TÉCNICO LISBOA

Poly-3-hydroxybutyrate production by *Halomonas elongata* using carbon-rich hydrolysates manufactured from residues of *Ulva lactuca* after protein extraction

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Thesis to obtain the Master of Science Degree in

Biological Engineering

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November 2021

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.

Preface

The experimental work reported in this thesis was performed at the Institute of Bioengineering and Biosciences (IBB) in Instituto Superior Técnico in Lisbon, Portugal between the period of March to October, under the supervision of Prof. Maria Teresa Ferreira Cesário Smolders and Prof. Pedro Carlos de Barros Fernandes.

”Da minha aldeia vejo quanto da terra se pode ver no Universo...
Por isso a minha aldeia é tão grande como outra terra qualquer
Porque eu sou do tamanho do que vejo
E não do tamanho da minha altura...
Nas cidades a vida é mais pequena
Que aqui na minha casa no cimo deste outeiro.
Na cidade as grandes casas fecham a vista à chave,
Escondem o horizonte, empurram o nosso olhar para longe de todo o céu,
Tornam-nos pequenos porque nos tiram o que os nossos olhos nos podem dar,
E tornam-nos pobres porque a nossa única riqueza é ver.”

Alberto Caeiro, in ”O Guardador de Rebanhos”

Acknowledgements

Firstly, I would like to thank my supervisors Prof. Maria Teresa Cesário and Prof. Pedro Fernandes for their guidance and for the availability to help me with the uprising issues throughout this work. Secondly, to Tânia Leandro I would like to express my gratitude for the patience and knowledge shared with me, regarding *Halomonas elongata* cultivation and to Diogo Faria for helping me with the SDS-PAGE gel as well. To my lab coworkers who made all the work extremely more enjoyable, I would like to give a special thank you.

To my family for all the shown support, particularly during the harder times, I would like to give my thanks and, especially to my sister Patrícia and Robert for all the help and availability shown. Lastly but not least, I would like to thank my friends for the support given, for the laughter and, in general, just for existing.

Obrigada,
Carolina Rodrigues

Abstract

As plastic production continues to rise, so does its generated pollution. In recent years, bioplastics such as polyhydroxyalkanoates (PHAs) are gaining more attention and, in an effort to lower production costs, alternative low-value bio-based feedstocks are being explored. Marine macroalgae are a rich source of carbohydrates, widely available in nature and, hence, a low-cost, renewable carbon source for the production of PHAs. Green macroalgae *Ulva lactuca*, involved in green tides observed worldwide, can be valued by using every fraction of its biomass for biorefinery processes.

In this work, *U. lactuca* residues after protein extraction were used as carbon source for the production of the biopolyesters - PHAs - by *Halomonas elongata* after hydrolysis of the carbohydrate fraction to simple sugars, thus applying the biorefinery concept to marine biomass. These residues were subjected to combined hydrolysis where acidic pre-treatments with different acids followed by enzymatic hydrolysis using various enzymatic cocktails were carried out. A thermo-acidic pre-treatment with 1.5% (w/v) HCl during 30 minutes at 121℃ followed by enzymatic hydrolysis with 0.25% (v/v) of cellulase and β-glucosidase mixture at 50℃ and pH 4.8 during 24 hours yielded 81.2% (g sugars hydrolysate/g sugars biomass) of total sugar recovery (83.2% in glucose, 87.2% in xylose and 60.4% in rhamnose) in a 6.3-fold scaled-up combined hydrolysis. Microbial growth inhibitor 5-hydroxymethylfurfural was detected in concentrations below 0.05 g/L, which did not significantly affect *H. elongata* growth. However, after hydrolysate concentration, this inhibitor was no longer detectable but a new compound with similar chemical composition was formed. Poly-3-hydroxybutyrate (P3HB) production by *H. elongata* was carried out using the concentrated hydrolysate in shake flask assays under unsterile conditions varying the nitrogen concentration in the medium to promote polymer production. Maximum productivity of 0.027 g/(L.h), polymer concentration of 4.5 g/L and yield of polymer on sugar consumed ($Y_{P/S}$) of 0.21 g P/g $S_{cons.}$ were attained by decreasing the glutamate and the ammonium concentrations. Cell growth was only observed after 72 h of incubation due to growth inhibition that may have resulted from the presence of the new compound formed during the hydrolysate concentration step. Results obtained show that is possible to produce high sugar yield hydrolysates of *U. lactuca* residues with mild combined hydrolysis conditions and that it is feasible to use them for P3HB production by *H. elongata* under unsterile nitrogen-limited conditions in shake flask fermentations.

Keywords: *Ulva lactuca*, *Halomonas elongata*, Polyhydroxyalkanoates, Poly-3-hydroxybutyrate, Enzymatic hydrolysis, Acidic pre-treatment

Resumo

Com o aumento da produção de plástico, a poluição que daí advém também aumenta. Nos últimos anos, bioplásticos como polihidroxialcanoatos (PHAs) estão a ganhar popularidade e, num esforço para diminuir o seu custo de produção, matérias-primas alternativas de baixo-custo e de origem biológica estão a ser exploradas. As macroalgas marinhas são uma fonte rica em carboidratos, amplamente disponí-vel na natureza e, por isso, uma fonte de carbono renovável e de baixo-custo para a produção de PHAs. A alga verde *Ulva lactuca*, conhecida por estar envolvida nas marés verdes a nível mundial, pode ser valorizada ao se aproveitarem todas as frações da sua biomassa.

Neste trabalho, resíduos de *U. lactuca* após extração de proteína foram utilizados como fonte de carbono para a produção de biopoliésteres - PHAs - a partir da bactéria *Halomonas elongata*, após hidrólise da fração de carboidratos a açúcares simples, aplicando, assim, o conceito de biorefinaria à biomassa marinha. Estes resíduos foram submetidos a um processo de hidrólise combinada, na qual foram testados pré-tratamentos acídicos com diferentes ácidos seguidos de hidrólises enzimáticas com vários *cocktails* enzimáticos. Um pré-tratamento com uma solução de 1,5% (w/v) HCl realizado durante 30 minutos a 121 ºC seguido por uma hidrólise enzimática com 0,25% (v/v) de uma mistura de celulase e β-glucosidase a pH 4.8 e temperatura de 50 ºC durante 24 horas, resultou num rendimento de 81,2 % (g açúcar hidrolisado/g açúcar biomassa) em açúcares totais recuperados (83,2 % em glucose, 87,2 % em xilose e 60,4 % em ramnose), após o scale-up de 6,3 vezes da hidrólise combinada. O inibidor de crescimento microbiano, 5-hidroximetilfurfural, foi detetado em concentrações inferiores a 0,05 g/L, o que não afeta significativamente o crescimento da bactéria *H. elongata*. No entanto, após o processo de concentração do hidrolisado, este inibidor deixou de ser detetado e notou-se a formação de um novo composto com propriedades químicas semelhantes às do 5-HMF. O hidrolisado produzido foi utilizado nos ensaios realizados visando a acumulação de P3HB a partir de *H. elongata*. Nestes, diferentes concentrações de azoto no meio de cultura foram testadas sob condições não estéreis. Obtiveram-se uma produtividade de 0,027 g/(L.h), uma concentração de polímero máximas de 4,5 g/L e um rendimento de polímero em substrato consumido de 0,21 g P/g $S_{cons.}$, reduzindo as concentrações de glutamato e de amónia do meio. O crescimento bacteriano foi só detetado 72 h após o início da fermentação, o que indica que ocorreu inibição de crescimento, possivelmente pela presença do novo composto formado no passo de concentração do hidrolisado. Os resultados obtidos neste trabalho mostram que é possível produzir hidrolisados a partir dos resíduos de *U. lactuca* com rendimentos em açúcares elevados e que, a partir dos mesmos, a produção de P3HB em frascos agitados com *H. elongata* em condições não estéreis e com azoto limitado é conseguida.

Palavras-chave: *Ulva lactuca*, *Halomonas elongata*, Polihidroxialcanoatos, Poli-3-hidroxibutirato, Hidrólise enzimátíca, Pré-tratamento acídico

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Abbreviations

BP	Biodegradable plastics
CBU	CelloBiase Unit
CDW	Cell dry weight
DTT	Dithiothreitol
dw	Dry weight
FPU	Filter Paper Unit
GC	Gas chromatography
HMF	5-hydroxymethylfurfural
HPLC	High-performance liquid chromatography
O.D.$_{600}$	Optical density at 600 nm
P(3HB-co-3HV)	Poly-3-hydroxymethylbutyrate-co-3-hydroxyvalerate
P3HB	Poly-3-hydroxybutyrate
PE	Polyethylene
PHA	Polyhydroxyalkanoate
PLA	Polylactic acid
PP	Polypropylene
Prod$_{max}$ (g/(L.h))	Maximal volumetric productivity (g/(L.h))
SDS	Sodium dodecyl sulphate
$Y_{P/S}$ (g P/g S_{cons})	Yield of product over substrate (g product/ g consumed substrate)

Chapter 1

Introduction

1.1 Context

Plastic has become indispensable in our daily lives. Throughout a rapid growth since 1950, with 2 million tonnes per year, plastic productions have increased 184-fold, reaching 368 million tonnes in 2019[1,2]. With the world population growing, plastic demand is still increasing and by 2050, it is estimated for plastic production to reach 1600 million tonnes[3]. To put into perspective, this is roughly 3.3 times the mass of today's world population (around 7.8 billion people[4]), considering an average world mass of 62 kg per person[5].

With plastic's desirable properties of strength, durability, stability, lightness and hydrophobicity, its usage spread rapidly and it is now widely utilized in many commercial and industry sectors, becoming globally ubiquitous[6]. However, these properties are the same that make plastic resistant to natural degradation, generating plastic waste and pollution which pose a threat to the environment and human health[7]. Despite being a global problem, waste mismanagement is largely seen in developing countries due to lack of resources and effective infrastructure to treat waste. Therefore, besides focusing on waste treatment solutions, it is necessary to address the issue at its source and rethink plastic production processes[8]. Nowadays, biodegradable plastics (BP) produced from renewable biomass are becoming popular. Even though these plastics have limitations due to not having properties as competitive as commonly used plastics, namely high plasticity and impact strength, they can be used as packaging materials, which is likely to have a remarkable impact on waste production[7].

About 30% of plastics in the world are consumed as packaging material, thus replacing oil-based plastics used in packaging by biodegradable plastics has a huge research and application market[6,7]. Polyhydroxyalkanoates (PHAs) are part of these biodegradable polymers and have properties close to petroleum-based plastics, which make them materials with high potential for replacing conventional oil-based plastics[9]. The most studied polymer in the PHA family is poly-3-hydroxybutyrate (P3HB). These exist in the form of granules inside bacteria as food and energy reserves and are produced in response to a nutrient limitation, such as nitrogen[10].

Biodegradable plastics only account for an insignificant percentage of total annual produced plastics.

The main reason is the cost. Oil-based plastics, namely PPs and PEs, can be 3 to 10 times cheaper than BP. One of the main causes for BP's higher costs is the raw material used, which can account for up to 50% of the plastic production cost[7, 11]. In this work, the residues of the green algae *Ulva lactuca* are used as a potential low-cost carbon-rich source for the production of P3HB by the halotolerant bacteria *Halomonas elongata*. This bacteria is an aerobic, gram-negative species, which can accumulate granules of P3HB, under conditions of excess of carbon and limitation of nitrogen, oxygen or phosphorus.

1.2 Objectives and Deliverables

Improving our understanding of the feasibility of producing the bio-based and biodegradable plastic P3HB by the halophile *Halomonas elongata*, utilizing low-cost carbon-rich hydrolysates from *Ulva lactuca* algae residues is the main focus of this master thesis. In order to achieve that, the following objectives have been defined:

- Characterization of *Ulva lactuca* algae residues after protein extraction, regarding its chemical composition.
- Optimization of hydrolysis conditions of the algae residue polysaccharides to monomers, by attaining high sugar yields and low inhibitor amounts.
- Determination of the chemical composition of the optimized hydrolysate, regarding its sugars, total nitrogen and inhibitor contents.
- Evaluation of the inhibitors effect on *Halomonas elongata*'s growth.
- Production of the optimized hydrolysate of *U. lactuca* residues in a scaled-up process.
- Optimization of P3HB accumulation conditions by *H. elongata* using the produced hydrolysate from *U. lactuca* residues, by varying the C/N ratio of the culture media, under unsterile conditions.

Chapter 2

State of the Art

2.1 Plastics

Mankind is often described according to the materials used to fill basic needs and to conceive new tools that improve our lifestyle. Throughout history the most commonly known periods are the Stone Age, Bronze Age and Iron Age. Nowadays, one could claim we are in the Plastic Age[12]. Plastics started a new era of development that currently shapes our society. They undoubtedly contributed enormously in improving food and water safety, health care and population safety, in macro- and microelectronics, transports and many other industry sectors[13]. Plastic's low cost combined with properties like strength, durability, lightness, corrosion-resistance and flexibility, allow it to be versatile and have a wide range of uses. Plastic is made of synthetic or semisynthetic organic polymers, which consist in long molecule chains of repeating units, composed of hydrocarbons mainly derived from fossil fuels[14].

There are 7 types of commercial available plastics based on their polymeric material (**Figure 2.1**): polyethylene terephthalate (PET), polyethylene (PE) which can be divided into high density polyethylene (HDPE) and low density polyethylene (LDPE), polyvinyl chloride (PVC), polypropylene (PP), polystyrene (PS) and others group (O) that includes several plastics such as polyactide, polycarbonate, fibreglass, nylon, etc[6].

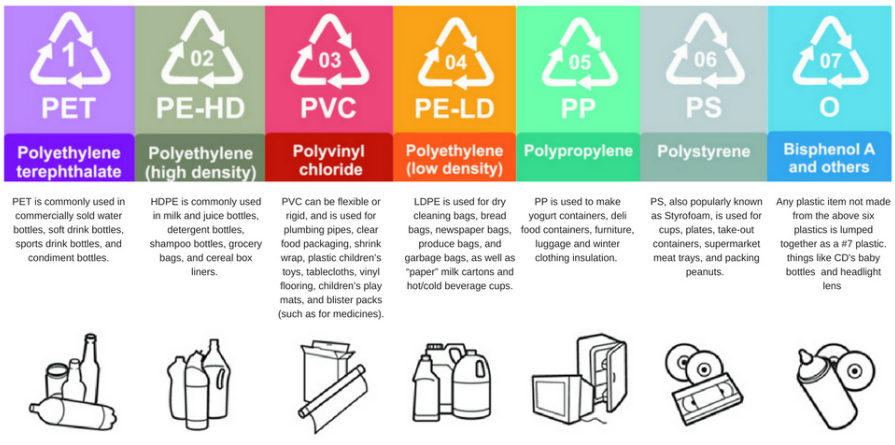


Figure 2.1: The 7 most common types of plastics described by Plastic Oceans[15]

Properties that make plastics so desirable are the same that cause their negative impact in the environment. Firstly, their durability and toughness cause them to be resistant to natural degradation. Secondly, their low cost increased the usage of single-use plastics over other materials like glass, wood or metal that were reused due to their sizeable cost, resulting in more waste accumulation[13].

2.1.1 Degradation of Plastic in Nature

Plastic is degraded by breaking down into small fragments that will grow even smaller over time, eventually reaching the size of microplastics ($<$ 5 mm) and even nanoplastic particles ($<$ 1000 nm), when further degradation occurs. This process starts with the mentioned fragmentation, that will introduce new chemical groups to the ends of the carbon chain, altering its composition. With further fragmentation, a mineralization process will occur, which consists in the conversion of carbon atoms into carbon dioxide and other inorganic chemicals, a process that is very lengthy. The degradation rate of the whole process depends on the type of plastic and to what and where it was exposed[14]. Since waste accumulation is of great impact to the marine environment, it has been studied throughout the years. Therefore, it is well-known that physical mechanisms contribute the most to plastic degradation in this environment. Mechanisms such as UV radiation with abrasion, wave action and turbulence, all result in the oxidation of the polymer matrix, leading to chemical bond breakage[16].

In addition to fragmentation, biodegradation also plays an important role in plastic degradation. This process begins with the attachment of microorganisms to the surface of floating plastic and the formation of a biofilm, containing distinct communities that could harbour organisms with potential to degrade plastic. Microorganisms secrete exoenzymes and endoenzymes into the biofilm that can potentiate plastic biodeterioration and, by fragmenting it, the microorganisms can assimilate this plastic as a carbon source and grow. As it occurs in the physical mechanisms, there is also a mineralization step, which consists in the release of final products like carbon dioxide, methane and water[6]. Polymer characteristics like high molecular weight, 3D structure and hydrophobic nature complicate microbial action. Environmental conditions like UV radiation, abrasion and wave action exposure facilitate biodegradation. However, the same does not happen the other way around. Biofilms may act as a barrier of UV radiation, reducing photo-degradation rates and may decrease the plastic's buoyancy, causing them to sink and hence, further reducing UV radiation and thus, degradation rates[14].

2.1.2 Waste Accumulation in the Marine Environment

Degradation of plastic can take up to hundreds of years and, with the increasing production rates, its accumulation in the oceans is estimated to quadruple by 2040 (if there is no change), reaching a value of 600 million metric tons[17]. Around 80% of plastics in the oceans come from land sources. Mismanagement of landfills and improper disposal of sewages as well as coastal landfills lead to plastic transportation by rivers and water streams into the oceans. The other 20% come from marine sources, such as fishing nets[18]. It is estimated that 5.25 trillion micro and nanoplastic particles contaminate the sea surface[19]. These can come from atypical sources such as washing of clothes or teabags. According

to Hernandez et al. (2019), a single cup of tea prepared with a plastic teabag can contain 11.6 billion microplastics and 3.1 billion nanoplastics[20].

Mismanaged waste includes all the litter that instead of being disposed off or managed in proper storage sites, is disposed in uncontrolled, open landfills, creating the risk of leakage to the surrounding environment via wind or waterways and thus, reaches the oceans. Low-to-middle income countries contribute the most to this kind of pollution by not having proper waste management infrastructures. In South Asia and Sub-Saharan Africa, 80 to 90% of plastic waste is mismanaged. This data is reflected in the top 20 polluting rivers being all from these regions, where Yangtze river, in China, is the most polluting one, having contributed with an estimated 333 thousand tonnes of plastic waste in 2015, which accounted for 4% of the annual ocean plastic pollution[8].

2.1.3 Waste Treatment

Currently, the most common ways to treat non-biodegradable plastic waste are through incineration, recycling and landfilling[7]. Incineration and landfilling can be grouped in the conventional waste treatment methods while recycling can be included in the advanced treatment technologies. Considering the first two methods, each face a specific bottleneck. Incineration of non-biodegradable plastics results in hazardous by-products such as carbon dioxide, acidic gases, heavy metals and organic compounds like furans and dioxins besides requiring a large amount of energy to take place, all of which contribute to global warming and to health issues, mainly of respiratory tract. Landfilling can be considered the worst non-biodegradable plastic treatment method since it gradually accumulates plastic, due to its continual disposal while its degradation happens slowly, leading to more land space being required. The leachates produced can also leak and harm the surrounding environment[21,22].

