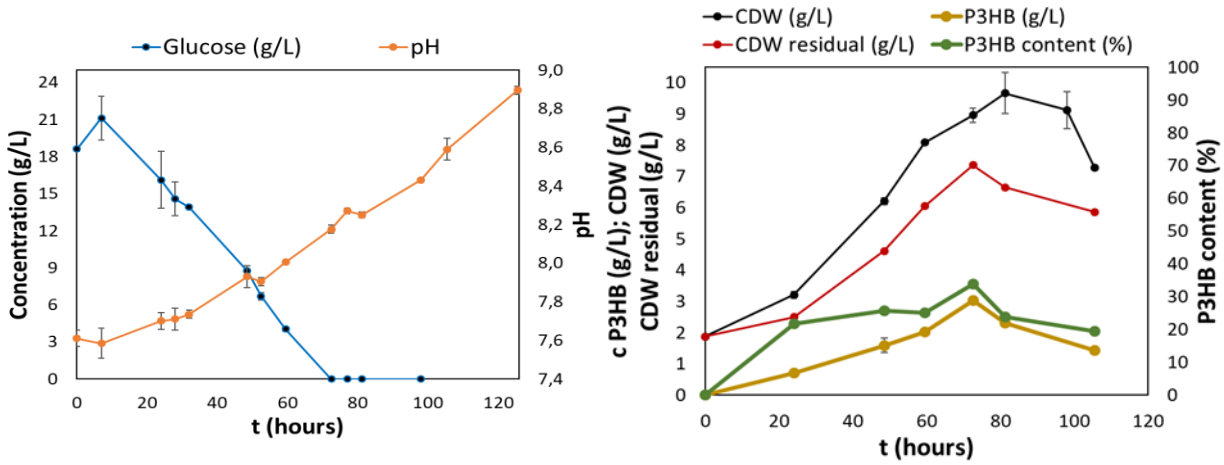


Figure 16 – *H. boliviensis* growth and P3HB accumulation on the production medium "A" supplemented with 18 gL^{-1} glucose as carbon source.

4.3.2.1 Optimization of production medium composition

In the following shake flask assays changes in the composition of medium "A" were made towards a higher polymer accumulation. It was expected that a reduction of the nitrogen content in the production medium, may improve the P3HB accumulation.

Aiming at this, different *MSG* and NH_4Cl and concentrations were tested (**Table 16**) on the algal hydrolysates.

The solutions of $NaCl$, magnesium salt and glucose were added separately (*3.3.2.2*). The final results of this assay are displayed in **Figure 17**. And the cultivation parameters are reported in **Table 17**.

Table 16 – Variable nitrogen content in the newly constructed P3HB production media.

Component (gL^{-1})	K_2HPO_4	$FeSO_4 \times 7H_2O$	NH_4Cl	*Tris*	***MSG***
Medium "B"	2.2	0.005	**1**	15	**0**
Medium "C"	2.2	0.005	**1**	15	**5**
Medium "D"	2.2	0.005	**1**	15	**10**
Medium "E"	2.2	0.005	**0**	15	**10**

Concerning, the cultivation parameters in **Table 17** an increase in the yield, productivity and P3HB content was achieved using production media "C,D and E" with comparison to the production medium "A". The combination of both CDW and P3HB content was the key feature to choose the most suitable production medium. Both cultivation media "D" and "E" yielded high CDW and polymer concentrations, however medium "E" was chosen for further assays due to the higher P3HB over the production medium "D".

Table 17 - Cultivation Parameters from H. boliviensis growth and P3HB accumulation on production media with variations of nitrogen source, the algal hydrolysates were used as carbon-rich feedstock

Cultivation Parameter (Unit)	$Y_{P/S}$ $(g\ P/\ g\ S_{cons.})$	$Prod_{max\ P}$ $(g\ L^{-1}h^{-1})$	CDW (gL^{-1})	$P3HB\ cont.$ (%)	$P3HB_{conc.}$ (gL^{-1})
Medium "B"	0.113	0.047	4.7	56.7	2.24
Medium "C"	0.149	0.057	7.1	52.2	3.02
Medium "D"	0.158	0.056	8.7	37.7	3.28
Medium "E"	**0.155**	**0.056**	**8.3**	**39.2**	**3.27**

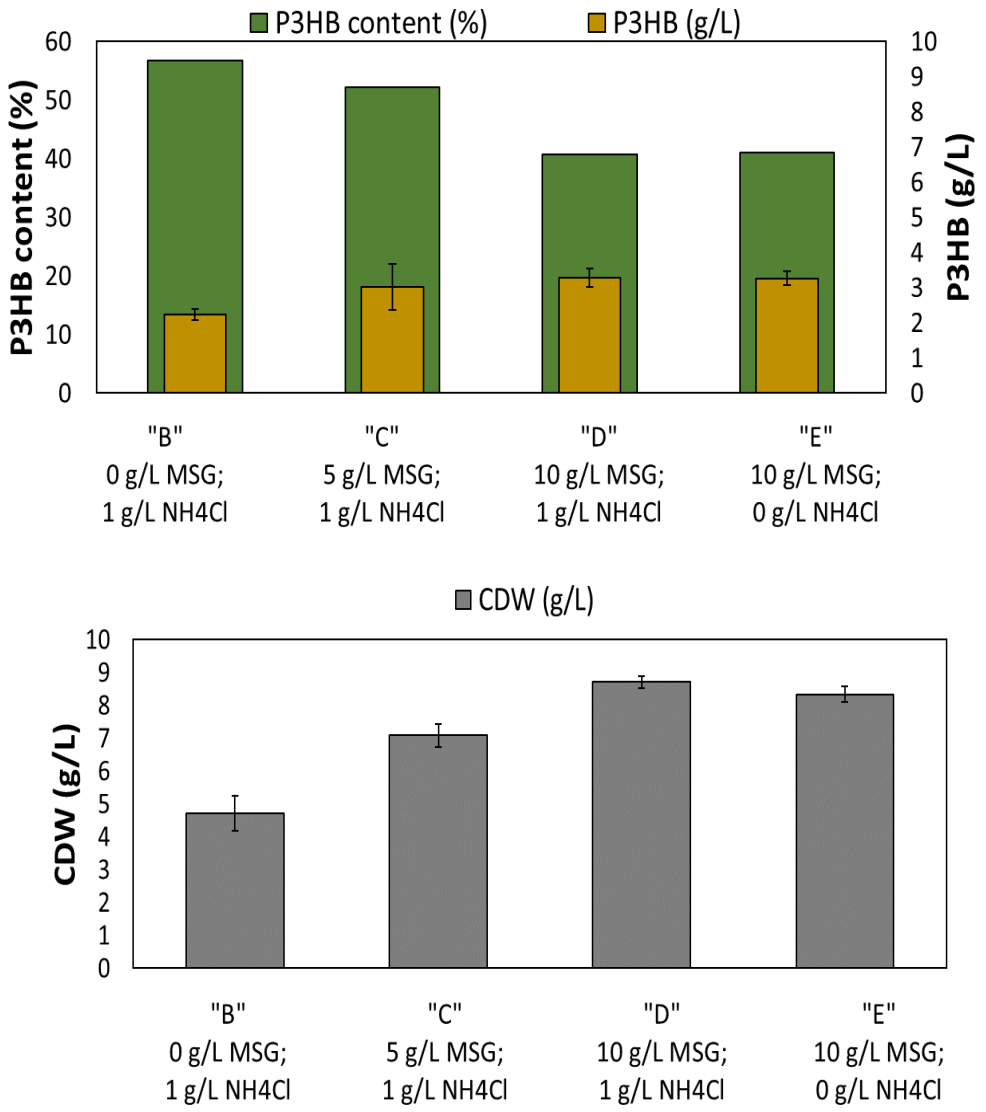


Figure 17 - *H. boliviensis* growth and P3HB production on production media with various nitrogen content.

To compare these results on algal hydrolysate using production medium “E” a simulation with medium “E” supplemented with glucose was conducted. Results are reported in **Figure 18**. (Results of *H. boliviensis* cultivation on production media “B”, “C” and “D” are enclosed in **Annexes**)

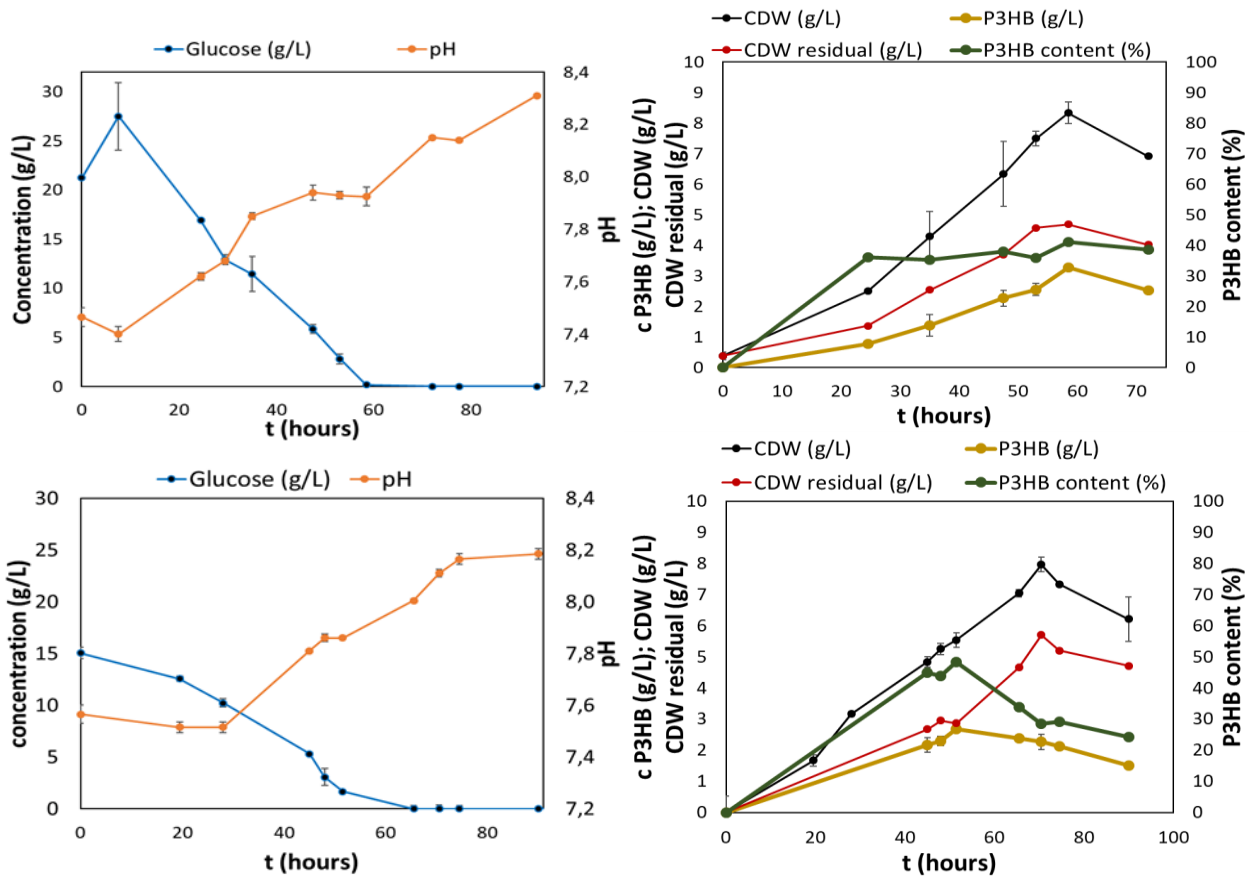


Figure 18 – *H. boliviensis* growth and P3HB accumulation on the algal hydrolysate (top) and on glucose as a carbon source (bottom) and with production medium "E".

Table 18 – Comparison between algal hydrolysate and glucose as a carbon source in cultivation parameters of *H. boliviensis* growth and P3HB production using production medium "E".

Parameter (Unit)	$Y_{P/S}$ (g P/ g $S_{cons.}$)	$Prod_{max\,P}$ ($g\,L^{-1}h^{-1}$)	CDW (gL^{-1})	P3HB cont. (%)	$P3HB_{conc.}$ (gL^{-1})
Algal hydrolysate	0.155	0.056	8.3	39.2	3.27
Glucose	0.199	0.052	5.5	48.4	2.68

Comparing the results (**Table 18**) obtained from *H. boliviensis* growth on algal hydrolysate and on glucose simulate, it can be concluded that an increase of the polymer accumulation, product yield and productivity were achieved in both cases on production

medium “E”. Nevertheless, a concentration of P3HB was lower in the case of the glucose simulate than in the study on algal hydrolysate, an explanation may be addressed to a lower initial concentration of glucose in shake flask, as in the simulate study the glucose concentration was 15 gL^{-1}, whereas in the assay with algal hydrolysate it was 21 gL^{-1}.

4.3.2.2 H. boliviensis growth on algal concentrates

To attain maximum polymer yields and productivities, bioreactor assays run in fed-batch should be carried out using concentrated *G. sesquipedale* hydrolysates. Two concentration techniques, namely vacuum rotatory evaporation (RE) and spray drying (SD) were carried out and the produced hydrolysates teste on the growth and P3HB production of *H. boliviensis*. The composition of both concentrates and the concentrations of both glucose and HMF in shake flasks are reported in **Table 19**. The production medium “E”. was used within this study case. The hydrolysate in the form of powder (after spray drying) was dissolved in demineralised water to attain a concentration of glucose similar to the glucose concentration in the hydrolysate by vacuum evaporation. This was done to conclude about the presence of inhibitors during the concentration step. Although the glucose concentration in both hydrolysates was not the same, it can be seen that the HMF concentration in the SP hydrolysate is lower.