In the advanced technologies scope, methods such as pyrolysis, photodegradation, thermodegradation and biodegradation are included. Zhang et al. (2020) categorize these advanced technologies into two larger groups: recycling and degradation. Both were further divided into physical recycling, energy recovery and resource recovery and into biodegradation and oxo-biodegradation, respectively. Energy recovery mainly includes incineration. Resource recovery includes chemolysis and thermolysis includes pyrolysis, hydrocracking and gasification. Oxo-biodegradation includes abiotic and biotic degradation, where the abiotic one includes photodegradation, thermodegradation, mechanochemical degradation and other forms of degradation[21]. This is schematized in **Figure 2.2**.

Focusing on recycling, the American Society for Testing and Materials (ASTM) separates it into four groups: primary recycling (ASTM I) in which only specific, uncontaminated plastics are recovered and reused without going through changes and are normally used for the same purpose they previously had; secondary (ASTM II), in which physical methods are used to reprocess the plastic after it has been separated from its contaminants (PET and PE plastics are practically the only types that can be mechanically recycled); tertiary recycling (ASTM III) is where chemical decomposition of plastics occurs, converting them into their building blocks, which can later be used to re-synthesize the original polymer or to produce new plastics, allowing for a cyclic flow. Lastly, quaternary recycling (ASTM IV) consists in

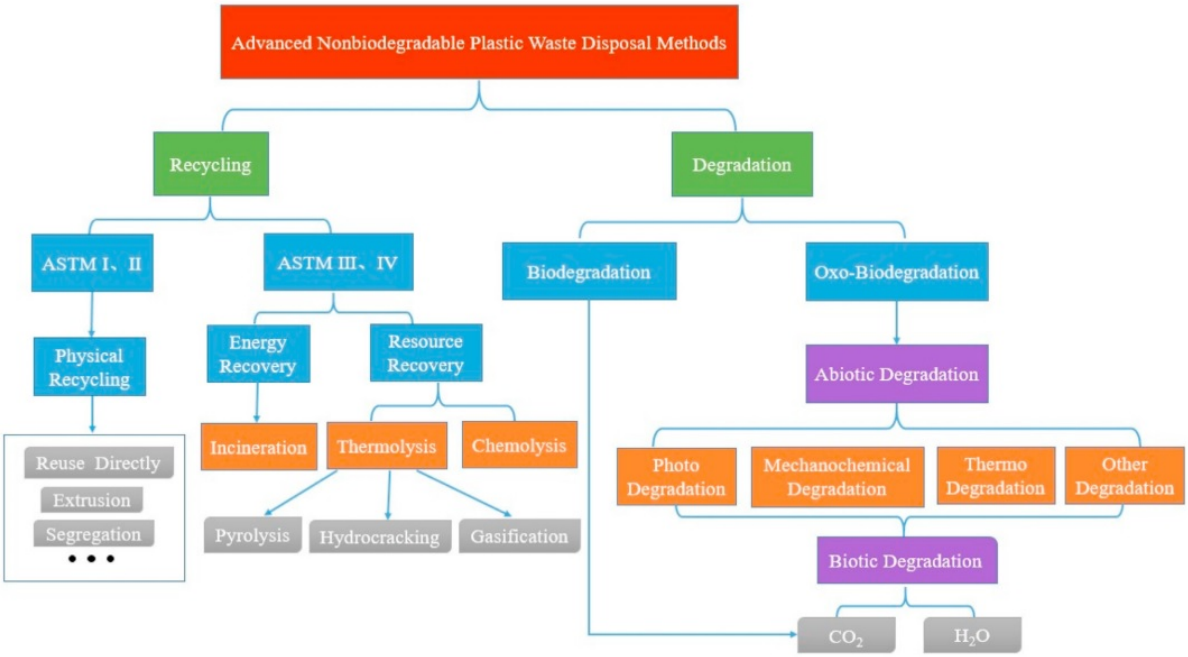


Figure 2.2: Categorization of the advanced technologies in non-biodegradable plastic waste treatment reported by Zhang et al.[21]

recovering energy from plastics by incineration processes[21,22].

Primary or secondary recycling deteriorate the polymer, which leads to low quality products after continuous recycling. Tertiary recycling is not applied to all plastics due to possible inefficiency or economically unsustainability, since it has high energy costs. Quaternary recycling gives further use to plastic wastes by incineration, since they have high calorific values. This is, however, not environmental friendly since it produces toxic products, as mentioned above[22].

The current recycling methods all have their flaws. Therefore, more promising solutions that reduce energy consumption and toxic components production in a sustainable and economic way and increase the value of the final product are required. Okan et al. (2018) propose many options on how to use plastic waste in sustainable ways. These are summarized in **Figure 2.3**.

Carbon capture using plastic waste in coal or gas burning power plants to sequestrate carbon dioxide generated during combustion could be a way to reduce CO_2 emissions in the atmosphere[23]. In order for it to work, polymers used for this purpose need to have selectivity towards CO_2, so they do not adsorb other flue gases; have a large sorption capacity in order for the process to be economically feasible; have physiochemical stability that allows them to endure moisture, heat and acidic gases and have an easily scalable synthesis process. Examples of these polymers can be seen in aminated PVC, which has high hydrophobicity (good for high moisture content environments) and has a scalable synthesis process since it is based on a single step reaction[24].

Uplcycling plastic waste is another form of sustainable management. This concept means giving value to plastic waste by converting it into a new product, that can be innovative and profitable. Examples of upcycling are synthesis of carbon microspheres and nanotubes from plastic waste that can be used for printers, paints, toners, batteries and tires.

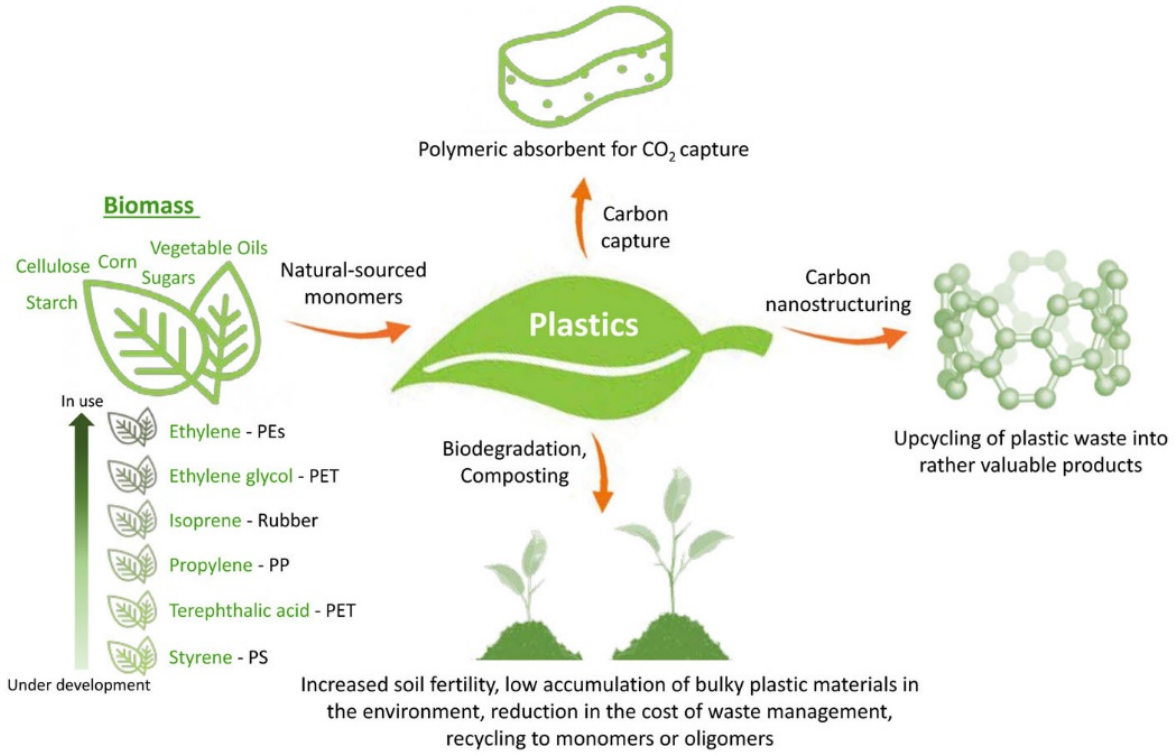


Figure 2.3: Summary of sustainable options in the management of plastic reported by Okan et al.[22]

Changing the source of plastic synthesis to natural based polymers is an obvious approach which aims to reduce fossil feedstocks usage, decreasing the negative effect of plastic waste in the environment. The rapid decrease of fossil resources and the escalating oil prices has triggered industries to focus their research on renewable alternatives[25]. These can be polysaccharides such as starch, cellulose, etc. and lipids from agro resources; polyhydroxyalkanoates produced by microorganisms or even polylactides, PBS (poly(butylene succinate)), PTT (poly(trimethylene terephthalate)), PPP (poly(p-phenylene)) and PE polymers from biotechnology produced via conventional synthesis[26]. Furthermore, if the alternative polymer is biodegradable it will help in reducing the generated waste accumulation, increase soil fertility or even in reducing waste management costs.

2.1.4 Biorefinery

Several issues concerning consumption of fossil fuel resources, namely rising demand and prices and dependency on the producing countries, gives rise to political concerns apart from the obvious environmental ones. Thus, renewable resources as alternatives are of great importance. The biorefinery concept appears as an integrated process analogous to the petrochemical refinery, in which fuel, power and other chemicals are produced utilizing plant-based materials as feedstock. Biomass is produced via photosynthesis in which atmospheric carbon dioxide and water are converted into sugars. These sugars are then used by plants to synthesize complex products that constitute the biomass[25,27]. To convert the biomass into valuable biorefinery products, thermochemical, biochemical, chemical and mechanical processes must be applied together. The latter is normally done in the beginning of the pathway to

reduce size, alter shape or to separate feedstock or biomass components. Chemical processes mostly include hydrolysis, to convert polysaccharides into their respective monomers, and lipids transesterification, which is currently the most common method to produce biodiesel[28]. Thermochemical processes include two main strategies. Gasification, consisting in keeping biomass at high temperatures and low oxygen levels to produce syngas: a mixture of hydrogen, carbon monoxide and dioxide and methane; and pyrolysis, in which bio oil, solid charcoal and light gases are produced in a process that utilizes intermediate temperatures in the absence of oxygen. Lastly, biochemical processes commonly involve fermentations or anaerobic digestions, which are performed at lower temperatures than the ones in the former processes. Fermentations use microorganisms, with or without the aid of exogenous enzymes, to mainly produce alcohols or organic acids, where ethanol remains the most demanded one[29]. Anaerobic digestion mainly results in biogas as the end product by biomass breakdown through bacterial action, in the absence of oxygen[30].

Feedstocks can be obtained from four different sectors: agriculture (from crops and residues), forestry, industry or households (from residues and leftovers or municipal solid waste and wastewater) and aquaculture (from algae or seaweeds). This allows the reduction of national dependence on oil importations since most of these materials are locally available and encourages the development of rural areas since biorefineries can combine different size facilities, contrarily to petrochemical ones[27].

2.2 Biodegradable Plastics

As discussed, plastic's degradation processes are slow and their rapid accumulation has great impact on the environment. Biodegradable plastics appear as a way to manage this accumulation and to reduce plastic pollution, moving towards a greener future. These are plastics whose properties allow them to remain unaltered during their shelf life but, after usage, can be broken down to CO_2, H_2O, CH_4 and biomass in nature, without releasing toxic substances[7]. These plastics can be divided into 2 categories according to their biodegradation degree and nature: completely biodegradable or destructive biodegradable plastics. The former, as the name suggests, refers to plastic that is fully degradable, made of natural polymers such as starch, cellulose or chitin, of microbial fermentation polymers, etc. The latter, made of natural polymers combined with synthetic ones, are not completely biodegradable, but the addition of the natural polymer will lead to the biodegradation of the polymer blend to some extent (oxo-biodegradable plastics)[31].

Bioplastics (bio-based and/or biodegradable) are currently applied as packaging material, catering products and in many other sectors, such as electronics, automotive, agriculture, textiles, etc., where packaging accounted for 53% of the total bioplastics market in 2019. They represent 1% of the total plastic produced in 2019 (368 million tonnes), however the market is growing. In 2024, bioplastic production is expected to reach around 2.43 million tonnes, compared with the 2.11 million tonnes reported in 2019. From these 2.43 million tonnes, over 55.5% are accounted for biodegradable plastics[32].

Although growth is seen in the market, biodegradable plastics are still a really small fraction of the annual plastic production. While plastic is cheap, used worldwide and its production process is well

developed, biodegradable plastics have a higher production cost, do not have all the properties that make conventional plastic a good material (P3HB has better barrier properties than PP making it better for packaging, however has low plasticity and impact strength) and there are still questions regarding its degradation.

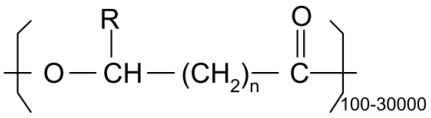
Biodegradation usually involves 3 steps: biodeterioration, biofragmentation and bioassimilation. These were already covered in **Subsection 2.1.1** and, just like in conventional plastics, this process is dependent on environmental conditions such as temperature, pH, oxygen levels, water content, polymer composition, etc[7]. Napper and Thompson (2019) reported that over the 3-year period of their experiment, none of the different types of tested plastics bags (biodegradable, oxo-biodegradable, conventional and compostable) exposed to open-air, soil and marine environment deteriorated enough to reduce the negative impacts of littering in the tested environments. The compostable bag was the only one to completely disappear within a 3-month period, but remained intact in the soil environment[31].

Another issue is when biodegradable plastics are disposed in an uncontrolled manner, their fragmentation can also lead to the formation of micro and nanoplastics. The consequences of these small particles are starting to be studied and adverse effects have been reported on diversity and growth of benthic communities (lower regions in water environments) caused by PLA, a corn or sugar based plastic that has been gaining popularity in the packaging industry[33]. P3HB, a biodegradable plastic produced by microbial fermentation, has also been the object of study. In 3 aquatic organisms, cyanobacterium *Anabaena* sp. PCC7120, green alga *Chlamydomonas reinhardtii* and crustacean *Daphnia magna*, the ecotoxicological effect of secondary nanoplastics released from P3HB microplastics by abiotic degradation were tested. Results showed they were harmful for the 3 organisms, which are primary producers (cyanobacterium and alga) and consumers (crustacean) in freshwater ecosystems, by decreasing the growth of the producers and inducing 85% of immobilization in the consumer after 3 and 2 days of exposure, respectively[34]. These data show there is still a lot of research to be done regarding the biodegradation of biodegradable plastics and that without change in human behaviour regarding littering, greenwashing practices and waste management approaches, biodegradable plastic will not become an important factor to plastic waste reduction[7].

2.2.1 Polyhydroxyalkanoates

A really important group of biopolymers is the PHA family. Polyhydroxyalkanoates (PHAs) are bio-based and biodegradable polyesters produced by many bacterial species, whose properties resemble those of conventional oil-based plastics[35]. These characteristics led them to gain attention in the recent years and, by 2024, their production is expected to triple[32]. PHAs' general structure is shown in **Figure 2.4**. The majority of them consist of R(-)-3-hydroxyalkanoic acid monomers that can range from C3 to C14 carbon atoms, varying in saturation, branching and side groups (aliphatic or aromatic)[36].

Approximately 300 bacteria are able to produce polyhydroxyalkanoates[37]. They synthesize and accumulate PHA as a food and energy reserve when there is excess of carbon and limitation of nutrients such as nitrogen, oxygen, phosphorus or magnesium in the environment (with some exceptions), pre-



n =1	R =	hydrogen	poly (-3-hydroxypropionate)
		methyl	poly (-3-hydroxybutyrate)
		ethyl	poly(3-horoxyvalerate)
		propyl	poly (-3-hydroxyhexanoate)
		pentyl	poly (-3-hydroxyoctanoate)
		nonyl	poly (-3-hydroxydodecanoate)
n = 2	R =	hydrogen	poly (-4-hydroxybutyrate)
n = 3	R =	hydrogen	poly (-5-hydroxyvalerate)

Figure 2.4: General structure of the monomer of the PHA family[37].

venting starvation. If the nutrient limitation is reverted, then intracellular depolymerases degrade the polymer into carbon and energy source ready to be used[10,36]. The accumulation of PHAs in the cell varies according to the bacterial species; an accumulation of 80-90% of cell dry weight in recombinant *Escherichia coli* has been reported, whereas a typical percentage for methylotrophs (microbes that use reduced one-carbon compounds as a carbon source) is 50-60% of cell dry weight[37]. Hence, it is of great importance to choose the right species in order to achieve higher productivity.

2.2.2 Poly-3-hydroxybutyrate

Poly-3-hydroxybutyrate (P3HB) is the most well characterized polymer from the PHA family. It was discovered in 1926 by Lemoigne in *Bacillus megaterium* cells[10]. Its structure corresponds to the one shown in **Figure 2.4** when n=1 and R is a methyl group. Regarding its properties, it has a melting point of 175℃ which is only slightly lower than its degradation temperature of 185℃, hindering its thermal processing. Its tensile strength is of 40 MPa, elongation to break of 6% and impact strength of 50 J/m, whereas polypropylene (PP) has similar tensile (34.5 MPa) and impact (45 J/m) strengths, but significantly higher elongation to break (400%)[36]. To improve P3HB mechanical properties, synthesis of the copolymer poly-3-hydroxybutyrate-co-3-hydroxyvalerate (P(3HB-co-3HV)) has been in development. As the -3HV fraction increases so does the impact strength and elongation to break, making the plastic tougher, more flexible and resistant to breakage. Furthermore, its melting temperature decreases allowing for easier thermal processing with no risk of degradation[36,37]. Thus, it is possible to control the plastic properties by adjusting the fraction of -3HV during fermentation. In 1982, a plastic named BIOPOL™, composed of the copolymer P(3HB-co-3HV) was developed by ICI, in England[10]. In 1996, its price reached $16/kg, whereas polypropylene prices were less than $1/kg[37]. On large scale, the fermentation, product recovery processes and substrate costs require high investments, which has been the cause for the lack of commercialization of these copolymers[10].

Recovery of P3HB processes start with cell collection after fermentation, through conventional methods such as centrifugation or filtration. Since P3HB is an intracellular material, cell disruption ensues. This process can be performed with the use of solvents, sodium hypochlorite or through aqueous enzymatic digestion. Solvent methods have been the most used processes, where a range of them in-

clude methylene chloride, chloroform, propylene carbonate and dichloroethane. These processes are not economically viable due to large volumes of solvent required[37]. Other methods such as digestion of non-PHA materials with sodium hypochlorite causes high PHA degradation although high purity is also achieved[36]. Aqueous enzymatic digestion consists in a three-step process where there is thermal treatment of the biomass, followed by enzymatic digestion and solubilization of non-PHA materials, by washing with an anionic surfactant[37].

Regarding P3HB metabolic pathway (**Figure 2.5**), there are 3 enzymes involved: 3-ketothiolase, acetoacetyl-CoA reductase and PHA synthase.