Table 19 – Composition of the concentrated algal hydrolysate (*conc.*) and an initial concentration of glucose and HMF in the shake flask (SF) assay of *H. boliviensis* cultivation on algal concentrates.

Parameter (Unit)	$c_{gluc.conc}\ (gL^{-1})$	$c_{\ HMF\ con.}\ (gL^{-1})$	$c_{gluc.SF}\ (gL^{-1})$	$c_{HMF.SF}\ (gL^{-1})$
RE Concentrate	181.8	1.347	17.86	0.135
SD Concentrate	153.4	0.903	17.31	0.105

Referring the results of HMF inhibitory effect on *H. boliviensis* (section *4.2.1.1*) the initial concentration of HMF in shake flasks (**Table 19**) for both conditions did not surpass 0.2 gL^{-1} concentration of HMF, therefore a significant limitation of the *H. boliviensis* growth was not expected. The result of *H. boliviensis* growth and P3HB production are shown in **Figure 19** and **20.**

Extracted data of the cultivation parameters of this particular study are reported in **Table 20**.

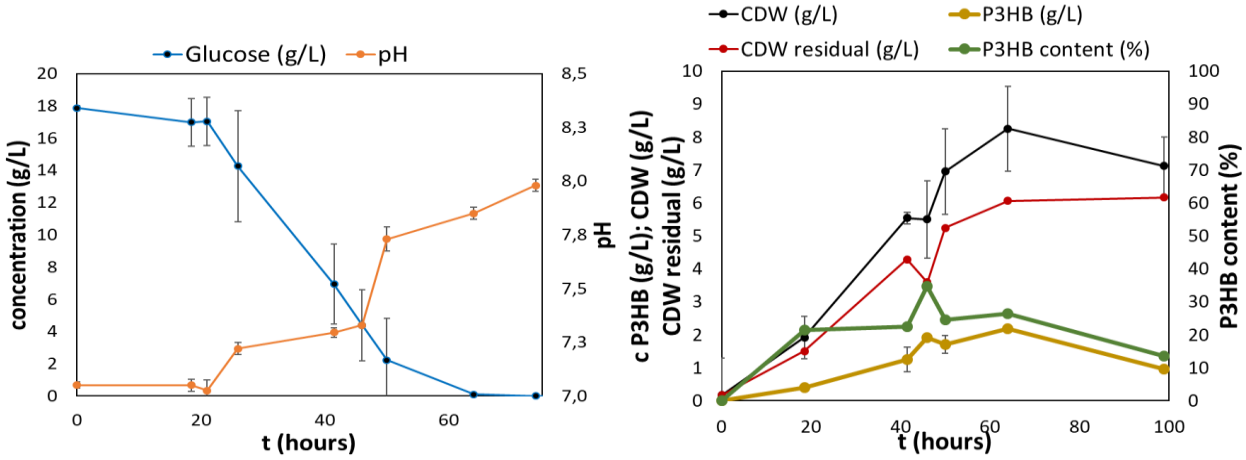


Figure 19 – *H. boliviensis* growth and P3HB production on the algal concentrate obtained by RE, using the production medium "E".

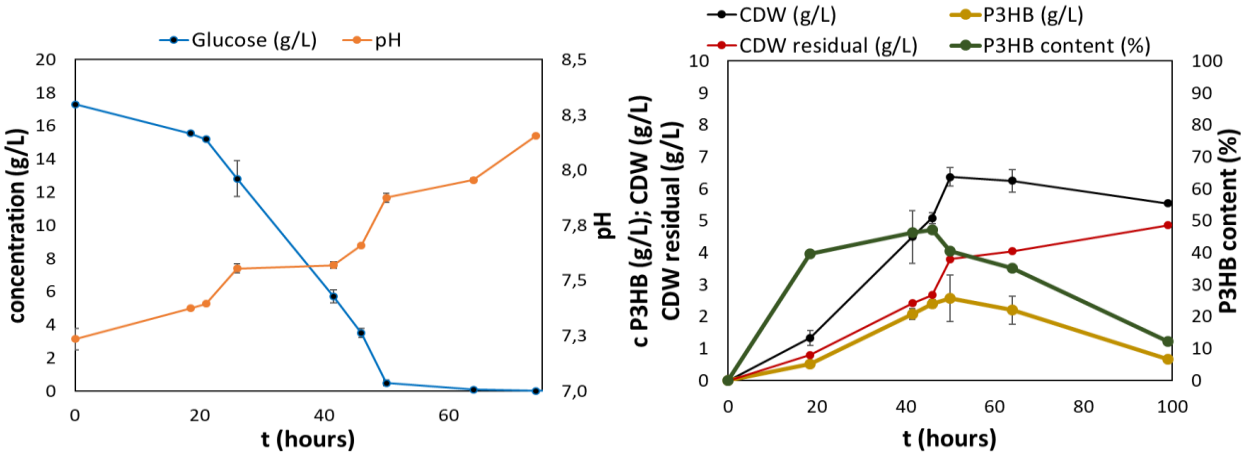


Figure 20 - *H. boliviensis* growth and P3HB production on the algal concentrate obtained by SD, using the production medium "E".

Table 20 – Cultivation parameters from shake flask assay of *H. boliviensis* growth and P3HB production on algal concentrates.

Parameter (Unit)	$Y_{P/S}$ $(g\ P/\ g\ S_{cons.})$	$Prod_{max\ P}$ $(g\ L^{-1}h^{-1})$	CDW (gL^{-1})	P3HB cont. (%)	$P3HB_{conc.}$ (gL^{-1})
RE Concentrate	0.123	0.034	8.3	26.5	2.19
SD Concentrate	0.153	0.051	6.4	40.4	2.58

Observing the results from *H. boliviensis* growth on algal concentrates (**Table 20**), a considerable difference between CDW in both conditions is evident , the highest CDW acquired was 8.7 and 6.4 gL^{-1} in the case of *RE* and *SD* concentrate, respectively. This disproportion affected the final $\mathrm{P3HB\ cont.}\,(\%)$ as in the condition with *RE* concentrate the highest $\mathrm{P3HB\ cont.}$ reached 26.5 %, while in the scenario with *SD* concentrate that value rose up to 40.4 %. Also, the highest $P3HB_{conc.}$ was attained with the *SD* concentrate, 2.58 gL^{-1} compared to 2.19 gL^{-1} with the *RE* concentrate.

Despite the limited microbial growth in the assay with *SD* concentrate, the production yield and productivity, were higher using this concentrate. *SD* proved thus to be a more useful concentration technique over *RE* , when preparing a concentrated algal hydrolysate for the cultivation of *H. boliviensis*.

However, the adequacy of such glucose-containing hydrolysate to evaporation should be studied more in depth as more degradation products may be formed during the concentration step.

4.4 Fed-batch Assays

To reach higher yields and P3HB productivities by *H. boliviensis, c*ontrolled cultivation conditions such as $DO\ (\%)$*;* pH and supply of substrate were accessed in 2L stirred-tank (STR) bioreactors operating in a fed-batch mode. To attain a high P3HB production upon nutrient limitation, two different strategies were attempted: i) polymer production triggered by P limitation and ii) production triggered by a dual limitation of N and oxygen (O_2). These strategies were first tested with glucose as carbon source and the best strategy applied using as feed the algal concentrate.

4.4.1 P3HB production triggered by P limitation

Based on the assays of Quillaguamán et al. (2008), the influence of P (K_2HPO_4) and N (NH_4Cl) limitation was examined on cell growth and P3HB production by *H. boliviensis* in a STR fed-batch cultivation. The authors achieved a CDW of 43.7 gL^{-1}, 81 % P3HB cont; 35.4 gL^{-1} $P3HB_{conc.}$ and 1.10 $Prod_{Vol\ P}$ ($g\ L^{-1}h^{-1}$), by nitrogen limitation with step-wise addition of two pulses of *MSG* during the batch phase (at 6 and 9 hours) [10]. The composition of the production medium and feeding solution(s) for the fed-batch assay is mentioned in **Table 21**.

Table 21 – The composition of cultivation media for batch phase and feeding solutions for fed-batch phase. $NaCl$, magnesium salt and glucose were prepared and added separately.

Component	Batch	Fed-Batch
K_2HPO_4	5 gL^{-1}	-
$FeSO_4 \times 7H_2O$	0.005 gL^{-1}	-
$NaCl$	45 gL^{-1}	-
$MgSO_4 \times 7H_2O$	50 mL/L of 100 gL^{-1}	-
MSG	10 gL^{-1}	200 mL of 250 gL^{-1}
Glucose	25 gL^{-1}	1L of 500 gL^{-1}

The results of fed-batch cultivation of *H. boliviensis* under P limitation are shown in **Figure 21** and **22** and the cultivation parameters from this study are displayed in **Table 22.** Compared to the results of Quillaguamán et al. (2008), much less attractive results of P3HB production were achieved. The exhaustion of phosphate could not be observed. This is due to a limitation of the HPLC technique used to monitor phosphate concentration. After a certain cultivation time due to an overlap of the phosphate peak with another compound in HPLC chromatogram, the phosphate concentration cannot be determined correctly and thus

phosphate exhaustion cannot be proven. Still, a decline of P3HB concentration (gL^{-1}) could be observed approximately after 70 hours of cultivation. .

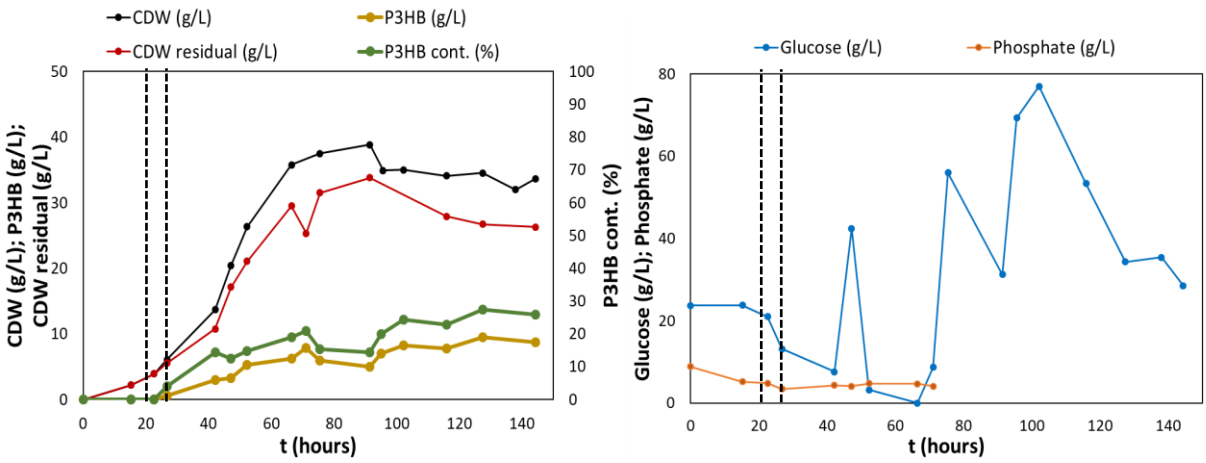


Figure 21 – The fed-batch cultivation of *H. boliviensis* growth and P3HB production triggered by P limitation. The DO (%) was kept at 20% with 2.0 $L\ min^{-1}$ of gas flow. Dotted lines represents the 1st (20 h.) and 2nd (27 h.) addition of MSG to the bioreactor.

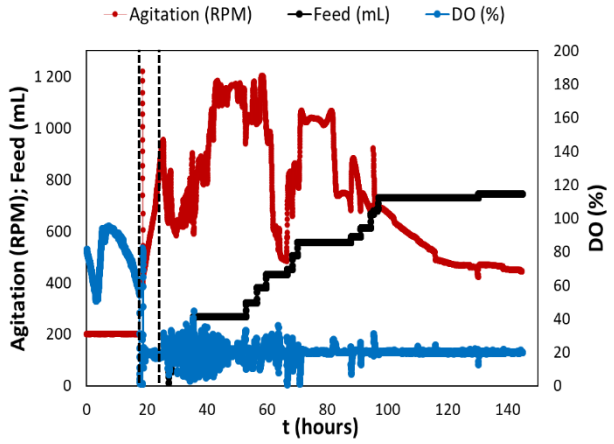


Figure 22 – An acquisition plot of the fed-batch cultivation of *H. boliviensis* and P3HB production triggered by P limitation. Dotted lines represents the 1st (20 h.) and 2nd (27 h.) addition of MSG bioreactor.