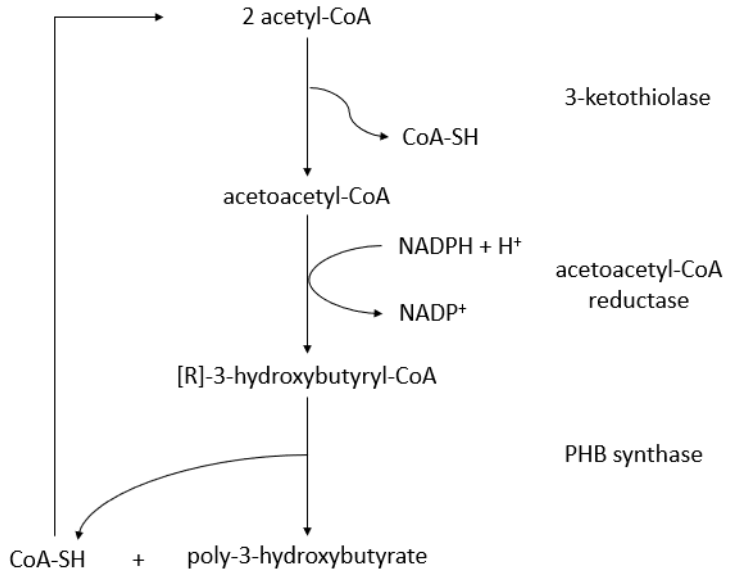


Figure 2.5: P3HB metabolic pathway[10].

When there is nutrient limitation, the pathway gets activated and acetyl units from the TCA cycle are diverted into the pathway. The ketothiolase will then catalyze the dimerization reaction of acetyl-CoA to acetoacetyl-CoA and, afterwards, the reductase will catalyze the hydrogenation of the latter into [R]-3-hydroxybutyryl-CoA, the monomer of P3HB. PHA synthase initiates and catalyzes the polymerization reactions. The latter enzyme is specific for monomers with [R] configuration, thus all natural PHAs are isotactic. When the nutrient is regenerated, the polymer can be reconverted to acetyl-CoA by a series of enzymatic reactions[10].

2.3 Alternative Carbon Sources

One of the main problems these biopolymers face against conventional plastics is their production cost. The carbon source used to feed the polymer producing bacteria can reach up to 50% of the overall production cost, thus research focused on using low-value substrates from industrial and agricultural wastes has been carried out in recent years[11]. For illustrative purposes, a few representative examples

are here presented. Hassan et al. (2018) attempted to optimize P3HB production with nonpathogenic *Bacillus subtilis* using agricultural and industrial wastes such as corn bran, corncob, wheat bran, rice bran, dairy waste and sugarcane molasses as the carbon source. Incubation was performed in agar plates at 30℃ for 5 days. Results showed rice bran achieved the highest production of 0.31 g/L of P3HB, corresponding to 30.4% of cell dry weight (CDW). After selecting the optimum cultural conditions (pH, incubation time and inoculum size influenced P3HB production the most), P3HB production reached 0.81 g/L, corresponding to 62.6% CDW[38]. Arumugam et al. (2019) aimed for low-cost production of PHA using cashew apple juice as substrate for *Cupriavidus necator*. Different nitrogen sources were tested and urea was selected as the best option for optimum microbial growth. After optimization of the production process, 15.78 g/L of 3-hydroxybutyrate-3-hydroxyvalerate copolymer were achieved in a batch stirred tanked reactor for a total reducing sugar concentration of 50 g/L, inoculum size of 50 mL/L and urea concentration of 3 g/L[39]. A really interesting approach is P3HB production through C1 carbon sources such as methane, methanol and CO_2, which play a huge role in the greenhouse effect, i.e., CO_2 can be used as carbon source by hydrogen-oxidizing bacteria such as *C. necator* or *Ideonella spp.* strain O-1, which were reported to produce 61.9 g/L (67,8% CDW) and 5.26 g/L (77.9% CDW) of P3HB, respectively under oxygen limitation[40, 41, 42]. A very promising alternative carbon source platform that has rapid growth, requires fewer resources and does not compete with food industry is algae, which is why the specific group of macroalgae will be covered in more detail in the following section.

2.4 Macroalgae

Marine macroalgae, also known as seaweeds, appear as a good substitute for agricultural feedstocks in biorefinery. The latter have been mainly derived from crops for biofuels production, as seen in **Section 2.3**, which led to an increasing competition for food and energy in the global market. Furthermore, water consumption and arable land requirements are higher when compared to macroalgae's that do not require land nor freshwater for their cultivation[43]. Marine algae have rapid growth rates derived from their higher photosynthetic efficiency and abundant available resources such as sunlight, CO_2 and inorganic matter, when compared to terrestrial biomass. Also, they contribute more for CO_2 remediation by having a higher fixation rate for this gas. Another aspect that makes them more favourable is their relatively easier degradation. Seaweed lack lignin in their composition, which is a structural component in terrestrial plants. Thus, they are easier to depolymerize, potentially reducing processing costs[44].

Macroalgae are categorized into 3 groups: green, red and brown macroalgae, according to their thallus colour, which depends on the pigments and types of chlorophylls they possess. Their main characteristics are summarized in **Table 2.1**.

Macroalgae's composition can be influenced by cultivation parameters, harvesting periods and habitat conditions such as light, temperature, salinity, nutrients availability, existing pollution and water motion. From these, light contributes the most to these variations since each seaweed has their respective pigments which absorb light in different wavelengths, ending up conditioning the environment they live in. Most algae live in coastal areas, whereas red algae can live in deep waters where sunlight's availabil-

Table 2.1: Macroalgae characteristics according to their classification type: green, brown or red. Adapted from Sudhakar et al.[45].

Type	Characteristics	Occurrence	Pigments	Relevant Species
Green	Multicellular; Simple thallus; Filamentous spongy fingers or paper-thin sheets; Cell wall contains cellulose	700-7000 species; Bays, estuaries, tide pools	Chlorophyll A, Chlorophyll B, carotenoids;	*Halimeda fragilis* *Ulva lactuca* *Codium tomentosum*
Brown	Olive green to dark brown colour; Can grow up to 100 meters; Complex thallus; Leathery; Cell wall contains align (or fucoidan) and pectin	1500 species; shallow and cold waters; rocky shores	Chlorophyll A Chlorophyll C, fucoxanthin	Kelps, Sargasso weed; *Saccharina latissima*; *Laminaria digitata*; *Ascophyllum nodosum*; *Undaria pinnatifida*; *Macrocystis pyrifera*;
Red	Filaments, sheets or cells; Parasites of other algae; Cell wall contains calcium carbonate	4000 species; Most abundant and widespread; Deep cold waters or warm shallow waters	Chlorophyll A, phycobilins	*Gracilaria verrucosa*; *Palmaria palmate*; *Asparagopsis armata*; *Gelidium sesquipedale*

ity is limited, since they have phycobilins, which absorb in wavelengths of radiation that can penetrate seawater to such depths[43].

2.4.1 *Ulva lactuca*

Ulva lactuca is a green macroalgae belonging to the *Ulva* genus. It is often associated with green tides formation due to eutrophication derived from human activity, which hinder marine ecosystems and local tourism[46]. Their rapid proliferation makes them well suited for cultivation, to particularly be applied in bioremediation of nutrient rich wastewaters. Their controlled cultivation, contrarily to natural harvesting, can achieve higher quality biomass and hence, higher quality bioproducts[47].

Table 2.2: *Ulva lactuca* species general composition[45, 48].

Ulva lactuca's composition	
Water	78-90 (%)
Ash	12-38 (% dw)
Proteins	10-33 (% dw)
Lipids	0-6 (% dw)
Carbohydrates	25-60 (% dw)

Within the carbohydrates content, *Ulva* species' starch represents 1-4% d.w. as a reserve polysaccharide, whereas ulvan, cellulose, xyloglucan and glucuronan represent around 38-54% d.w. as cell wall polysaccharides[48, 49].

When processing this algae it is important to know its structure in order to know the existent linkages between polymer molecules and depolymerize them more easily. For it to be used as a carbon source,

its polysaccharides need to undergo saccharification into their sugar monomers. As mentioned, most of these polysaccharides are cell wall components, which are the hardest to depolymerize, since they have a structural support role and are, thus, more resistant. *Ulva* species cell wall contain cellulose and β-1,4-D-xyloglucan linked by hydrogen bonds in the outer layers, whereas ulvan and β-1,4-D-glucuronan crosslink them through ionic interactions[49].

Cellulose is composed of linear D-glucose repeating units linked by β-1,4 glycosidic bonds. Xyloglucan (hemicellulose) has a backbone of β-1,4-linked D-glucose residues, mainly branched with β-1,6-linked D-xylose residues[50]. Glucuronan consists of repeating β-1,4-linked glucuronic acid residues[47]. Ulvan is one of the main cell wall polysaccharides and is composed of rhamnose (5.0-92.2 mol%), xylose (0.0-38.0 mol%), glucuronic acid (2.6-52.0 mol%) and iduronic acid (0.6-15.3 mol%). Its antimicrobial, antioxidant, anticancer and immunostimulatory properties allow for potential applications in the biomedical field for wound dressing, tissue engineering, biofilm prevention and excipients or in food and pharmaceutical industries[47,49]. Thus, it could be interesting to use this algae for ulvan extraction and add value to the remaining fractions for biorefinery processes. Regarding ulvan's structure (see **Figure 2.6**), it is composed by two major disaccharide repeating units, named ulvanobiuronic acids type A and B and by minor disaccharide repeating units, named ulvanobioses (type U). Ulvanobiuronic acid type A consists of β-D-glucuronic acid (1,4)-linked to α-L-rhamnose 3 sulphate, while type B consists of α-L-iduronic acid (1,4)-linked to α-L-rhamnose 3-sulphate. Ulvanobioses distinguish themselves in type U_{3s}, which consists of β-D-xylose (1,4)-linked to α-L-rhamose 3-sulphate, and type $U_{2's,3s}$, which has β-D-xylose 2-sulphate (1,4)-linked to α-L-rhamnose 3-sulphate[47].

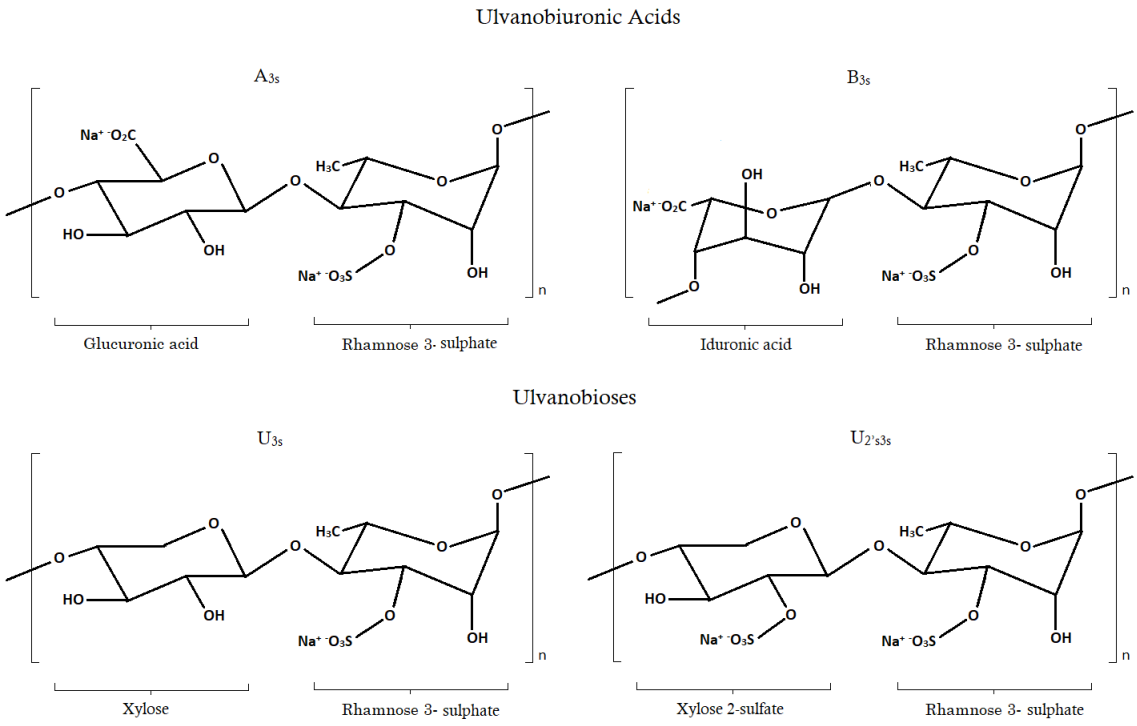


Figure 2.6: Structural representation of the main repeating disaccharide units of ulvan. Adapted from[47].

2.4.2 Chemical pre-treatment

An efficient saccharification of macroalgae should consider a pretreatment process, in which there is an increase of biomass accessibility to enzymes. Pre-treatments can be of chemical, physical or biological nature or a combination of these[51]. Lime, AFEX (ammonia fiber expansion) and acid pre-treatments show the greatest potential from an economical perspective. However, when considering ethanol production, processes like wet oxidation, sodium hydroxide treatment and steam explosion are more promising[52]. Alternative methods such as solvent-based ones like organosolv (with alcohols or organic acids), ultrasounds, microwaves or even white-rot fungi treatment are also used[52, 51]. Acidic pre-treatments are generally performed with dilute acids since concentrated acids enhance sugar degradation and corrosivity of the equipment used[53]. Dilute acid hydrolysis combines a low pH and a high temperature, which mainly affects hemicellulose and can partially degrade cellulose[54]. For this purpose, commonly used conditions are 0.5-4 %(w/w) sulphuric acid at 120-210 ℃ during 5-30 minutes[52]. Karimi et al. (2006) reported a hydrolysis yield of xylan to xylose of 78.90% with 0.5% sulphuric acid in a second-stage pretreatment, whereas the highest obtained yield of glucose was of 46.6%[55]. Soliman et al. (2018) performed thermochemical hydrolysis of *U. lactuca* with sulphuric acid and hydrochloric acid at different concentrations (0.5, 1, 2, 3, 4 and 5% w/v) at 100 or 120 ℃ during 30 or 60 minutes. Results showed higher total sugar yields for HCl treatments than for H_2SO_4, without following a specific trend. However, these yields decreased with the harsher condition of 5% HCl solution at 120 ℃ and 60 minutes[56]. Alkaline hydrolysis can partially dissolve hemicellulose while swelling the structure, which increases accessibility to enzymes. McIntosh and Vancoc (2010) reported reaching 5.6 times higher sugar yields using 2% sodium hydroxide at 121 ℃ for 60 minutes than with a pretreatment in the same conditions but without the alkali[57]. Greetham et al. (2020) attempted a novel seawater based seaweed hydrolysis process that followed one of either three pre-treatment approaches: dilute acid, alkaline or hydrothermal pre-treatment. All of the solutions were prepared either with reverse osmosis (RO) water or with filtered seawater and, to ensure total salt content was the determining factor in the treatment, the algae were also treated with synthetic seawater (SW) that was prepared with RO water only contained main reported seawater salts. Results showed higher concentration of sugars when seawater was used, i.e, a dilute acid treatment with 1% sulphuric acid in seawater released 41.80% of total sugars, whereas the same treatment with RO water resulted in 36.20% of total released sugars[58]. This could be interesting for future considerations when developing an industrial-scale process that makes use of locations by the sea to acquire available seawater for processing algae biomass, where the latter could be cultivated or harvested in the region.

2.4.3 Microbial growth inhibitors - furans

As previously mentioned in **Subsection 2.4.2**, harsher acidic hydrolysis conditions (low pH, high temperatures and long hydrolysis time) will intensify monomeric sugar degradation. This will result in the formation of by-products such as furans, whose growth inhibitory properties will hinder the fermentation steps later on. Monomers with six carbon atoms (i.e. glucose) can be degraded into 5-

hydroxymethylfurfural (5-HMF) while monomers with five carbon atoms (i.e. xylose) can be degraded into furfural, by dehydration reactions[52]. Alkali pre-treatments, AFEX, wet oxidation are performed at high pH, resulting in non-significant amounts of these species[53]. Other by-products can be formed during hydrolysis, derived from non-sugar compounds present in hemicellulose, such as uronic acid and acetic acid. Further degradation stimulates the dehydration of furans which results in the formation of furoic acid, formic acid and levulinic acid. Once more, in alkali hydrolysis, AFEX and wet oxidation, furoic and levulinic acids are almost non-existent, whereas formic acid can be present, although from a different origin[52, 53].

Furans cause inhibition by reducing specific growth rates and productivity of cells. In *Saccharomyces cerevisiae* cells, furfural inhibits dehydrogenase enzymes involved in glycolysis as well as alcohol, aldehyde and pyruvate dehydrogenases. It is also associated to an increase of reactive oxygen species which, in turn, damage the mitochondria and vacuole membranes as well as cytoskeleton and nuclear chromatin. 5-HMF also inhibits dehydrogenases, although to a minor extent[52]. Interestingly, some bacteria have the ability to reduce both furfural and 5-HMF into furfuryl alcohol and 5-hydroxymethyl furfuryl alcohol, respectively, which have a less inhibitory effect than the former[59, 60].

2.4.4 Enzymatic hydrolysis

A typical subsequent process after chemical pre-treatment is enzymatic hydrolysis. Together, they are usually referred to as combined hydrolysis. Enzymatic hydrolysis requires less energy and milder environment conditions than the chemical pre-treatment and thus, produces negligible fermentation inhibitory components. As discussed, chemical pre-treatment's goal is to improve the accessibility of the biomass to enzymes by disrupting its chemical structure, since cellulose and hemicellulose form a complex network and some enzymes can even be absorbed by lignin if lignocellulosic biomass is treated[61]. Cellulose is completely degraded into glucose monomers by the synergistic action of three enzymes: β-1,4-endoglucanase, cellobiohydrolase/β-1,4-exoglucanase and β-glucosidase. Hemicellulose composed of a backbone of xyloglucan is degraded by xyloglucan-active β-1,4-endoglucanase and β-1,4-glucosidase[50]. In *Ulva* species, ulvan has great potential as a sugar source. However, its complex structure (**see Figure 2.6**) requires different enzymes that can be used with different approaches. Currently, there are four known enzymes with ulvanolytic activity: endo-ulvan lyase and β-glucuronidase, which break (1,4)-linkage between rhamnose 3-sulphate and uronic acids; ulvan lyase, that breaks osidic bonds formed between ulvanobiuronic acids and glucuronan lyase, which acetylates and deacetylates glucuronans in general[62].

Trivedi et al. (2013) investigated the saccharification effect of four different cellulases (Novozymes NS 22119, NS 22128, NS 22086 and Viscozyme L) on *U. lactuca* at a temperature of 45℃, pH 4.8 and during 48 hours. Results showed a higher conversion efficiency of biomass into reducing sugars for cellulase 22119, with a sugar yield of 215 mg/g[63]. Thygesen et al. (2020) tested enzymatic hydrolysis in algal biomass (*Ulva fasciata, Chaetomorpha linum* and *Caulerpa taxifolia*) with Cellic® CTec2 (contains cellulases and β-glucosidase) at 50℃ and pH 5.0 during 0, 6, 24 and 48 hours. For all seaweed

samples, saccharification into glucose increased over time and levelled off after 24 hours of incubation time. For *U. fasciata*, enzymatic saccharification resulted in glucose yields of 38% and of 99%, without and with pre-autoclaving, respectively[64].