Table 22 – Cultivation parameters from the fed-batch production of P3HB by *H. boliviensis* triggered P limitation. The CDW is appropriate to the highest $\mathrm{P3HB\ cont.}$

Parameter (Unit)	$Y_{P/S}$ $(g\ P/\ g\ S_{cons.})$	$Prod_{max\ P}$ $(g\ L^{-1}h^{-1})$	CDW (gL^{-1})	$P3HB\ cont.$ (%)	$P3HB_{conc.}$ (gL^{-1})
This study	0.186	0.074	34.5	27.5	9.47
Quillaguamán et al. (2008)	*N.R.*	1.100 **	43.7	81.0	35.4

* *N.R.*: not reported

** Reported as a $Prod_{volumetric\ P}(g\ L^{-1}h^{-1})$

4.4.2 P3HB production triggered by N and O_2 limitation

Garcia-Torreiro et al. (2016) achieved the highest P3HB accumulation 73% (w/w) using *H. boliviensis* fed-batch cultivations under a dual limitation by nitrogen and oxygen [99]. Using this approach, a single limitation by nitrogen and a dual limitation by nitrogen and oxygen were tested (**Figure 23** and **24**) in the fed-batch cultivation of *H. boliviensis*. The composition of the production medium and feeding solution(s) are mentioned in **Table 23.** During the batch phase the pH was controlled by $10\ \%\ NH_4OH$ and $1M\ HCl$, however, to initiate the N limitation, the NH_4OH base was substituted by $2.5M\ NaOH$.

Table 23 – Composition of the cultivation media and feeding solutions for productions assay using N and N+O_2 limitation. $NaCl$, magnesium salt and glucose were prepared and added separately.

Component	**Batch**	**Fed-Batch**
K_2HPO_4	15 gL^{-1}	-
$FeSO_4 \times 7H_2O$	0.005 gL^{-1}	-
$NaCl$	45 gL^{-1}	-
$MgSO_4 \times 7H_2O$	50 mL/L of 100 gL^{-1}	-
MSG	10 gL^{-1}	-
Glucose	25 gL^{-1}	1L of 500 gL^{-1}

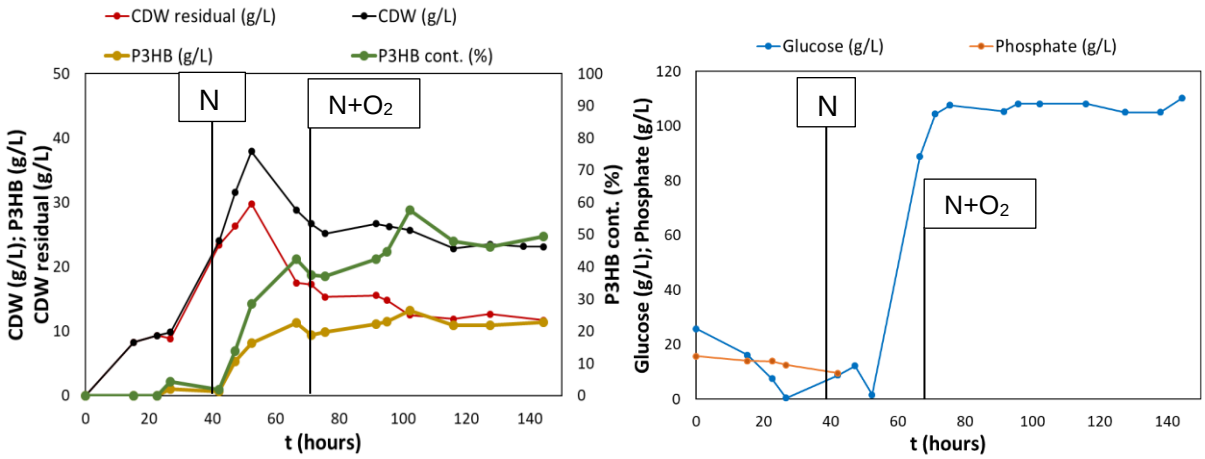


Figure 23 – The fed-batch of *H boliviensis* growth and P3HB production triggered by single N limitation (40 h.) and dual limitation by N and O_2 limitation (72 h.). An DO (%) setpoint was put at 20% with 2.0 $L\,min^{-1}$ of gas flow. After 72 h. the DO setpoint was decreased to 2% with 0.8 $L\,min^{-1}$ of gas flow.

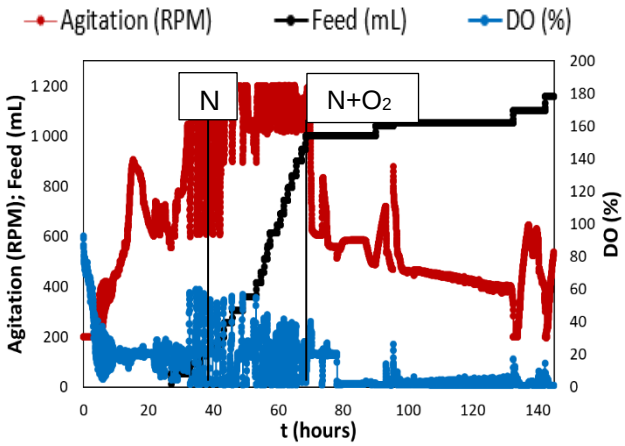


Figure 24 - The acquisition plot of fed-batch cultivation of *H. boliviensis* and P3HB production triggered by single N limitation (40 h.) and by dual N and O_2 limitation. The straight lines represents the start of single N-limitation (40 h.) and the dual N and O2 limitation (72 h.).

Table 24 – Cultivation parameters from the fed-batch production of P3HB by *H. boliviensis* triggered by single N limitation and by dual N and O_2 limitation. The CDW is appropriate to the highest $\mathrm{P3HB\ cont.}$

Parameter (Unit)	$Y_{P/S}$ $(g\ P/\ g\ S_{cons.})$	$Prod_{max\ P}$ $(g\ L^{-1}h^{-1})$	CDW (gL^{-1})	$P3HB\ cont.$ (%)	$P3HB_{conc.}$ (gL^{-1})
This study	0.214	0.129	25.7	57.6	13.15
Garcia-Torreiro (2016)	1.200*	1.320	48.0	73.0	35.0

* $Y_{P/S}$ unit reported as $[Cmol \cdot Cmol^{-1}]$

A first look on results (**Table 24**) obtained from the fed-batch cultivation of *H. boliviensis* under dual limitation of N and O_2 suggests that a significant improvement in the $\mathrm{P3HB\ cont.}$ (57.6 %) was achieved regarding the previous study with P limitation (27.5 %) assay. Despite, an increase of the P3HB production, promising results obtained by Garcia-Torreiro (2016) were not attained. The highest P3HB concentration of 13 gL^{-1} was achieved after 102 h. of cultivation, whereas Garcia-Torreiro (2016) achieved the highest 35 gL^{-1} $P3HB_{conc.}$ within 40 h.

In **Figure 23** a sharp decline in the *H. boliviensis* CDW can be observed and this pattern corelates well with a substantial increase of the glucose concentration. The decrease of CDW is thus caused by a dilution effect due to an over feeding overnight. This has caused an increase of the glucose concentration in the broth which may have surpassed the inhibitory glucose treshold. For this reason, growth has probably stopped.

Comparing both P3HB production strategies, the limitation by N was more promising than the P limitation. Although, the effect of a dual limitation N+O_2 was not conclusive, it was chosen as production strategy in further assay with carbon-rich concentrates from *Gelidium* residues as feeding.