2.5 *Halophiles* in PHA production

Halophilic microorganisms should have optimal growth with 5% (w/v) NaCl or higher concentrations and should be able to tolerate NaCl concentrations of, at least, 10% (w/v). To cope with osmotic pressure, these prokaryotes have developed two strategies to keep NaCl outside the cells. One is mainly utilized by archaea microorganisms, which will accumulate KCl to equivalent concentrations to that of NaCl in the extracellular environment. The other, performed by most halophilic bacteria and eukarya, is carried out by accumulation of small organic compounds, named osmolytes. These can be amino acids or their derivatives, sugars or some other polyols. Their role is to maintain cell volume, pressure and concentration of electrolytes in the cell, during osmotic stress[65]. An important osmolyte produced by most species of the *Halomonadaceae* family is ectoine. This osmolyte has protective properties acting as a cell stabilizer against UV radiation or cytotoxins induced stresses as well as some other inflammations. These properties make ectoine a valuable commercial compound in health care and skin products, with an industrial production scale of tons per year. Ectoine is produced in a biotechnological process using *Halomonas elongata*[66]. This halophilic γ-proteobacterium can tolerate salt concentrations between the range of 3% to 20% NaCl by producing ectoine. In addition, it is also known for producing intracellular PHA granules under nutrient stress conditions. In order to reduce PHA production costs, another approach that could be carried out along with the utilization of low cost feedstocks as carbon sources is the combined production of PHA and ectoine[67, 65]. This was, however, assumed to be unfeasible due to three main reasons: firstly, PHA and osmolyte producing pathways are induced through different ways and a bi-factorial stress fermentation would need to be carried out; secondly, ectoine contains 19.7% (w/w) nitrogen and could act as a source of nitrogen for cell growth, hindering PHA synthesis and thirdly, producing both PHA and ectoine promotes competition for energy and carbon resources[67]. Despite this, Mothes et al. (2008) co-produced ectoine and P3HB in *H. elongata* in fed-batch using a medium with 10% (w/v) NaCl under N-limiting or P-limiting conditions. It was observed that ectoine contents were nearly constant under P-limiting conditions whereas, under N-limiting ones, ectoine contents seemed to decrease. P3HB yield reached a maximum value of 55% (w/v) within 120 hours, during N limitation, while ectoine content was approximately 6% of the cell dry weight. However, its maximum content reached 12.5% after 45 hours of incubation time[67].

Another approach to decrease PHA production costs utilizing halophiles is by operating under unsterile conditions, since a medium with high salt concentration inhibits growth of non-halophilic microorganisms[65]. Within this perspective, Tan et al. (2011) attempted to develop an unsterile and continuous fermentation process with *Halomonas* TD01, consisting in two open fermentor tanks, where the cells were grown in the first fermentor under optimized growth conditions with continuous feeding and, after reaching a concentration of 40 g/L, were continuously pumped to the second fermentor, which was fed

with a nitrogen deficient medium. No contaminating microorganism was detected and a yield of 60% P3HB in cell dry weight was achieved in the first fermentor, whereas a P3HB level of 65% to 70% of cell dry weight was maintained in the second fermentor. By producing P3HB in a continuous process, production costs are also reduced over batch and fed-batch processes[68].

Other studies in halophilic microorganisms showed that, in *Haloferax mediterranei*, PHA accumulation could reach 65% of the cell dry weight in batch cultures under phosphorous limitation conditions, using starch or glucose as carbon sources, whereas in continuous cultures, 46% PHA could be attained[69]. Furthermore, in fed-batch cultures, *H. mediterranei* produced a maximum PHA content of 48.6% and a volumetric productivity of 0.36 g/(L.h) using glucose as the carbon source. With treated corn starch as the carbon source, in the same mode of operation, a volumetric productivity of 0.29 g/(L.h) was attained[70,71]. Interestingly, PHA produced by this microorganism is the copolymer P(3HB-co-3HV), which, as mentioned in **Subsection 2.2.2**, has better mechanical properties than P3HB[70].

In summary, halophiles can be used as a mean to produce valuable commercial compounds as by-products during PHA accumulation processes; PHA production can be achieved under unsterile conditions due to high salt concentrations in the media, preventing other microbial growth and, as a result, lowering production costs. Finally, some halophiles have the ability to produce the copolymer P(3HB-co-3HV) that, by having closer properties to conventional used plastics, expands the range of uses of bio-based and biodegradable plastics in plastic industry.

Chapter 3

Materials and Methods

3.1 Materials

3.1.1 Raw materials

Ulva lactuca, batches U1.00618M, U1.00319M and U1.00321MB2401, supplied by ALGAplus, were submitted to protein extraction at pilot scale (as described in subsection **3.2.1**). The resulting algae residues were oven dried to 50-60℃ for at least 72 hours. The dried residues were then stored in closed buckets at room temperature.

Figure 3.1: Sample of *Ulva lactuca* residues after protein extraction as described in subsection **3.2.1**.The particle's size varies from approximately 1 mm to 60 mm. Throughout this work, residues with no more than 11 mm were used, as depicted above.

3.1.2 Synthetic seawater

The synthetic seawater (SW) was used to prepare the acidic solutions of HCl and H_2SO_4 applied in *Ulva lactuca*'s chemical pre-treatment, in order to assess the influence of seawater salts on the algae acidic hydrolysis. For this, solutions containing 2.7% NaCl, 0.33% $MgSO_4$, 0.25% $MgCl_2$, 0.1% $CaCl_2$ and 0.07% KCl were prepared using Milli Q® water to make up the total volume[58].

3.1.3 Enzymes and chemicals

The commercial enzymes used for this work were cellulase complex (NS 22086), β-glucosidase (NS 22118), xylanase (NS 22083) and xylanase/β-glucanase mixture (NS 22002), all from Novozymes® (Bagsværd, Denmark). NS 22086 had a filter paper activity of 111.2 Filter Paper Unit (FPU)/mL, while NS 22118 had a cellobiase activity of 20.1 CBU/mL[72]. The chemicals used were sulphuric acid >95% (Fischer Chemical), hydrochloric acid 37% (Honeywell Fluka), sodium hydroxide (Fischer Chemical), dextrose monohydrate (COPAM, Portugal), glycerol (ACROS Organics), calcium chloride dihydrate (Merck), magnesium chloride hexahydrate (Merck), ammonium chloride (Merck), magnesium chloride 99% (Fagron), di-potassium hydrogen phosphate 99% (PanReac AppliChem), potassium chloride (Merck), magnesium sulphate hepta-hydrate (LabChem), sodium chloride 99.5% (Fischer Chemical), calcium carbonate min. 99% (Merck), Tris (Eurobio Scientific) and 5-hydroxymethylfurfural 98% (Biosynth Carbosynth).

3.1.4 Microorganism

Throughout this work, the halotolerant bacteria *Halomonas elongata* DSMZ 2581 was used, since it is a strain capable of using the sugars present in *Ulva lactuca*'s hydrolysate to accumulate P3HB.

3.2 Methods

3.2.1 Production of *Ulva lactuca* residues upon protein extraction

Protein extraction of *U. lactuca* was carried out in pilot scale, in which 70 g/L of *U. lactuca* flocks were processed per batch and it consisted in four stages: 2 aqueous extractions (> 16 hours) and 2 alkaline (0.1 M NaOH, 1 hour) ones performed with stirring and at room temperature. After this, the extract was separated from the *U. lactuca*'s residues through press filtration and the latter got oven dried at 50-60 ℃ for at least 72 hours. These dried residues were stored in the lab, in closed buckets, at room temperature, ready to use.

3.2.2 Chemical pre-treatment of algal residues

Chemical pre-treatment of algal residues with dilute acid solutions is an essential step before enzymatic hydrolysis in order to improve biomass accessibility to enzymes by changing its structural characteristics and to obtain higher saccharification yields [53]. In this work, different concentrations of dilute sulphuric acid and dilute hydrochloric acid solutions were tested, using either synthetic seawater or Milli Q® water to prepare the solutions. The composition of the synthetic seawater is described in subsection **3.1.2**. The chemical hydrolysis was performed at 121 ℃ for 15 or 30 minutes in an autoclave. The tested parameters and experimental conditions are described in **Table 3.1** .

To compare the effect of the acid and the hydrolysis yield, acid concentrations units of normality (N) were used. Previous assays with sulphuric acid used 1.0 and 1.5 %(w/v) concentrations. These were recalculated to N giving values of 0.20 and 0.31 N, respectively. For hydrochloric acid, 0.20 and 0.31

Table 3.1: Overview of the conditions used for chemical pre-treatments tested in *Ulva lactuca* residues

Catalyst	Solvent	Concentration %(w/v)	Time (min)	Temperature (℃)
Sulphuric Acid Hydrochloric Acid	SW and Milli Q®	0.25; 0.5; 1.0 and 1.5	15 and 30	121

N each correspond to, approximately, 0.74 and 1.12 %(w/v). The hydrolysis was run at 121℃ in an autoclave for 15 minutes. These conditions are described in **Table 3.2**.

Table 3.2: Overview of the conditions used to directly compare the effect of H_2SO_4 and HCl on the algae residues' hydrolysis yield

Catalyst	Solvent	Concentration %(w/v)	Concentration (N)	Time (min)	Temperature (℃)
Sulphuric Acid Hydrochloric Acid	SW and Milli Q®	1.0 and 1.5 0.74 and 1.12	0.20 and 0.31	15	121

The algal biomass was weighted into 100 mL *Schott* flasks and 40 mL of acid were added, resulting in a biomass concentration of 50 g/L. After each hydrolysis, the flasks were cooled down in room temperature and the pH was adjusted between the range of 3-6 with NaOH 8M to avoid HPLC column damage. Samples were taken and prepared for sugar quantification in HPLC as described in **3.2.10.1.1**. All trials were done in duplicate.

3.2.3 Combined hydrolysis

Combined hydrolysis was carried out with chemical pre-treatment prior to enzymatic hydrolysis, using the best pre-treatment condition, which corresponds to the one with higher saccharification yields and less inhibitor concentration. After the chemical pre-treatment, described in **3.2.2**, the pH was adjusted to 4.8 using NaOH 8M right after flask cooling and Milli Q® water was added to make up a biomass concentration of 44.4 g/L, under sterile conditions. Before and right after adding the enzymes, a sample is taken from each flask and is prepared for HPLC quantification, allowing the subtraction of sugar contribution from the enzyme preparations.

The enzymatic hydrolysis was then carried out by adding either cocktail A, B or C in each of the testing concentrations over the chemically pre-treated biomass in order to assess the most suitable option. This information is described in more detail in **Table 3.3**.

Table 3.3: Tested concentrations in %(v/v) used in *Ulva lactuca*'s combined hydrolysis for each enzymatic cocktail and their respective contained enzymes

Concentrations %(v/v)	0.25	0.50	1.00
Cocktail A	Cellulase, β-glucosidase		
Cocktail B	Cellulase, β-glucosidase, xylanase		
Cocktail C	Cellulase, β-glucosidase, β-glucanase and xylanase		

The process was conducted at 50℃ and stirring speed of 600 rpm in an incubator with magnetic stir plates. Samples were collected at different times and prepared for HPLC analysis throughout a period

of 48h to check upon the optimum time for the enzymatic hydrolysis. This time will correspond to a maximum stabilized concentration of glucose.

3.2.4 Scale-up of combined hydrolysis

In order to produce higher quantities of hydrolysate to test in *H. elongata* fermentations, combined hydrolysis was carried out. In the chemical pre-treatment, 250 mL of 1.5%(w/v) of HCl prepared with Milli Q® water were added to a 500 mL *Schott* flask containing *Ulva lactuca* algae residues, making up 50 g/L of algal biomass and thermal treatment at 121 ℃ for 30 minutes was followed. For the enzymatic hydrolysis, water was added to make up a biomass concentration of 44.4 g/L, after pH adjustment at 4.8 with NaOH and the hydrolysis was conducted for 24h at 50 ℃. The procedure was carried out in duplicates and 2 batches were produced.

3.2.5 Concentration and storage of the hydrolysate

Following the combined hydrolysis of the algae residues, the hydrolysate was further processed in order to separate the biomass suspensions from the liquid hydrolysate which was then concentrated to reach higher sugar concentrations. Thus, the hydrolysate was centrifuged for 10 minutes at 4347 g (Centrifuge 5810 R, Eppendorf) and then vacuum filtered with a Bückner funnel and a 900 µm paper filter (brand). To concentrate the hydrolysate, an oven was used at 60 ℃ for 82 hours and afterwards the pH was adjusted to 7.5 with NaOH 8M. The filtrate was stored in an 100 mL *Schott* flask in the lab, taking in consideration the avoidance of UV light.

3.2.6 Strain storage

Cultures of *H. elongata* were stored at -80 ℃ in refrigeration chambers within 2 mL cryovials containing a volume of 900 µL of an inoculum from mid-exponential phase and 900 µL of a 30% sterile glycerol solution, to obtain a 15% glycerol concentration in the end. For the inoculum medium, a modified HM medium (Quillanguamán et al., 2004) was prepared by sequentially adding the following components to water: 45 g/L NaCl, 0.25 g/L $MgSO_4.7H_2O$, 0.5 g/L KCl, 5 g/L peptone, 10 g/L yeast extract, 1 g/L glucose and 0.09 g/L $CaCl_2.2H_2O$. The medium's pH was adjusted to 7.5 using KOH 1M and afterwards the medium was sterilized by incubation at 121 ℃ for 20 minutes. The cultures for storage were prepared in 100 mL of the described medium in 500 mL Erlenmeyer flasks and the medium was inoculated with 1.8 mL of a *H. elongata* glycerol stock and incubated at 30 ℃ for 24 hours.

3.2.7 Inoculum medium preparation

For the bacterial assays tested within this work, the inoculation medium consisted in 45 g/L NaCl, 2.5 g/L $MgSO_4.7H_2O$, 20 g/L glucose, 3 g/L $K_2.HPO_4$, 4 g/L NH_4Cl, 8.9 g/L $MSG.H_2O$, 1 mL/L trace elements and 15 g/L Tris. Firstly, Tris was dissolved in Milli Q® water and 37% (w/w) HCl was added to adjust the pH to 7.5. Then, the medium was prepared by sequentially adding the components in the following

order: NaCl, $K_2.HPO_4$, NH_4Cl, $MSG.H_2O$ and trace elements. This trace elements solution is composed of 10 mL/L 25% (w/w) HCl, 0.19 g/L $CoCl_2.6H_2O$, 0.1 g/L $MnCl_2.4H_2O$, 1.5 g/L $FeCl_2.4H_2O$, 0.07 g/L $ZnCl_2$, 0.062 g/L H_3BO_3, 0.036 g/L $NaMoO_4.2H_2O$, 0.024 g/L $NiCl_2.6H_2O$ and 0.017 g/L $CuCl_2H_2O$[73]. Stock solutions of $MgSO_4.7H_2O$ (100 g/L) and of glucose (500 g/L) were prepared separately to avoid, respectively, precipitation and enhanced thermal degradation[74] during sterilization in the autoclave. The inoculum medium was prepared in 500 mL Erlenmeyer flasks for a total volume of 100 mL corresponding to: 93.5 mL of culture medium, 2.5 mL of 100 g/L $MgSO_4.7H_2O$ and 4 mL of 500 g/L glucose. A 2 mL cryovial of *H. elongata* prepared as described in subsection **3.2.6** was added. Inoculum incubation was carried out in an AGITORB 200 orbital shaker, supplied by Aralab®, at 30 ºC and 200 rpm for 17-18 hours where, after it, the $O.D._{600}$ was measured in order to calculate the volume of inoculum needed to add to the respective assay in study. The incubation conditions remained the same throughout every other assay, unless stated otherwise.

3.2.8 Effect of HMF concentration on cell growth

Hydroxymethylfurfural acts as a bacterial growth inhibitor. To avoid this while using the algae hydrolysate, an assay testing different HMF's concentrations was done to assess its influence in *Halomonas elongata*'s maximum specific growth rate.

The culture medium for this assay consisted in 45 g/L NaCl, 2.5 g/L $MgSO_4.7H_2O$, 20 g/L glucose, 3 g/L K_2HPO_4, 4 g/L NH_4Cl, 8.9 g/L $MSG.H_2O$, (0, 0.05, 0.1, 0.2, 0.5 or 1) g/L HMF, 1 mL/L trace elements and 15 g/L Tris. The components dissolution was done as described in the Inoculum medium preparation (**Subsection 3.2.7**). A 50 g/L HMF stock solution was separately prepared, sterilized with a $0.22\,\mu m$ sterile filter and stored at 4 ºC, to avoid possible thermal degradation. Glucose and $MgSO_4.7H_2O$ were also prepared separately as stock solutions with concentrations of 500 g/L and 100 g/L, respectively, and sterilized at 121 ºC for 20 minutes in the autoclave. The culture medium was prepared in 500 mL Erlenmeyer flasks to a final volume of 100 mL which contained: 86.5 mL of the previously described medium, 4 mL of glucose (500 g/L), 2.5 mL of $MgSO_4.7H_2O$ (100 g/L), (0, 0.2, 0.4, 1 or 2) mL of a 50 g/L HMF solution, 2.5 mL of the inoculum with an $O.D._{600}$ of 4 (thus starting the assay with a 0.1 $O.D._{600}$) and the adequate volume of Milli Q® water to make up to the final volume. The experiment was done in duplicates and a sample was taken every hour for 10 hours to follow bacterial growth, by measuring the optical density at 600 nm.

3.2.9 Effect of C/N ratio on the production of P3HB

In order to assess the effect of the C/N ratio on the production of P3HB, an assay was conducted testing various C/N ratios, where the concentration of the accessible nitrogen components (NH_4Cl and $MSG.H_2O$) in the mineral medium was altered while the sugar concentration remained unchanged. The composition of this culture medium consisted in: 45 g/L NaCl, 2.5 g/L $MgSO_4.7H_2O$, 3 g/L K_2HPO_4, (0 or 1) g/L NH_4Cl, (0, 1.0, or 8.9) g/L $MSG.H_2O$, 1.0 mL/L trace elements and 15 g/L Tris. The trace elements solution's composition is described above in subsection **3.2.7**. Stock solutions of NH_4Cl (250

g/L) and MSG.H_2O (500 g/L) were prepared separately in order to adjust the adding volume according to the tested assays. The stock solutions and the culture medium were sterilized in the autoclave at 121℃ for 20 minutes. The sugar source was supplied by the optimized concentrated hydrolysate produced from the combined hydrolysis of *U. lactuca* residues treated with 1.5% (w/v) HCl solution prepared with Milli Q® for 30 minutes at 121℃ in the autoclave, followed by an enzymatic hydrolysis with a 0.25% (v/v) cellulase complex and β-glucosidase mixture (Cocktail A) at 50℃ and pH 4.8 for 24 hours.

The assays were prepared in duplicate under sterile conditions in a laminar flow chamber (BioAir Instruments, aura 2000 M.A.C). The culture medium was prepared in 250 mL Erlenmeyer flasks to make up a final volume of 50 mL. Note that the hydrolysate remained unsterile to infer about the feasibility of P3HB accumulation by the halophile *H. elongata* under unsterile conditions, since the high salt concentration in the culture medium should prevent the growth of contaminants present in the air. Samples were taken periodically to measure cell dry weight *CDW*, pH, sugar and P3HB content.

3.2.10 Analytical Methods

3.2.10.1 Characterization of the algae residues

To characterize *Ulva lactuca*'s residues, total carbohydrates quantification as well as ash, moisture and total solids content took place.

3.2.10.1.1 Quantification of total carbohydrates in biomass

The determination of total carbohydrates of *Ulva lactuca* residues was done according to an adaptation of the National Renewable Energy Laboratory (NREL) protocol "Determination of Total Carbohydrates in Algal Biomass" [75].

This method consists in a two-step acidic hydrolysis where, in the first step, triplets of 0.5 g of biomass residues are weighted out in Erlenmeyer flasks and 5 mL of 72% (w/w) sulphuric acid are added in each. Then, the flasks are incubated at 30C and 100 rpm for one hour in an orbital shaker. The second step consists in autoclaving the samples at 121C for one hour after diluting the hydrolysate to a final 4% (w/w) sulphuric acid concentration with 139 mL of Milli Q® water. After cooled down to room temperature, 10 mL of each triplet are taken into Falcon tubes to then get neutralized to pH 6-8 with calcium carbonate. To separate the suspended solids, centrifugation at 1932 g for 10 minutes is done and the supernatant is recovered and prepared for HPLC carbohydrate quantification. This preparation consists in filling *Eppendorf* tubes with each hydrolysate supernatant and centrifuging them at 9167 g for 5 minutes where, again, the supernatant is removed and subjected to a 2-fold dilution with 50 mM sulphuric acid. Then, a last centrifugation at 9167 g for 5 minutes is carried out and the supernatant is diluted by 10-fold with 50 mM sulphuric acid.

3.2.10.1.2 Ash, moisture and total solids

To determine the ash, moisture and total solid contents, the protocol "Determination of Total Solids and Ash in Algal Biomass"[76] from NREL was followed. This method consists in pre conditioning crucibles by drying them overnight at 575℃ in the muffle furnace and weighing them after cooling down in room temperature in a desiccator. Afterwards, 100 mg of sample were weighed in each crucible (done in triplicate) and left drying in a convection drying oven at 60℃ for at least 18 hours. The crucibles with the samples were then weighed again and sample's total solids and moisture were calculated according to equations **3.1** and **3.2**. For the ash content, the same crucibles with the already dried samples were burnt in a muffle furnace following a ramping program that consisted in: ramping from room temperature to 105℃ and hold for 12 minutes, then ramping to 250℃ at 10℃/minute and hold for 30 minutes and finally ramping to 600℃ at 14℃/minute and hold for 16 hours, allowing the temperature to drop back to 105℃ in the end, before removing the samples. The crucibles with the ashes were weighed and the ash content calculated according to the equation **3.4**.

$$\boldsymbol{Total\ Solids}\,(\%) = \frac{(\boldsymbol{Weight_{crucible+dry\ sample}} - \boldsymbol{Weight_{crucible}})}{\boldsymbol{Weight_{sample\ as\ received}}} \times \mathbf{100} \tag{3.1}$$

$$\boldsymbol{Moisture}\,(\%) = \mathbf{100} - \left(\frac{(\boldsymbol{Weight_{crucible+dry\ sample}} - \boldsymbol{Weight_{crucible}})}{\boldsymbol{Weight_{sample\ as\ received}}} \times \mathbf{100}\right) \tag{3.2}$$

$$\boldsymbol{OvenDryWeight}\,(\%) = \boldsymbol{Weight_{dried\ sample}} \times \boldsymbol{Total\ Solids}\,(\%) \tag{3.3}$$

$$\boldsymbol{Ash}\,(\%\boldsymbol{dw}) = \frac{(\boldsymbol{Weight_{ashed\ sample+crucible}} - \boldsymbol{Weight_{crucible}})}{\boldsymbol{ODW_{sample}}} \times \mathbf{100} \tag{3.4}$$

3.2.10.2 Quantifications by High Performance Liquid Chromatography

Sugars as glucose, xylose and rhamnose as well as glucuronic acid and HMF were quantified using a Hitachi LaChrom Elite High Performance Liquid Chromatography (HPLC). The column used in this equipment is a Rezex ROA Organic acid H+ 8% 30 mm x 7.8 mm, the autosampler is a Hitachi LaChrom Elite L-2200, the pump is a Hitachi LaChrom Elite L-2130 and the detectors are Hitachi L-2420 UV/VIS and Hitachi L-2490 RI. An external heater (Croco-CIL 100-040-220P 40 cm x 8 cm x 8 cm, 30-99℃) kept the column at 65℃. The mobile phase consisted of 5 mM H_2SO_4 run at a flow rate of 0.500 mL/min.

Sample preparation consisted in two-time successive dilutions: 2-fold and 10-fold, to reach 20-fold, both with 50 mM H_2SO_4. Firstly, at least 400 µL of sample were taken into *Eppendorf* tubes and centrifuged at 9167 g for 5 minutes (SIGMA 1-15P, Sartorius) where, 200 µL of supernatant were transferred to new *Eppendorf* tubes and the 2-fold dilution was done. The diluted sample was vortexed and centrifuged again in the same conditions and 100 µL of supernatant were transferred to HPLC vials already filled with 900 µL of 50 mM H_2SO_4, thus diluting the sample by 10-fold, resulting in a 20-fold dilution. The samples were then ready to be analysed by HPLC and the concentrations were calculated through calibration curves previously done for each component.

3.2.10.3 Nitrogen quantification in the algae hydrolysate

Total nitrogen in the algae residues hydrolysate was quantified using the standard LCK338 Laton Total Nitrogen test kit from Hach. It is important to quantify this parameter when using the hydrolysate for P3HB synthesis, since its accumulation depends on the C/N ratio.

3.2.10.4 Protein analysis by SDS-PAGE

The enzyme samples analysed by SDS-PAGE were firstly diluted by 100 and 200-fold with Milli Q® water. Then, 25 µL of Laemmli buffer from Bio-Rad (277.8 mM Tris-HCl pH 6.8, 4.4% LDS, 44.4% (w/v) glycerol, 0.02% bromophenol blue) and 5 µL of 1 M of dithiothreitol (DTT) were added into 20 µL of sample. The resulting sample solutions were boiled for 10 minutes in water bath at 90 ℃ and loaded into each gel well. The molecular markers Precision Plus Protein™Standards Dual Colour from Bio-Rad were also loaded into one well. Electrophoresis at 90V for 2 hours took place in SDS-PAGE gel with 12% T, 2.67% C resolving gel and 4% T, 2.67% C stacking gel filled with running buffer (192 mM glycine, 25 mM Tris and 0.10% (w/v) SDS, pH 8.3) and was only stopped after the bromophenol blue had reached the bottom of the gel. Staining was carried out with Coomassie Phast Gel (Pharmacia AB Laboratory Separations®).

3.2.10.5 Protein quantification via Bradford Assay

To quantify the commercial enzymes from Novozymes®, Bradford Assay was performed for protein quantification, following a Thermo Fisher Scientific protocol for the working range of 1-25 µg/mL[77]. Overall, 150 µL of the diluted BSA standards or of the sample to quantify, were pipetted to the microplate wells and 150 µL of the Coomassie reagent was added on each well. Afterwards, the microplate was mixed for 30 seconds on a plate shaker and was left to rest at room temperature for 10 minutes. The absorbance was then read at 595 nm in a SpectraMax Plus 384 microplate reader (Molecular Devices ©) and the data was gathered using SoftMax® Pro Software version 5.4.1. The concentration was calculated based on the bovine serum albumin (BSA) calibration curve, used as the standard. The assay was done in duplicates.

3.2.10.6 P3HB quantification

To quantify the accumulated P3HB produced by *H. elongata*, an acidic methanolysis was done in order to convert the polymer into hydrocarboxylic acid methyl esters, which are then analysed through Gas Chromatography (GC). 1.2 mL samples were taken to *Eppendorf* tubes and, after centrifugation at 9167 g for 5 minutes (SIGMA 1-15P, Sartorius), the supernatant was discarded and the pellet washed with 1 mL Milli Q® water and centrifuged again in the same conditions. The supernatant was again removed and the pellet was stored at -20 ℃ until further use. The acidic methanolysis reaction consists in the addition of 1 mL of chloroform to the stored pellets, resuspension of the cells until complete detachment from the bottom of the *Eppendorf* tubes, sample transfer to Pirex glass tubes and addition to each tube of 1 mL of ”solution A”, which contains 97 mL methanol, 3 mL 96% H_2SO_4 and 330 µL hexanoic

acid. The tubes were then vortexed and incubated at 100 ℃ for 5 hours in an oven and throughout this time the samples were resuspended once. Afterwards, the samples were neutralized by adding 1 mL of 60 g/L Na_2CO_3 and centrifuged at 432 g for 5 min in a Heraeus Labofuge 200 from Thermo Scientific. More Na_2CO_3 solution was added if necessary to balance the tubes in the centrifuge. Finally, 200 µL of the organic phase (bottom phase) were taken and the P3HB concentration was determined using Gas Chromatography (GC).

3.2.10.7 Quantifications by Gas Chromatography

P3HB samples were analyzed through Gas Chromatography after being prepared as described in subsection **3.2.10.6**. The GC column used was a HP-5 from Agilent JW Scientific with a length of 30 meters and 0.32 mm of internal diameter. A gas chromatograph Agilent Technologies 5890 series II with a 7683B injector and a FID detector was used. The temperatures were set to 60 ℃, 120 ℃ and 150 ℃ for the oven, the injector and the detector, respectively. The software used for data acquisition and analysis was Shimadzu CBM-102 communication Bus Module and Shimadzu GC solution software (Version 2.3). For P3HB quantification, an internal standard of 3-methylhydroxybutyrate was used.

3.2.10.8 Biomass quantification

To evaluate the cell growth of the bacterial assays both the optical density at the wavelength of 600 nm as well as the cell dry weight (CDW) were measured. For the optical density, 1 mL aliquots were used for the spectrophotometer Hitachi U-200. In the HMF assay the samples were measured against Milli Q® water while in the P3HB fermentation assays, extra samples were centrifuged and the supernatant used as the blank. Dilution of the blank was the same as used for the sample. For the cell dry weight, 1.2 mL samples were taken into overnight dried and weighed *Eppendorf* tubes and were centrifuged at 9167 g for 5 minutes in a SIGMA 1-15P centrifuge from Sartorius. The supernatant was removed and the pellet washed with 1 mL Milli Q® water and the sample was centrifuged again in the same conditions. The supernatant was removed and the *Eppendorf* tubes with the pellet were put to dry at 60 ℃ for at least 48 hours and afterwards the samples were weighed and cell dry weight (CDW) was calculated.

Chapter 4

Results and Discussion

4.1 Characterization of the *Ulva lactuca* residues

The analysis of the composition of *Ulva lactuca* residues after protein extraction was essential to follow up the hydrolysis steps. For the acidic pre-treatment, a mixture of residues from batches U1.00618M and U1.00319M was used while for the combined hydrolysis and the remaining work a mixture of the residues from all three batches was used. **Table 4.1** displays these results.

Table 4.1: Chemical composition of the residues of *Ulva lactuca* after protein extraction from both batches U1.00618M and U1.00319M and from all of the three batches U1.00618M, U1.00319M and U1.00321MB2401

Batches U1.00618.M and U1.00319M		**Batches U1.00618M, U1.00319M and U1.00321MB2401**	
Parameter (Unit)	**Value**	**Parameter (Unit)**	**Value**
Moisture (%)	6.94 ± 0.75	Moisture (%)	11.14 ± 1.81
Total Solids (%)	93.04 ± 0.75	Total Solids (%)	88.86 ± 1.81
Ash (% dw)	25.32 ± 0.70	Ash (% dw)	21.88 ± 0.54
Total Carbohydrates (% dw)	32.67 ± 0.40	Total Carbohydrates (% dw)	44.02 ± 1.01
Glucose (% dw)	13.30 ± 0.27	Glucose (% dw)	28.66 ± 0.98
Xylose (% dw)	4.32 ± 0.11	Xylose (% dw)	3.96 ± 0.15
Rhamnose (% dw)	15.05 ± 0.27	Rhamnose (% dw)	11.39 ± 0.17
Glucuronic Acid (% dw)	21.76 ± 0.056	Glucuronic Acid (% dw)	21.15 ± 0.052

Jard et al. (2013) reports similar results for total solid and total sugar contents for *Ulva lactuca* of 83.3% and of 31.4% dw, respectively[78]. Amamou et al. (2018) reports a slightly higher total solid content of 90% while a lower total sugar content of 25.8% dw. It is important to note that the reported results were obtained for the residues of *U. lactuca* after protein extraction and not for the entire algae, meaning there should be a higher value of total carbohydrates in the latter, which account for the soluble carbohydrates

that were removed in the process. For ash content, Amamou et al. reports 32% dw, similar to the results obtained. Glucose, xylose and rhamnose were reported to constitute 15.2 ± 1.01% dw, 3.1 ± 0.18% dw and 7.5 ± 0.13% dw of *U. lactuca*'s content, respectively[79]. It is important to note that the chemical composition of macroalgae can vary due to many uncontrolled factors such as geographical origin, harvest season, environment growth or even the method utilized to quantify their composition.

A significant increase of the glucose content can be observed when the residues from all three batches are mixed, indicating that the algae from batch U1.00321MB2401 has a higher glucose content than the other two batches, possibly due to variations in the growth conditions imposed by ALGAplus. This will result in a discrepancy of results when comparing the values obtained for glucose concentrations in the acidic pre-treatment with the ones obtained in the pre-treatment of the combined hydrolysis.

4.2 Chemical pre-treatment of the algae residues

Degradation of the algae residues began with acidic hydrolysis. To optimize the conditions before proceeding to the combined hydrolysis, different concentrations of HCl and H_2SO_4 solutions prepared either with Milli Q® water (RO) or synthetic seawater (SW) and durations of thermal treatment were tested (see in **Table 3.1**).

Comparing both thermal treatments in **Figure 4.1**, it is seen that both glucose and rhamnose reach the highest concentrations for 1.5% and 1.0% of HCl, but in terms of yield, xylose seems to be the most released sugar. In the 15 minutes thermal treatment (left panel), the highest sugar concentrations are seen for the 1.5% HCl prepared with Milli Q® water, reaching 3.95 g/L of glucose, 1.57 g/L of xylose and 3.84 g/L of rhamnose, which correspond to yields of 63.7%, 78.3% and 54.9%, respectively. In the 30 minutes thermal treatment (right panel), 1.5% HCl treatment has the highest values, where the synthetic seawater HCl solution hydrolysis seems to achieve slightly higher sugar concentrations than the RO one. Hence, glucose reaches 4.25 g/L, xylose 1.58 g/L and rhamnose 4.36 g/L, corresponding to a yield of 68.7%, 78.9% and 62.3%, respectively. The concentration of HMF is slightly higher when the pre-treatment is done with HCl prepared with synthetic seawater, indicating that salt could play a role in degrading glucose altogether with this type of chemical treatment. Overall, the 30 minute treatment seems better since the release of sugars is higher. Here, the difference between the usage of synthetic seawater and Milli Q® water is not significant. However, considering 1.5% HCl condition, HMF's concentration is lower for the RO water (0.038 g/L) than for the SW (0.043 g/L) and thus, the 30 minute 1.5% (w/v) HCl prepared with Milli Q® water thermal treatment was considered the best option.

For the H_2SO_4 treatments, in **Figure 4.2,** HMF was only quantifiable and different from zero in the 1.5% (w/v) SW treatment, reaching 0.019 g/L for both 15 and 30 minutes thermal treatment in the autoclave. This corroborates with the results obtained for the HCl treatment, where salt seems to stimulate the production of HMF.

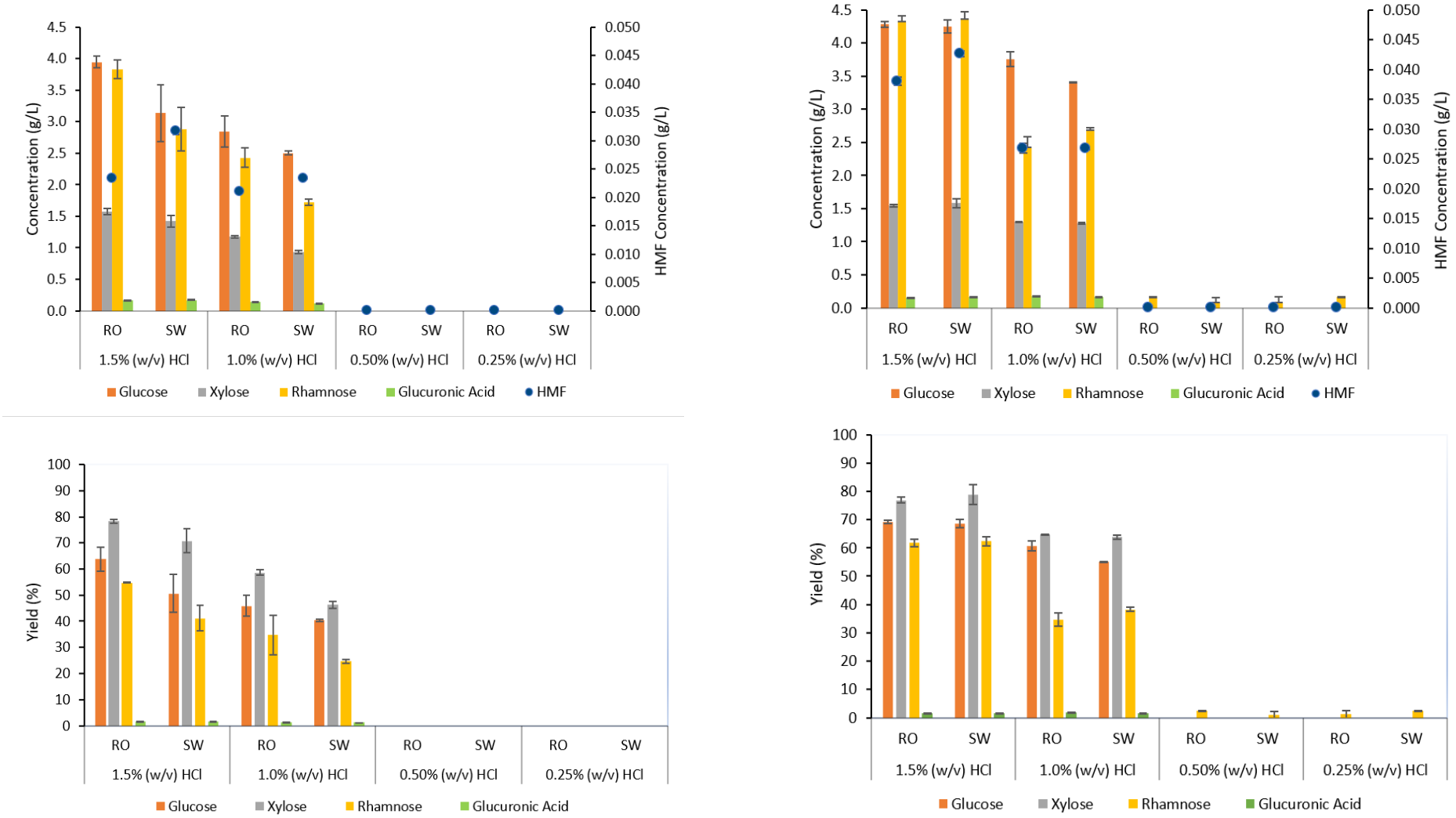


Figure 4.1: Chemical pre-treatment of *Ulva lactuca*'s residues performed with HCl solutions of 0.25, 0.50, 1.0 and 1.5% (w/v) concentrations prepared either with Milli Q® (RO) water or with synthetic seawater solution (SW) for a biomass concentration of 50 g/L tested for 15 or 30 minutes in the autoclave at 121℃. At the top are depicted the concentrations of released algae components of interest for 15 minutes of thermal treatment in the autoclave (top left) and for 30 minutes (top right) and in the bottom is each process components respective yields (bottom left corresponding to 15 minute process and bottom right 30 minute's one).