4.4.3 P3HB production by *H. boliviensis* on carbon-rich concentrates from *G. sesquipedale* residues.

A concentrated hydrolysate from *G. sesquipedale* residues was used as feeding solution in fed-batch assays the results from that assay were compared to a similar cultivation using as feeding a glucose solution with the same concentration as the hydrolysate (**Table 25**).The medium and feeding solution(s) are in **Table 26.**

Table 25 – Composition of feeding solutions for the fed-batch assay of *H. boliviensis* on algal concentrated hydrolysate and on the respective glucose simulate.

Parameter (Unit)	$V\ (mL)$	$c_{\ glucose}\ (gL^{-1})$	$c_{\ HMF}\ (gL^{-1})$
Feed: Carbon-Rich Concentrate	420	165.1	0.086
Feed: Glucose	420	160.0	-

Table 26 – Composition of the cultivation media for the fed-batch assays using algal concentrated hydrolysate as feeding . $NaCl$, magnesium salt and glucose were prepared and added separately.

Component	**Batch**	**Fed-Batch**
K_2HPO_4	15 gL^{-1}	-
$FeSO_4 \times 7H_2O$	0.005 gL^{-1}	-
$NaCl$	45 gL^{-1}	
$MgSO_4 \times 7H_2O$	50 mL/L of 100 gL^{-1}	-
MSG	10 gL^{-1}	-
Glucose	25 gL^{-1}	**Table 25**

In the end of the batch phase, the base $10\,\%\ NH_4OH$ was substituted with $2.5M\ NaOH$ to initiate the N limitation. Simultaneously to access O_2 limitation, the $DO\ (\%)$ setpoint was reduced from 20.0 to 2.0% as well as the gas flow from 2.0 to 0.8 $L\ min^{-1}$. The final results from fed-batch cultivation and acquisition plots are showed in **Figure 25** and **26**, respectively.

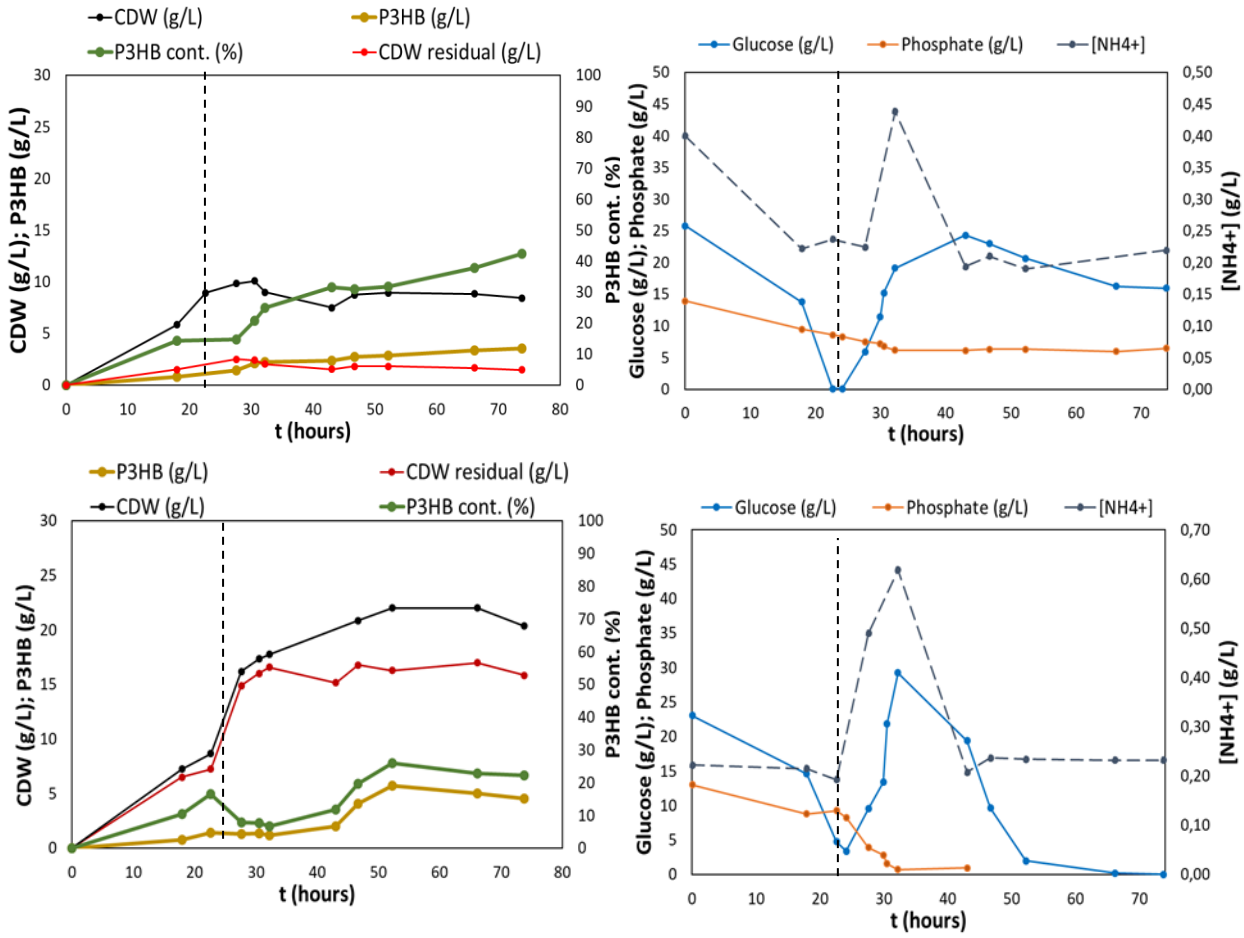


Figure 25 – The fed-batch cultivation of *H boliviensis* growth and P3HB production triggered by dual N+O_2 limitation (25 h.), start of the dual N+O_2 limitation (25 h.) is visualized by dotted lines. The top two figures represents the feeding with 160 gL^{-1} glucose and the bottom ones represents the feeding by a concentrated hydrolysate from *G. sesquipedale* residues, containing 165 gL^{-1} glucose.