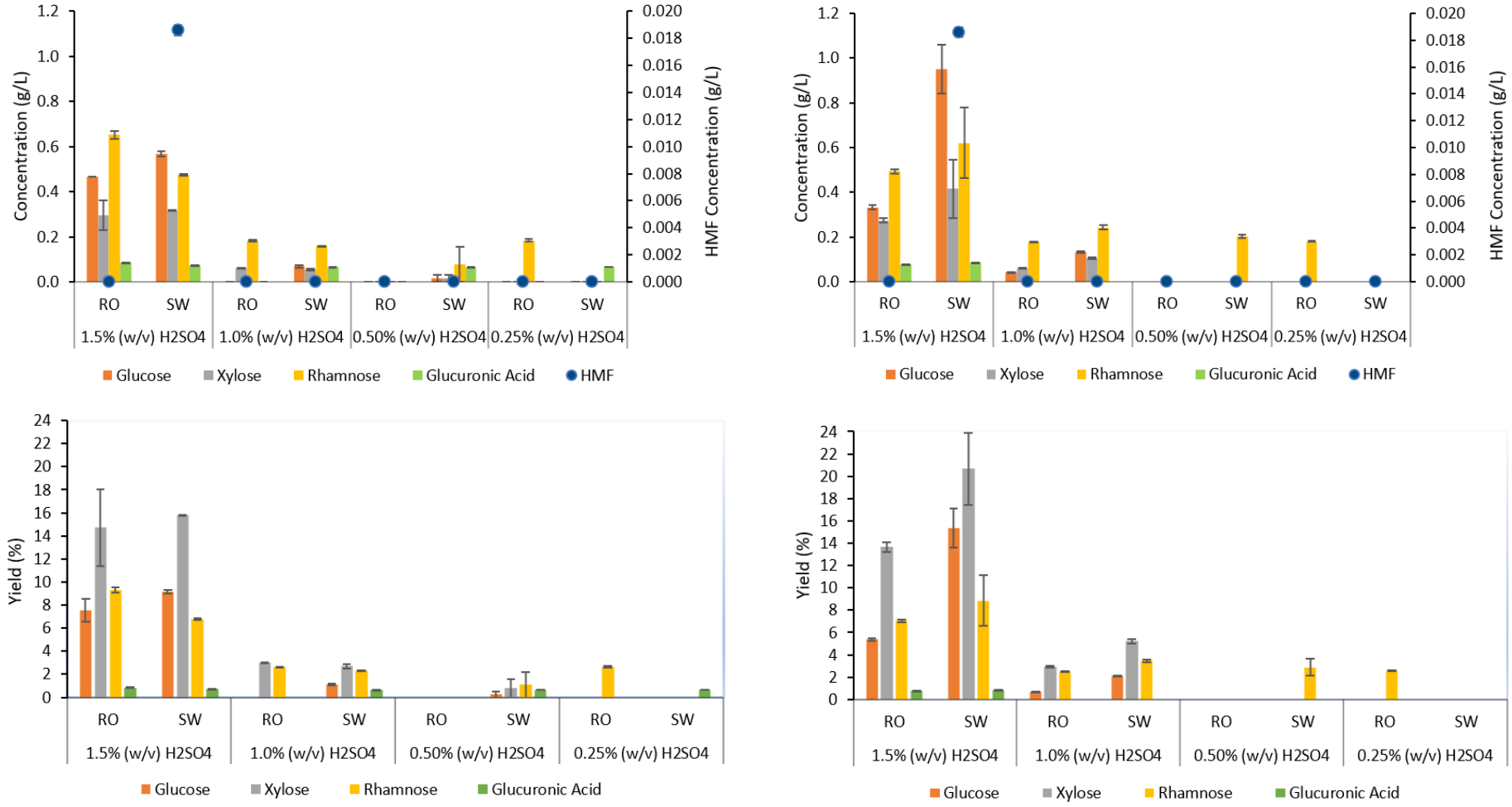


Figure 4.2: Chemical pre-treatment of *Ulva lactuca*'s residues performed with H_2SO_4 solutions of 0.25, 0.50, 1.0 and 1.5% (w/v) concentrations prepared either with Milli Q® (RO) water or with synthetic seawater solution (SW) for a biomass concentration of 50 g/L tested for 15 or 30 minutes in the autoclave at 121℃. At the top are depicted the concentrations of released algae components of interest for 15 minutes of thermal treatment in the autoclave (top left) and for 30 minutes (top right) and in the bottom is each process components respective yields (bottom left corresponding to 15 minute process and bottom right 30 minute's one).

Regarding sugar release, as expected, 1.5% H_2SO_4 conditions have the highest concentration values where, in the 15 minute treatment, the SW one reaches a slight higher value for glucose concentration, 0.57 g/L (9.2%), than the RO water one, which reaches 0.47 g/L (7.5%), although for rhamnose, the opposite happens, reaching a concentration of 0.47 g/L (6.8%) in SW and of 0.65 g/L (9.3%) in RO. For the 30 minute process, SW reached the highest values of released sugar, where glucose reached 0.95 g/L, xylose 0.42 g/L and rhamnose 0.62 g/L, corresponding to yields of 15.4%, 20.7% and 8.9%, respectively. Hence, between the 15 minute and the 30 minute processes, the latter seems to be better when using the 1.5% H_2SO_4 solution prepared with seawater. Greetham et al. (2020) tested diluted acid pre-treatments in 10% (w/v) *Ulva linza sp* with 1%, 2%, 3% and 5% H_2SO_4 prepared with either RO water or SW and, autoclaved for 15 minutes at 121 ℃[58]. For every acid concentration, SW was reported to have a higher sugar release than the one seen in RO water results, which, in this study, is observed for the 30 minute treatment with sulphuric acid, however, not in the 15 minute one.

Looking to **Figures 4.1** and **4.2**, an acidic treatment with HCl seems to hydrolyse the algae residues better than one done with H_2SO_4. However, to be able to claim that one acid is better than the other in the algae residues degradation, an assay testing the concentration of released sugars in normality (N) units was done, using 1 and 1.5% (w/v) H_2SO_4 as reference (see **Table 3.2**).

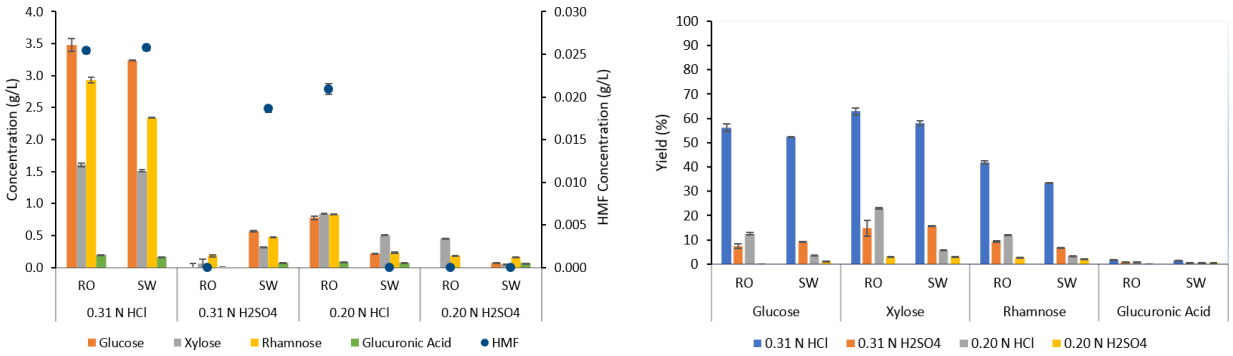


Figure 4.3: Comparison of chemical pre-treatments of *Ulva lactuca*'s residues performed with both HCl H_2SO_4 solutions of 0.20 N and 0.31 N prepared either with Milli Q® (RO) water or with synthetic seawater (SW) for a biomass concentration of 50 g/L and tested for 15 minutes in the autoclave at 121 ℃. H_2SO_4 concentrations of 1.0% w/v (0.20 N) and 1.5% w/v (0.31 N) were used as a reference. In the left side are depicted the concentrations of released algae components of interest and in the right is each process components respective yields.

It is seen that, for the same concentrations, HCl further hydrolyses the algae residues, always reaching higher concentrations of released sugars and thus higher HMF concentrations, making it a better choice than H_2SO_4, since the HMF concentrations are rather low for both acids in the tested conditions. Hence, 30 minute thermal treatment done with 1.5% HCl prepared with Milli Q® water was chosen for the next stage of combined hydrolysis.

4.2.1 Inhibitory effect of HMF on *Halomonas elongata* growth

To check the impact that HMF has on *Halomonas elongata*'s growth, an assay with different HMF concentrations was conducted and the specific growth rate for each HMF concentration calculated.

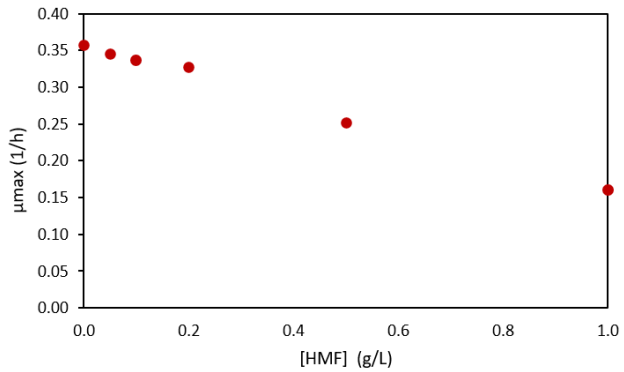


Figure 4.4: Inhibitory effect of HMF on *H. elongata*'s growth where the maximum specific growth rate (μ_{max}) is shown for the different tested HMF concentrations of 0.0 g/L, 0.05 g/L, 0.1 g/L, 0.2 g/L, 0.5 g/L and 1.0 g/L, all done in equal medium as the inoculum (see subsection **3.2.7**) for 20 g/L of glucose. To determine each μ_{max}, O.D.$_{600}$ was measured over a period of 10h covering the exponential growth phase and a plot with the logarithmic values of the O.D.$_{600}$ for the points in exponential phase was drawn. The slope from each linear regression obtained gave the μ_{max} values.

From the results obtained, a decrease of the maximum specific growth rate with HMF is observed, as expected. This decrease is rather low until 0.2 g/L of HMF is reached. However, at 0.5 g/L of HMF, the decrease of μ_{max} is noticeable. It can be then concluded that above 0.05 g/L of HMF and until 0.2 g/L of HMF, *H. elongata* growth is affected but not significantly, for these conditions.

4.3 Bradford assay and SDS-PAGE gel analysis

To quantify the protein content in the commercial enzymes utilized in this work, the Bradford assay was conducted with BSA as a standard. The quantified commercial enzymes were then run in a SDS-PAGE gel to evaluate on enzyme purity, shown in **Figure 4.5**.

Table 4.2: Results from Bradford assay performed for protein quantification of the commercial enzymes

	Celulase (NS 22086)	**β-Glucosidase (NS 22118)**	**Xylanase (NS 22083)**	**Xylanase/ β-glucanase (NS 22002)**
Concentration (mg/mL)	202.7 ± 7.8	124.7 ± 1.0	196.5 ± 6.1	109.3 ± 1.3

From the gel, it is seen that except for lanes 7 and 8, there are many bands in each lane, which indicates a rather low purity. The darker and blurrier bands from cellulase an xylanase lanes have a higher protein concentration and will probably belong to the respective enzyme. β-glucosidase lanes show 3 distinguishable bands with good resolution, which show this commercial enzyme has a higher purity than cellulase and xylanase, but no supposition can be done regarding the probability of which band could correspond to β-glucosidase. Finally, xylanase/β-glucanase mixture seems to have the highest purity, since there can only be seen 3 distinguishable bands and there are, at least, 2 enzymes in the mixture. It is known from Novozymes® that xylanase/β-glucanase is extracted from *Humicola insolens*.

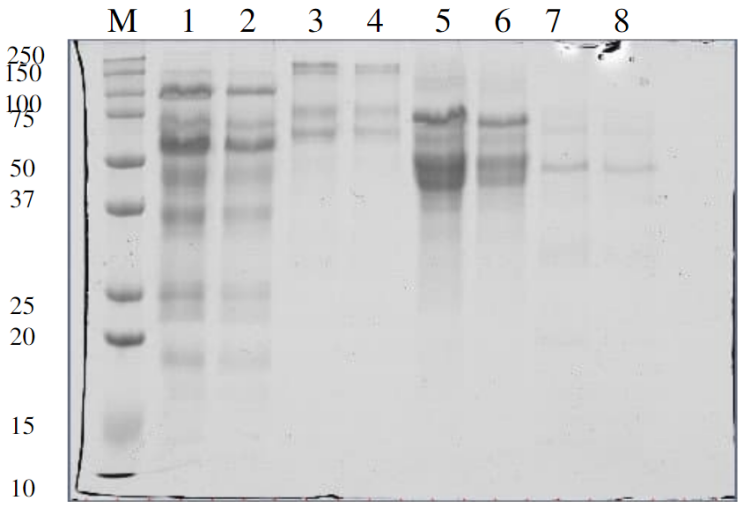


Figure 4.5: SDS-PAGE gel of commercial enzymes used in this work. Lane M represents the marker from Bio-Rad, where the standard protein molecular weights are represented in the left side, in kDa; lanes 1 and 2 are from cellulase (NS 22086), lanes 3 and 4 from β-glucosidase (NS 22118), lanes 5 and 6 from xylanase (NS 22083) and lanes 7 and 8 from xylanase/β-glucanase mixture (NS 22002). For each enzyme a 100-fold and 200-fold dilutions were ran, respectively, i.e. lane 1 is a 100-fold dilution of cellulase while lane 2 is a 200-fold one.

Comparing with other molecular weights found in literature, for β-1,4-glucanase, purified *HicelI6C* from *H. insolens* Y1 showed a molecular weight of approximately 50 kDa when run in SDS-PAGE gel[80]. As for xylanase, *XynW* also purified from *H. insolens* Y1 was found to have a molecular weight of 44 kDa when run in a SDS-PAGE gel[81] while Xyn11A and Xyn11B (xylanases also produced from *H. insolens* Y1) were found to have, respectively, 22.8 and 29.1 kDa[82]. From these results, one can conclude that the commercial enzymes used are not pure, being thus a mixture of different proteins. For this reason, the SDS-PAGE test cannot be used to determine the molecular weight of the enzymes of interest in this work.

4.4 Combined hydrolysis of *Ulva lactuca* residues

To further increase the amount of released sugars during the hydrolysis, the combined hydrolysis of the algae residues was performed. With the best condition already chosen for the chemical pre-treatment, the optimization of enzymatic hydrolysis was tested. The tested conditions are described in **Section 3.2.3**. These were tested with the mixture of algae residues from 3 different batches, thus the increase seen in released sugars at time t=0 hours, corresponds to the chemical pre-treatment. The biggest changes between batches were in glucose concentration, where, in the 3 batches mix, it increased significantly. The new concentrations and yields of released carbohydrates and concentration of HMF after chemical pre-treatment are shown in **Table 4.3**.

Regarding the enzymatic cocktails, it is expected that cocktail A increases the amount of released glucose from the algae residues, while cocktail B, in addition, is expected to increase the amount of xylose. Cocktail C is used to test if further cellulose degradation may occur by β-glucanase hydrolytic action, apart from glucose and xylose release due to cellulase, β-glucosidase and xylanase activity.

Table 4.3: Concentrations and yields of components of interest after chemical pre-treatment of *Ulva lactuca* residues from 3 the batches mixture

Parameter (Unit)	Glucose	Xylose	Rhamnose	Glucuronic Acid	HMF
Concentration (g/L)	7.92 ± 0.49	1.09 ± 0.12	2.73 ± 0.21	0.14 ± 0.00	0.044 ± 0.014
Yield (%)	66.8 ± 4.1	66.5 ± 7.4	58.0 ± 4.5	1.63 ± 0.03	-

Rhamnose release concentration is not expected to increase since no enzyme that breaks the bonds between glucuronic acid and rhamnose and iduronic acid and rhamnose, such as β-glucuronidase, nor xylose and rhamnose bonds, such as ulvan lyase, are used. In **Figure 4.6**, there is an increase of released glucose for every cocktail while rhamnose concentration remains unchanged, as expected. Xylose concentration reaches the highest values in cocktail B, which might be due to a higher concentration of xylanase present in commercial enzyme NS 22083 that could influence the results when using lower enzyme concentrations such as 0.25% (v/v).

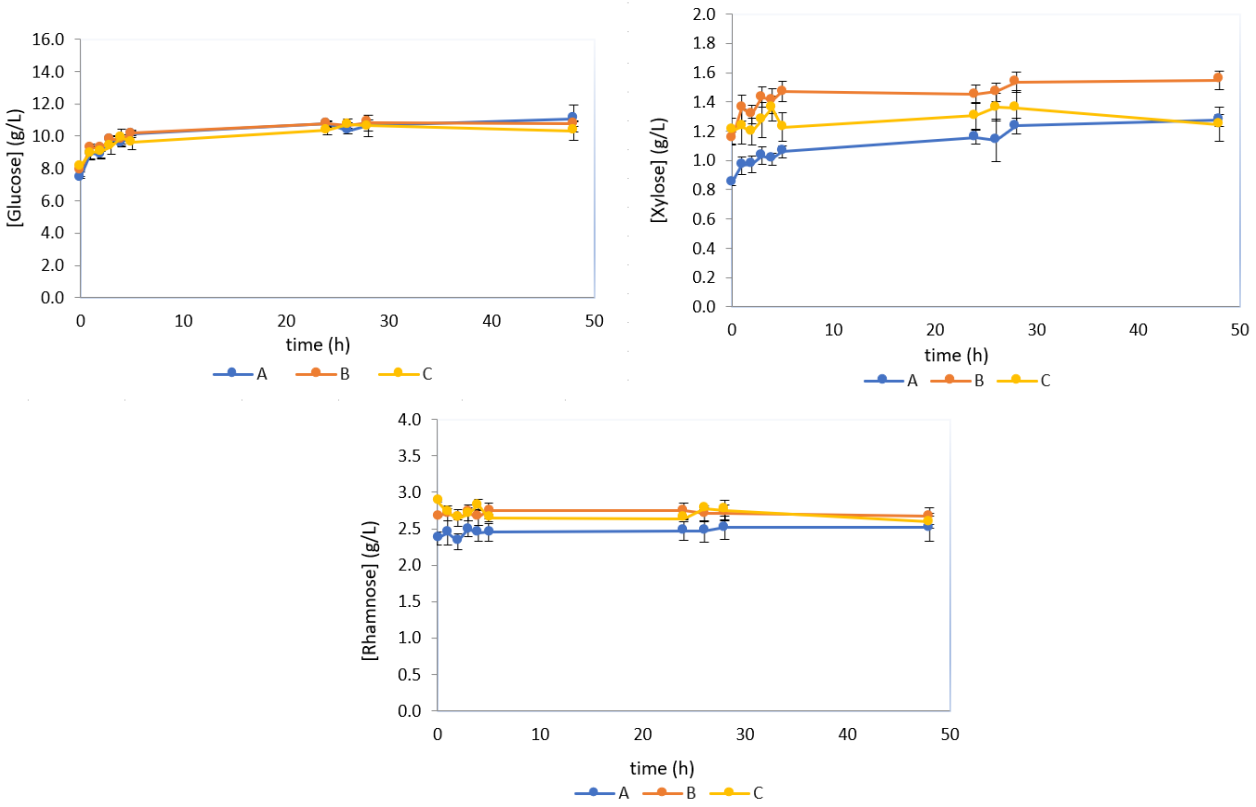


Figure 4.6: Combined hydrolysis of 5% (w/v) *Ulva lactuca* residues performed with Cocktails A (cellulase and β-glucosidase), B (cellulase, β-glucosidase and xylanase) and C (cellulase, β-glucosidase and β-glucanase/xylanase mixture) at 0.25% (v/v) of each enzyme during 48 hours at pH 4.8 and temperature of 50 ℃, after pre-treatment performed with 1.5% (w/v) HCl prepared with Milli Q® water for 30 minutes at 121 ℃. At time t=0 h, immediately prior to enzyme addition, each sugar concentration, in g/L, after chemical pre-treatment is represented. For times beyond 0 h, sugar contribution from the enzyme formulations was subtracted.

In **Figure 4.7**, Cocktail C seems to release more glucose than the other cocktails, which is supported by **Table 4.5** data. As previously mentioned, further xylose release was expected when cocktails B and C were used, although this was not the case with cocktail B. Comparing the standard deviations for xylose,

Table 4.4: Maximum reached concentrations of released sugars in the 0.25% (v/v) enzymatic hydrolysis step of combined hydrolysis and their respective incubation times and achieved recovery yields. Sugar concentrations and recovery yields account solely for the outcome of enzymatic hydrolysis.

Sugar	Cocktail	Concentration$_{max}$ (g/L)	time (h)	Recovery Yield (%)
Glucose	**A**	3.66	48	30.9
	B	3.01	28	24.6
	C	2.55	26	18.5
Xylose	**A**	0.38	28	25.6
	B	0.39	28	24.3
	C	0.16	26	9.9
Rhamnose	**A**	0.15	28	3.2
	B	0.07	24	0.0
	C	0.00	0	0.0

cocktail B has the largest ones for every point, which may be a reason for these results. Rhamnose concentration has low variation and comparing with the data in the same table, the recovery yields are either 0% or near it, as expected by the absence of an enzyme that cleaves the bonds associated with this sugar.

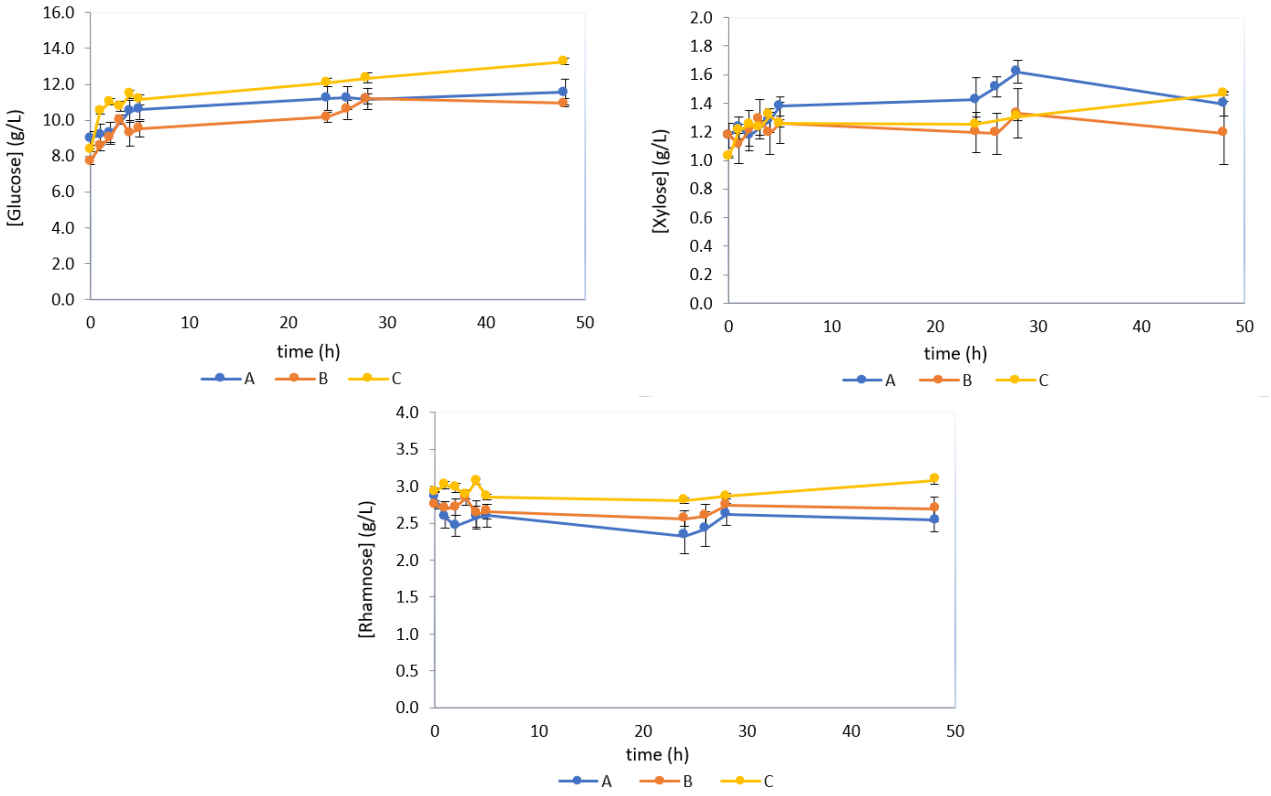


Figure 4.7: Combined hydrolysis of 5% (w/v) *Ulva lactuca* residues performed with Cocktails A (cellulase and β-glucosidase), B (cellulase, β-glucosidase and xylanase) and C (cellulase, β-glucosidase and β-glucanase/xylanase mixture) at 0.50% (v/v) of each enzyme during 48 hours at pH 4.8 and temperature of 50 ℃, after pre-treatment performed with 1.5% (w/v) HCl prepared with Milli Q® water for 30 minutes at 121 ℃. At time t=0 h, immediately prior to enzyme addition, each sugar concentration, in g/L, after chemical pre-treatment is represented. For times beyond 0 h, sugar contribution from the enzyme formulations was subtracted.

Table 4.5: Maximum reached concentrations of released sugars in the 0.50% (v/v) enzymatic hydrolysis step of combined hydrolysis and their respective incubation times and achieved recovery yields. Sugar concentrations and recovery yields account solely for the outcome of enzymatic hydrolysis.

Sugar	Cocktail	Concentration$_{max}$ (g /L)	time (h)	Recovery Yield (%)
Glucose	**A**	2.55	48	21.5
	B	3.45	28	27.2
	C	4.96	48	41.9
Xylose	**A**	0.45	28	13.4
	B	0.16	28	9.50
	C	0.44	48	27.0
Rhamnose	**A**	0.00	0	0.0
	B	0.10	3	0.0
	C	0.16	48	3.3

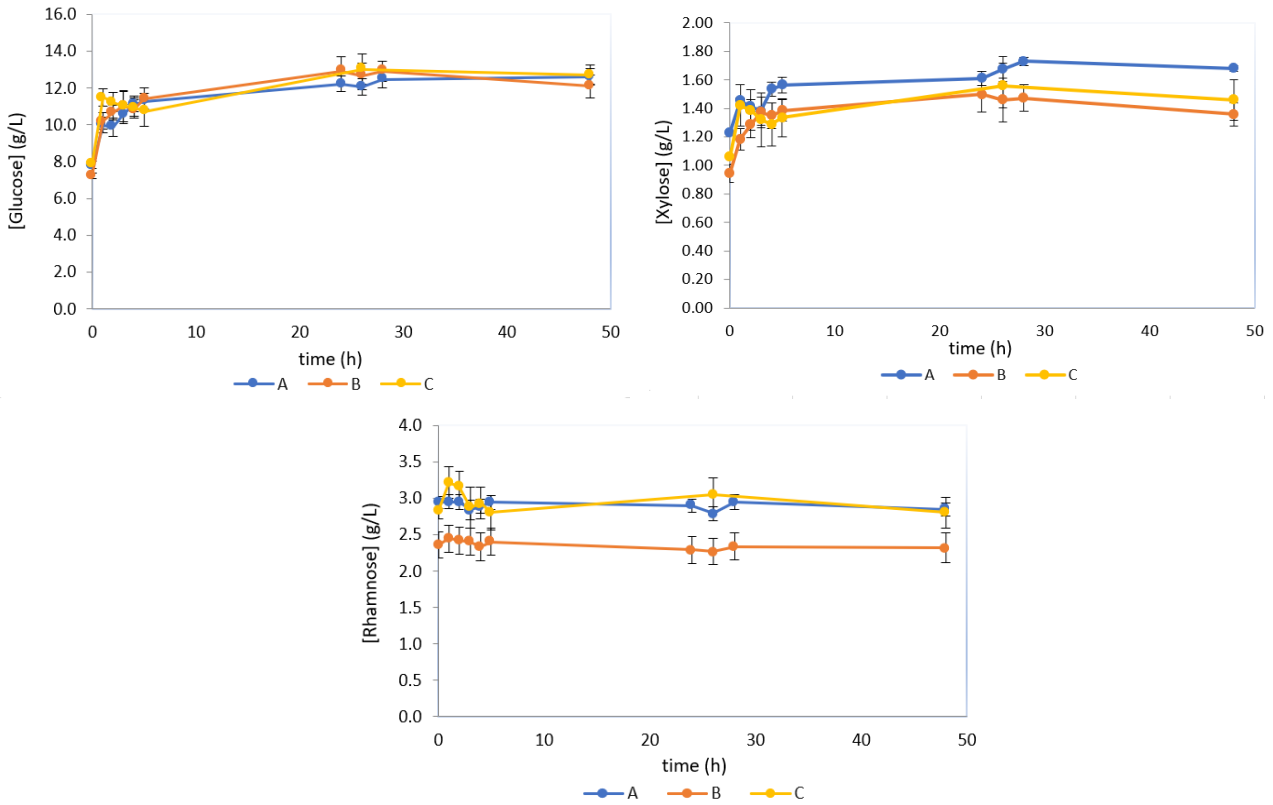


Figure 4.8: Combined hydrolysis of 5% (w/v) *Ulva lactuca* residues performed with Cocktails A (cellulase and β-glucosidase), B (cellulase, β-glucosidase and xylanase) and C (cellulase, β-glucosidase and β-glucanase/xylanase mixture) at 1.0% (v/v) of each enzyme during 48 hours at pH 4.8 and temperature of 50 ℃, after pre-treatment performed with 1.5% (w/v) HCl prepared with Milli Q® water for 30 minutes at 121 ℃. At time t=0 h, immediately prior to enzyme addition, each sugar concentration, in g/L, after chemical pre-treatment is represented. For times beyond 0 h, sugar contribution from the enzyme formulations was subtracted.

For 1% (v/v) enzymatic treatment, cocktail C reached higher maximum concentrations for every sugar in the enzymatic hydrolysis step, although in rhamnose, this difference was observed for incubation time of t=1h, which does not imply there was an increase of released sugar as it can be seen at time t=48h, when rhamnose concentration is equal as the one at time t=0h. Glucose release profile seems similar for every cocktail and, after 48 hours of incubation, each glucose concentration is almost the same.

Cocktail A reaches a higher concentration of xylose after 48 hours of hydrolysis, although it does not reach the highest concentration obtained with the enzymatic hydrolysis step.

Table 4.6: Maximum reached concentrations of released sugars in the 1.0% (v/v) enzymatic hydrolysis step of combined hydrolysis and their respective incubation times and achieved recovery yields. Sugar concentrations and recovery yields account solely for the outcome of enzymatic hydrolysis.

Sugar	Cocktail	Concentration$_{max}$ (g /L)	time (h)	Recovery Yield (%)
Glucose	**A**	4.85	48	40.9
	B	5.70	24	41.0
	C	6.28	28	41.1
Xylose	**A**	0.50	28	27.5
	B	0.55	24	25.1
	C	0.70	28	24.3
Rhamnose	**A**	0.01	1	0.0
	B	0.08	1	0.0
	C	0.39	1	0.0

In conclusion, the cocktails action is very similar and the increase in enzyme concentration only increases sugar concentration in a range of 0 to 2.93 g/L for glucose and of 0 to 0.41 g/L for xylose, when comparing the work of each enzymatic cocktail. This difference can be easily compensated in the subsequent hydrolysate concentration step and thus, in order to choose the most economically viable option, 0.25% (v/v) cocktail A was selected to operate during 24h.

4.5 Scale-up of combined hydrolysis

After optimizing the combined hydrolysis conditions, the production of the hydrolysate to be used in P3HB production fermentation assays was performed as described in **Subsection 3.2.4**, in a 6.3-fold scaled up combined hydrolysis. Results of released sugar yields of the whole process are shown in **Table 4.7**.

Table 4.7: Released sugar and inhibitor concentrations in g/L and sugar yield in % and of total released sugars in g/g dw and sugar recovery yield in % (g recovered sugar/ g sugar in biomass) after the 6.3-fold scaled up combined hydrolysis of *Ulva lactuca* residues (44.4 g/L biomass).

Sugar	Concentration (Unit)	Sugar recovery yield (Unit)
Glucose	9.85 ± 0.24 (g/L)	83.2 (%)
Xylose	1.43 ± 0.03 (g/L)	87.2 (%)
Rhamnose	2.84 ± 0.07 (g/L)	60.4 (%)
HMF	0.036 ± 0.005 (g/L)	-
Total Sugars	0.357 ± 0.001 (g/ g dw)	81.2 (%)

4.6 Concentration of hydrolysates produced from algae residues

Before initiating the concentration step, the produced hydrolysate from the 6.3-fold scaled-up combined hydrolysis was centrifuged and vacuum filtered to remove most of suspended particles in the mix-

ture and the filtrate was distributed among 6 *Schott* flasks of 100 mL. To concentrate, the hydrolysate was then placed in an oven at 60℃ for 82 hours. The processes results are shown in **Table 4.8**.

Table 4.8: Summarized results of centrifugation and filtration and of concentration processes related to 2 batches of hydrolysate from *Ulva lactuca* residues

Centrifugation and Filtration		**Concentration**	
Results (per batch)			
Parameter (Unit)	**Value**	**Parameter (Unit)**	**Value**
Initial Volume (mL)	563	Initial Volume per flask (mL)	80 (approx.)
Final Volume (mL)	484	Final Volume (mL)	41
Yield (%)	85.97	Concentration Factor	11.80
[Glucose]$_{final}$ (g/L)	11.33 ± 0.17	[Glucose]$_{final}$ (g/L)	119.02 ± 0.19
End Colour	Light gold	End Colour	Dark brown

Two batches of hydrolysate were produced beginning with a total volume of 1126 mL (563 mL per batch) resulting in a total volume of 82 mL of concentrated hydrolysate, after the whole process. Each 100 mL *Schott* had around 7 mL of concentrate at the end, which altogether resulted in a glucose concentration of 119.02 ± 0.19 g/L. After sample analysis by HPLC, it was noticed that the peak corresponding to HMF, that had previously been analysed at the end of the combined hydrolysis, was no longer in the chromatogram but instead, a peak near HMF's retention time had appeared. This could indicate that a component with similar molecular structure of HMF could have possibly been produced with HMF's degradation by heat exposure. In order to decide the temperature for the concentration process, previous tests were made with the combined hydrolysis hydrolysates from all the cocktails and concentrations previously tested, where, after centrifugation and vacuum filtration, they were concentrated at 60℃ in the oven in 100 mL *Schott* flasks. The flasks were filled with approximately 80 mL of hydrolysate and ended up with 20 mL after 48h in the oven. Samples were taken over the 48h period and ran in HPLC. Analysing these results, it was seen an increase on HMF's concentration following a similar concentration factor as glucose's, which led to the hypothesis that glucose and HMF were not being degraded at this temperature. These results are not shown in this thesis due to lack of rigorous data and since there were too many uncontrolled variables. The main differences between these tests and the results shown in this work are the different exposure times to heat and different final volumes, besides the different scale and conditions of combined hydrolysis.

4.7 P3HB production assays

The production of P3HB by *H. elongata* with the prepared hydrolysate was tested in 250 mL shake flasks, where the nitrogen content varied in each experimented condition to check upon its influence on the polymer production. According to the results obtained with LCK338 Laton Total Nitrogen test kit,

Table 4.9: Properties of the concentrated hydrolysate produced from the algae residues. Total nitrogen quantification was performed using one standard LCK338 Laton Total Nitrogen test kit, thus the lack of calculated standard deviation.

Hydrolysate Properties	
Parameter (Unit)	**Value**
Volume (mL)	82
pH	7.5
[Glucose] (g/L)	119.02 ± 0.19
[Xylose] (g/L)	14.77 ± 0.10
[Rhamnose] (g/L)	29.76 ± 0.84
[Total Nitrogen] (g/L)	4.8
[HMF] (g/L)	undetectable
Appearence	dark brown fluid liquid

the hydrolysate had 4.8 g/L of total nitrogen, however, part of that N might not be available for bacterial intake. Since a reduced nitrogen content increases P3HB production, the lesser available nitrogen in the hydrolysate the better. The worst case scenario for P3HB production when supplementing the culture mineral medium (see **subsection 3.2.9**) with only hydrolysate and no external nitrogen sources (condition A), would be a C/N ratio of 11.5, if all of the 4.8 g/L of N in the hydrolysate were to be available for bacterial intake. Four fermentation conditions were tested for P3HB production and each medium component is described in **Table 4.10**.