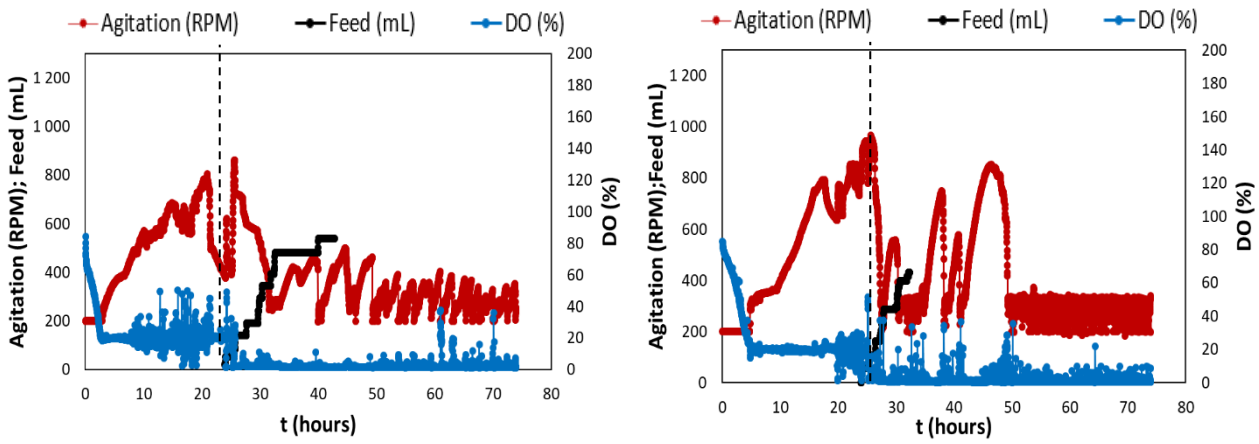


Figure 26 - Acquisition plots of the fed-batch cultivation of *H. boliviensis* and P3HB production triggered by dual N and O_2 limitation (25 h.). On the left is the acquisition plot extracted from the assay with feeding by glucose and on the right is the feeding with carbon-rich concentrate from *G. sesquipedale*. Dotted lines represents the start of the dual N and O_2 limitation (25 h.).

Table 27 – Cultivation parameters from the fed-batch production of P3HB by *H. boliviensis* triggered by dual N and O_2 limitation and with feeding by glucose and concentrated hydrolysate from *G. sesquipedale*. The CDW value corresponds to the highest P3HB cont.

Parameter (Unit)	$Y_{P/S}$ (g P/ g $S_{cons.}$)	$Prod_{max\,P}$ ($g\,L^{-1}h^{-1}$)	CDW (gL^{-1})	$P3HB\ cont.$ (%)	$P3HB_{conc.}$ (gL^{-1})
Feed: Carbon-Rich Concentrate	0.237	0.109	22.0	26.0	5.72
Feed: Glucose	0.275	0.048	8.4	42.3	3.56

Regarding the results acquired from the latest study (**Table 27**) shows a very high $Y_{P/S}$, that were attained in both cultivations. Surprisingly, 5.72 gL^{-1} P3HB concentration was achieved in the case of feeding by concentrated hydrolysate, which was almost 1.5 times higher than in the study with feeding by glucose 3.56 gL^{-1}. Although, a low volume and glucose concentration did not allow to continue with this process longer than for 42 h., the production of P3HB was steadily continuing in the case of glucose feeding, whilst in the case of hydrolysate the P3HB production it seemed to have halted. However, due to a very slow

production in the assay with glucose feeding, the $Prod_{max\,P}$ favours the feeding with hydrolysate.

A huge disproportion can be observed between the $CDW\ (gL^{-1})$ in the two feeding conditions. This might be caused by the hydrolysate composition. As already before in section **4.2.4**, the formation of precipitates, probably originated from both protein and oligosaccharide fraction, could clog the final concentrate. Thus, by feeding with such a solution a change in the value of $CDW\ (gL^{-1})$ is inevitable. It is difficult or almost impossible to predict the $CDW\ (gL^{-1})$ corresponding to microbial growth only. To avoid this problem, a next centrifugation step should be implemented to minimize the presence of precipitates in concentrates from algal hydrolysates. For this reason, also the $P3HB\ cont\ (\%)$ differs significantly between these assays. However, the attained polymer concentration using the hydrolysate as feeding was higher than feeding by glucose and these are promising results.

Nevertheless, it was verified that the whole process starting with *G. sesquipedale* residues biomass to the feeding carbon-rich concentrate for fed-batch production by *H. boliviensis* is a feasible strategy. In further assays the conditions of feeding and limitation should be improved to optimize the results of P3HB production based on algal hydrolysates.

5 Conclusion and Future Prospects

The waste material resulting from the extraction of agar from *Gelidium sesquipedale* is an unexplored and yet valuable material still containing nonnegligible amounts of carbohydrates (44.80 ± 1.90 % dw.). To produce a sugar-rich hydrolysate from the waste biomass of *G. sesquipedale* with low or no sugar degradation products, different acid catalysts and mild reaction conditions were tested in the hydrothermal pre-treatment. The pre-treated biomass was then subjected to enzymatic hydrolysis with a cocktail of cellulolytic enzymes. The produced hydrolysates contained merely one degradation product that was identified as 5-hydroxymethylfurfural (HMF). This compound showed a substantial influence on the growth of *Halomonas boliviensis*, above a concentration of 0.1 gL^{-1} of HMF. At this concentration the μ_{max} of *H. boliviensis* dropped circa 92.8 % in comparison to the control with no HMF. To produce *G. sesquipedale* hydrolysates with minimum HMF and a high sugar concentration a two-step process was chosen: the chemical pre-treatment by 0.5% (w/v) sulfamic acid at 121 °C in an autoclave for 30 mins with subsequent enzymatic hydrolysis using an enzyme load of 2.8 mg cellulase complex/g biomass and 0.7 mg β-glucosidase/ g biomass at 50 °C for 30 hours. Under these conditions and with biomass concentration of 86.4 gL^{-1}, the HMF concentration was less than 0.2 gL^{-1} and the glucose ranged from 24 – 26 gL^{-1} correspond to approximately 30% g_{sugars}/g_{dw}. Under these combined hydrolysis conditions, a insignificant amount of galactose was released. This is due to an incomplete hydrolysis of the residual agar (7.3 % dw) still present in the residues by the mild sulfamic acid. Due to the low agar content of the residues the saccharification of agar and the release of galactose was sacrificed to avoid the formation of inhibiting HMF concentrations. This is acceptable with waste *Gelidium* due to the low agar content, the same not being true if whole *Gelidium* biomass was intended to be used as carbohydrate platform. The scale-up feasibility was confirmed up to 700 mL working volume without substantial differences in the hydrolysis yield.

In order to upgrade the hydrolysis of algal biomass, a more innovative technique for the chemical pre-treatment should be taken into consideration. Instead of using an autoclave with no mixing, a stirred vessel in which both chemical pre-treatment and enzymatic hydrolysis could take place at different working conditions would provide an easier control of the reaction conditions and a higher yield of the released sugars

The main core of this work was to employ the hydrolysates from *G. sesquipedale* as a carbon-rich feedstock for the production of biodegradable polymer, poly-3-hydroxybutyrate by the halotolerant bacteria *H. boliviensis*. Biological cultivation of *H. boliviensis* was examined in shake flask and bioreactor scale. Shake flask assays showed that *H. boliviensis* was able to consume glucose and produce P3HB. The polymer accumulation is triggered by a lack of an essential nutrient such as nitrogen, phosphorus or oxygen. Hence, a shake flask study using *G. sesquipedale* hydrolysate was conducted on production media with varying concentrations of NH_4Cl (0 or 1 gL^{-1}) and *MSG* (0; 5; 10 or 20 gL^{-1}) as nitrogen sources. The best growth and P3HB production by *H. boliviensis* on *G. sesquipedale* hydrolysate were attained using the production medium with 10 gL^{-1}*MSG* and no other nitrogen source. The highest obtained $Y_{P/S}$; $Prod._{max\,P}$; CDW; $P3HB_{conc.}$ and $P3HB$ (%) were 0.155 ($g_{product}/g_{substrate}$); 0.056 $g\ L^{-1}h^{-1}$; 8.3 gL^{-1}; 3.27 gL^{-1} and 39.2 %, respectively. The comparison of these results with the growth on production medium supplemented with glucose: 0.199 ($g_{product}/g_{substrate}$) $Y_{P/S}$; 0.052 $g\ L^{-1}h^{-1}$ $Prod._{max\,P}$; 5.5 gL^{-1} CDW; 2.68 gL^{-1} $P3HB_{conc.}$ and 48.4 $P3HB$ (%). Therefore, it can be concluded that *G. sesquipedale* hydrolysates have the potential to be a sustainable carbon source for the P3HB accumulation by *H. boliviensis*.

To improve the production of P3HB on *G. sesquipedale* hydrolysates, it is vital to analyse the hydrolysates in terms of elementary composition. A broad knowledge about the composition of the elements in the hydrolysates will help understanding the microbial behaviour under limiting conditions, and therefore a more suitable production medium could be proposed.

To attain higher polymer productivities based on algal hydrolysates, fed-batch bioreactor assays were carried out. The carbon-rich algal hydrolysates were used as feeding solution. To prepare the concentrated feeding solution from *G. sesquipedale* hydrolysates, an evaporation step was implemented. Two widely used evaporation techniques were used, namely a vacuum rotatory evaporator and a spray-dryer. Although, both techniques were successful to fulfil the evaporation task, the spray-drying proved to be a more suitable option as it requires less energy and the obtained material is a dry powder, enabling a long-shelf storage. Results from the study of *H. boliviensis* growth and P3HB production on the concentrates obtained by both techniques, showed a better performance of the concentrate produced by spray-drying the hydrolysate. The following cultivation parameters were obtained: 0.153 ($g_{product}/g_{substrate}$) $Y_{P/S}$; 0.051 $g\ L^{-1}H^{-1}$ $Prod._{max\,P}$; 6.4 gL^{-1} CDW ; 2.58

gL^{-1} $P3HB_{conc.}$ and 40.4 $P3HB$ (%) However, due to the stickiness properties of glucose powder only 62.56 % of the expected glucose in the hydrolysate was recovered due to the attachment of glucose solids to the walls of the spray chamber. Moreover, part of the solids formed poorly soluble agglomerates, making the dissolution of the glucose powder to a concentrated solution difficult.

The concentration step is a crucial operation affecting product yields and possibly increasing the number and concentration of degradation products formed. Therefore, care should be payed to the concentration process to avoid issues like the glucose stickiness and minimize the potential risk of further degradation reactions of carbohydrates. The use of pressure-driven membranes should be taken into consideration as with membranes detoxification of hydrolysates and recovery of the enzymes could be aimed at.

When a sufficient volume of the concentrated hydrolysate was attained (V_{final} 420 mL; $c_{glucose}$ 165.0 $g\ L^{-1}$ and c_{HMF} 0.086 gL^{-1}), it was used as feeding in the fed-batch cultivation with *H. boliviensis*. Firstly, an optimal cultivation strategy needed to be chosen to attain high polymer yields. This was carried out with a commercial glucose solution. Higher polymer contents (42.3 $P3HB$ (%)) and productivities ($Prod._{max\ P}$= 0.048 $g\ L^{-1}h^{-1}$) were attained using a dual limitation of nitrogen and oxygen to promote P3HB production. A similar assay using algal concentrates as feeding was performed yielding a volumetric productivity $Prod._{max\ P}=$ 0.109 $g\ L^{-1}h^{-1}$ and 26.0 of $P3HB$ (%). Although a higher polymer productivity was attained using the algal hydrolysate, a lower polymer cell content was observed. This is hugely influenced by the presence of insoluble precipitates in the concentrated hydrolysate used as feeding, which were generated during the evaporation of *G. sesquipedale* hydrolysates. This caused a gradual increase of the CDW along the cultivation time while feeding was being added. Therefore, the CDW and $P3HB$ (%) were not comparable using the two types of feeding. Nevertheless, the maximum produced $P3HB_{conc.}$ was almost 1.5 times higher on the algal hydrolysates compared to feeding with glucose. In the future, an extra centrifugation step for preparing the algal hydrolysate feeding should be considered. Additionally, next fed-batch assays should involve a deeper study of the *H. boliviensis* behaviour under different DO (%) and feeding rates to promote higher polymer yields and productivities.

Later, an examination of self-sterile ability of saline environment during the *H. boliviensis* cultivation should be verified.

In the end, this study showed the feasibility of using *G. sesquipedale* residues as a

sustainable carbon platform for the production of the biodegradable polymer P3HB by *Halomonas boliviensis.*

6 References

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Annexes

1) The HPLC quantification of monosaccharides, phosphate (RI detector) and 5-hydroxymethylfurfural (UV/VIS detector) was accomplished according to following equations:

$$Glucose\ (gL^{-1}) = 0.0000072575 \times A_{glucose} - 0.07224687\ (r^2 = 0.9999)$$

$$Galactose\ (gL^{-1}) = 0.0000070576 \times A_{galactose} - 0.0700507649\ (r^2 = 0.9999)$$

$$Phosphate\ (gL^{-1}) = 0.0001 \times A_{phosphate} - 0.0997\ (r^2 = 0.9995)$$

$$5 - hydroxymethylfurfural\ (gL^{-1}) = \frac{A_{HMF} + 29603.87}{4906074.41}\ (r^2 = 0.9994)$$

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2) The GC quantification of poly-3-hydroxybutyrate was accomplished according to following equation:

$$P3HB\ (gL^{-1}) = \frac{A_{P3HB}}{A_{Internal\ standard}} \times 7.9239 + 0.3998\ \ (r^2 = 0.9939)$$

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3) The ammonia quantification by spectrophotometer was accomplished according to following equation:

$$[NH_4^+]\ (gL^{-1}) = 0.76 \times OD\ + 5.92 \times 10^{-2}\quad (r^2 = 0.9839)$$

4) An assay of production medium composition. From top - production medium “B”, “C” and “D” on the bottom.