Table 4.10: P3HB production media used for each tested condition in *Halomonas elongata* fermentation.

P3HB Production Media							
Condition	**Tris (g/L)**	**MSG.H_2O (g/L)**	**K_2HPO_4 (g/L)**	**$MgSO_4.7H_2O$ (g/L)**	**NH_4Cl (g/L)**	**NaCl (g/L)**	**Trace elements (ml/L)**
A	15	0	3.0	2.5	0	45	1.0
B	15	8.9	3.0	2.5	0	45	1.0
C	15	0	3.0	2.5	1.0	45	1.0
D	15	1.0	3.0	2.5	1.0	45	1.0

To follow cell growth, neither optical density at 600 nm nor cell dry weight quantification were adequate methods due to the interference of suspended particles present in the hydrolysate and thus, are not represented in **Figure 4.9**. If protein levels were also to be quantified as an attempt to measure biomass growth, the problem would persist since nitrogen content from the hydrolysate would also interfere. It was observed, however, an increase of biomass over time through the pellet of the centrifuged samples with a clear distinction between the hydrolysate (brown appearance) and biomass (white appearance).

Analysing **Figure 4.9**, a significant glucose consumption is observed only after 72h of fermentation for every condition and the pH does not change significantly until after 72h of incubation time as well. *Halomonas elongata* growth seem to be inhibited for at least 3 days, although, after 72h, condition A

already showed signs of biomass growth through pellet observation. This could be the reason why a higher P3HB production in condition A is observed, while the other conditions showed a negligible P3HB production after 96h of cultivation. Growth inhibition is probably due to the unknown component, similar to HMF which is present in the medium (as mentioned in **Section 4.6**). It is hypothesized that this component might be levulinic acid because it results from HMF degradation after heat exposure and also inhibits microbial growth[83].

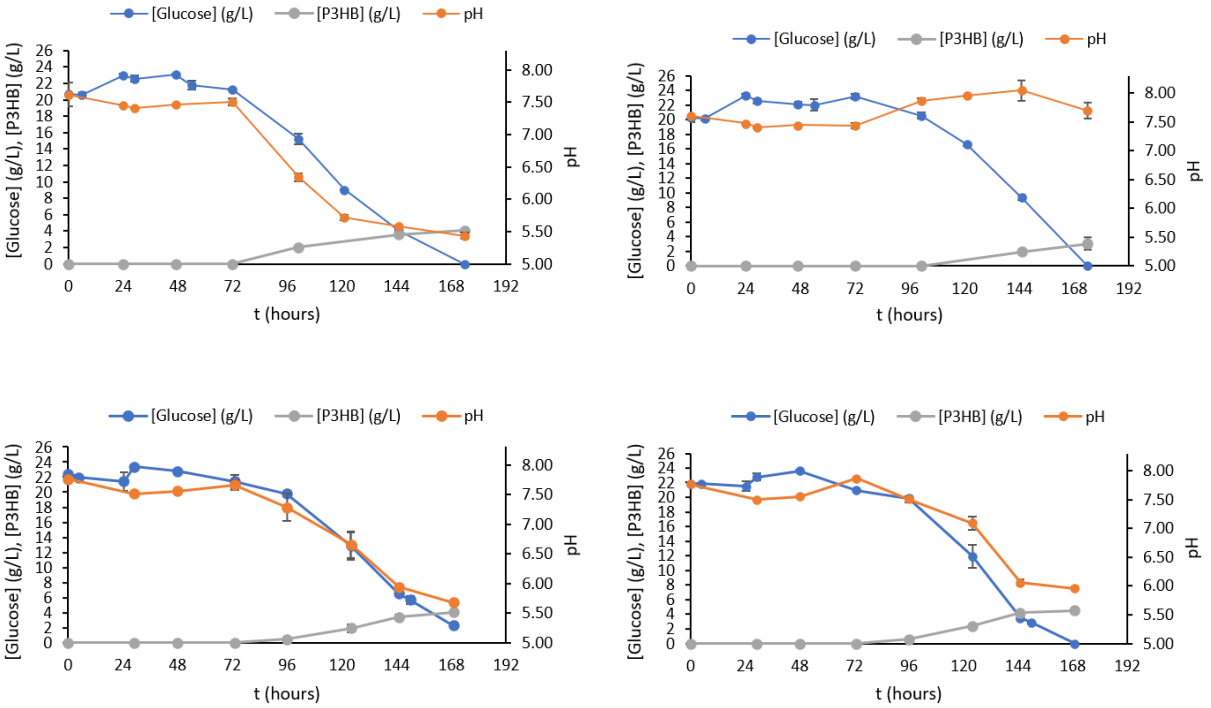


Figure 4.9: P3HB production by *Halomonas elongata* using *Ulva lactuca* residue hydrolysate as a carbon rich source, replacing the use of pure glucose. In the left axis the glucose and polymer concentrations are represented, in g/L, while in the right axis the pH values are represented throughout the fermentation time. The top part figure represents Conditions A (top left) and B (top right) which were carried out simultaneously in parallel, while the bottom part represents tested Conditions C (bottom left) and D (bottom right), also carried out simultaneously in parallel. Condition A consists in a fermentation using hydrolysate and mineral media described in **Subsection 3.2.9** without MSG and NH_4Cl; Conditions B, C and D are similar to Condition A, but the MSG and NH_4Cl concentrations are, respectively, 8.9 g/L and 0.0 g/L, 0.0 g/L and 1.0 g/L, and 1 g/L for each. The assays were performed at 30 ℃ with 200 rpm shaking frequency.

Table 4.11: Comparison between each medium used to produce P3HB by *Halomonas elongata* using algae residues hydrolysate in terms of yield of product over substrate, in g P/ g $S_{cons.}$, maximum productivity, in g/(L.h), and maximum P3HB concentration, in g/L, respectively.

Parameter / Condition	**$Y_{P/S}$ (g P/g S_{cons})**	**$Prod_{max}$ (g/(L.h))**	**[P3HB] (g/L)**
A	0.20	0.023	4.07
B	0.15	0.018	3.06
C	0.18	0.024	4.07
D	0.21	0.027	4.53

Results from **Table 4.11** show the yield of polymer over substrate, the maximum productivity and the maximum P3HB concentration for each condition. Conditions A and C show the same P3HB maximum concentration, almost the same maximum productivity and similar yields. The yields diverge more than the productivity due to conditions A and B starting with higher glucose concentration (approx. 22 g/L) than conditions C and D (approx. 20 g/L). Condition D has the highest values in each parameter while condition B has the lowest. It is clear that MSG influences polymer production the most within the tested conditions. The lower polymer production attained in condition B seems to be caused by the high MSG concentrations and thus higher N concentrations in the cultivation medium. Similar results were attained by Tůma et al. (2020), using *Gelidium sesquipedale* residues and *Halomonas boliviensis*[72]. Condition D, however, with 1 g/L of MSG and 1 g/L NH_4Cl, increases the nitrogen content over the already existent one from the hydrolysate and still has higher P3HB production than condition A and C. Therefore, MSG has a greater impact than NH_4Cl in the polymer production and, to optimize it, there should be a balance between the C/N ratio and the added MSG content, since condition A has a higher C/N ratio (11.5 considering hydrolysate's total nitrogen availability) than condition D (8.41 considering hydrolysate's total nitrogen availability). Tůma et al. (2020), reported 2.7 g/L of P3HB produced for 1 g/L NH_4Cl and 0 g/L MSG for *H. boliviensis* using a similar production medium with *Gelidium sesquipedale* residue hydrolysate as a carbon rich source. Within their tested conditions, the ones with lower nitrogen content that still contained MSG reached higher P3HB concentrations, corroborating the obtained results[72].

Chapter 5

Conclusions

Biodegradable plastic production is a market in expansion. Research towards greener alternatives to oil-based plastics has led to the development of biorefinery techniques. However, production costs of bio-based and biodegradable plastics remain high compared to oil-based ones. A solution towards this problem goes by employing low-cost carbon-rich materials as feedstocks, such as agriculture or industrial wastes.

In this work, residues of *U. lactuca* algae after protein extraction were used as a carbon-rich feedstock to produce P3HB by *Halomonas elongata*. In order to obtain carbon-rich hydrolysates from these residues, different chemical pre-treatments were initially tested. Highest sugar yields were attained using 1.5 %(w/v) HCl for 30 minutes at 121 ºC, with a yield of 63.7% in released glucose. Pre-treated residues were then subjected to enzymatic hydrolysis using different enzyme cocktails and concentrations, where it was decided to choose the most economically efficient condition. This was verified for the cocktail with 0.25 %(v/v) of cellulase (278 FPU/L) and β-glucosidase (50.3 CBU/L). To produce higher amounts of hydrolysate for the P3HB production assays, a 6.3-fold scale-up of the combined hydrolysis with the optimized conditions was carried out. Total sugar recovery yield of the whole process was 81.2 % (g sugar/ g total sugar biomass). During this process, 0.04 g/L hydroxymethylfurfural (HMF) was formed. According to the effect of HMF on the specific growth rate of *H. elongata*, concentrations of HMF between 0.05 g/L and 0.2 g/L were not inhibitory. However, after solvent evaporation at 60 ºC to concentrate the hydrolysate to be used as carbon source in shake flasks assays, HPLC results showed HMF was no longer visible but instead, a new peak with a retention time close to that of HMF was detected, indicating that a new compound was formed through the thermal degradation of HMF.

For P3HB production assays, the produced concentrated hydrolysate was used and different concentrations of nitrogen source in the medium were tested under non-sterile environment. Maximum productivity and polymer concentration were obtained with 1.0 g/L of MSG.H_2O and 1.0 g/L of NH_4Cl, resulting in 0.027 g/(L.h) and 4.5 g/L, respectively. This rather low productivity is caused by the 72 h lag period due to growth inhibition possibly caused by the presence of the compound formed after HMF's thermal degradation. The hypothesis of this compound being levulinic acid is plausible since it is reported to result from HMF's degradation after heat exposure and to inhibit microbial growth.

Overall, it was demonstrated that high yields of released sugars with low titers of HMF can be obtained through combined hydrolysis of *U. lactuca* residues using mild HCl conditions in the chemical pre-treatment. The resulting hydrolysate proved feasible as carbon source for the production of P3HB by *H. elongata* in unsterile shake flask fermentations.

5.1 Future Work

In the immediate future, in order to avoid HMF thermal degradation, the concentration step by solvent evaporation should be further tested either by vacuum-evaporation or by reducing heat exposure time of the hydrolysate. Other concentration methods such as membrane processes could also be explored to avoid thermal degradation of sugars and to lower processing costs. Nanofiltration, reverse osmosis, osmotic or membrane distillations are typical examples of these processes, used to concentrate sugar syrups[84].

On a later stage, batch and fed-batch modes of operation in bench-scale stirred bioreactors should be addressed for P3HB production by *H. elongata* using the optimized hydrolysate conditions, inferring about production costs reduction.

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Appendix A

Equations

A.1 Equations used for carbohydrate and HMF quantification

A.1.1 Glucose concentration for a range between 0.05 g/L and 3.64 g/L

$$Glucose\,(g/L) = 5.80 \times 10^{-6} \cdot Peak\,Area + 2.91 \times 10^{-2},\ R^2 = 1 \tag{A.1}$$

A.1.2 Glucose concentration for a range between 0.5 g/L and 100 g/L

$$Glucose\,(g/L) = 5.72 \times 10^{-6} \cdot Peak\,Area - 7.10 \times 10^{-2},\ R^2 = 1 \tag{A.2}$$

A.1.3 Xylose concentration for a range between 0.1 g/L and 4.0 g/L

$$Xylose\,(g/L) = 6.14 \times 10^{-6} \cdot Peak\,Area + 3.09 \times 10^{-2},\ R^2 = 1 \tag{A.3}$$

A.1.4 Rhamnose concentration for a range between 0.2 g/L and 20 g/L

$$Rhamnose\,(g/L) = 6.18 \times 10^{-6} \cdot Peak\,Area + 1.52 \times 10^{-1},\ R^2 = 1 \tag{A.4}$$

A.1.5 Glucuronic acid concentration for a range between 0.1 g/L and 10 g/L

$$Glucuronic\,Acid\,(g/L) = 2.25 \times 10^{-7} \cdot Peak\,Area + 6.77 \times 10^{-2},\ R^2 = 1 \tag{A.5}$$

A.1.6 5-hydroxymethylfurfural for a range between 0.01 g/L to 2.56 g/L

$$HMF\,(g/L) = 1.80 \times 10^{-7} \cdot \ Peak\,Area \ + \ 1.57 \times 10^{-2},\ R^2 = 1 \tag{A.6}$$

A.2 Equation used for P3HB quantification

$$P3HB\,(g/L) = \left(7.924 \cdot \ \frac{P3HB\ Peak\ Area}{IS\ Peak\ Area} \ + \ 0.399\right) \div 1.2 \tag{A.7}$$

where IS stands for internal standard which in this case is 3-methylhydroxybutyrate and 1.2 is the sample volume, in mL.

Appendix B

Supplementary illustrations

B.1 Hydrolysate before and after the concentration step

Figure B.1: Centrifuged and filtered hydrolysate after scaled-up combined hydrolysis distributed among the 6 *Schott* flasks right before the concentration step

Figure B.2: Concentrated hydrolysate used in the P3HB production assays

B.2 Chromatograms

B.2.1 Sugars chromatogram after scaled-up combined hydrolysis

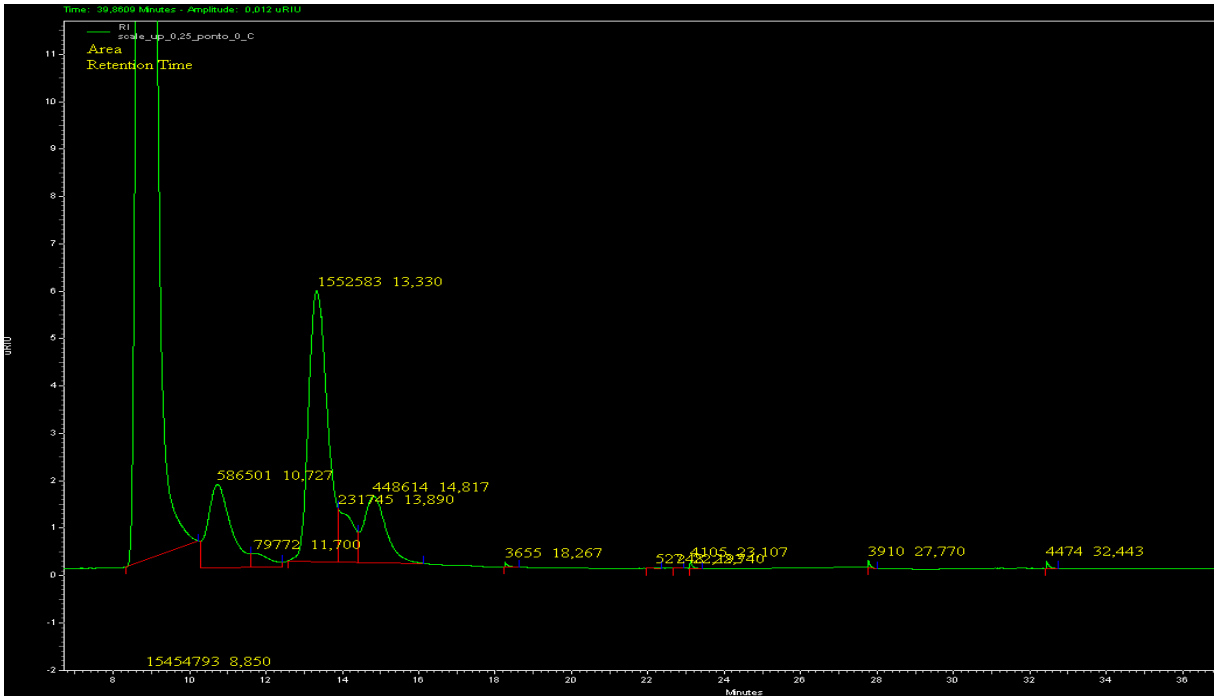

Figure B.3: Example of a chromatogram (RI) of a sample ran in the HPLC after the scaled-up combined hydrolysis. The peaks with retention times of 13.330 min, 13,890 min and 14.817 min correspond to glucose, xylose and rhamnose, respectively.

B.2.2 UV-range chromatogram after scaled-up combined hydrolysis

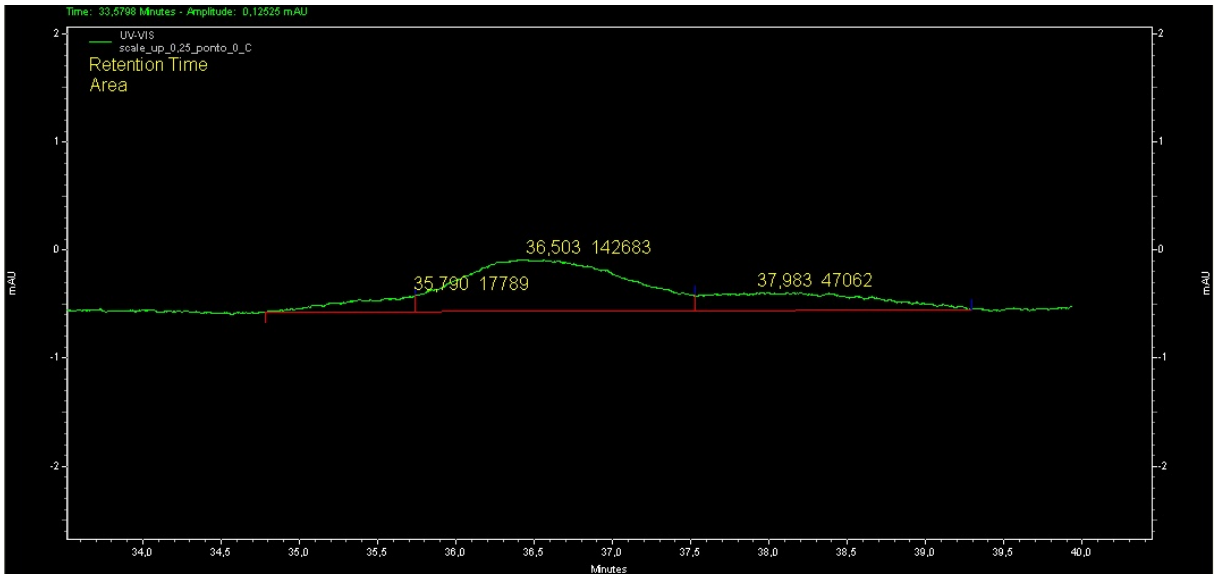

Figure B.4: Example of a chromatogram (UV-VIS) of a sample ran in the HPLC after the scaled-up combined hydrolysis. The chromatogram was redimensioned to present the peaks between the range of 33.5 min to 40.5 min where 3 peaks are observed. Only the peak at a retention time of around 36.5 min was identified and it corresponded to HMF.

B.2.3 Sugars chromatogram after the concentration step

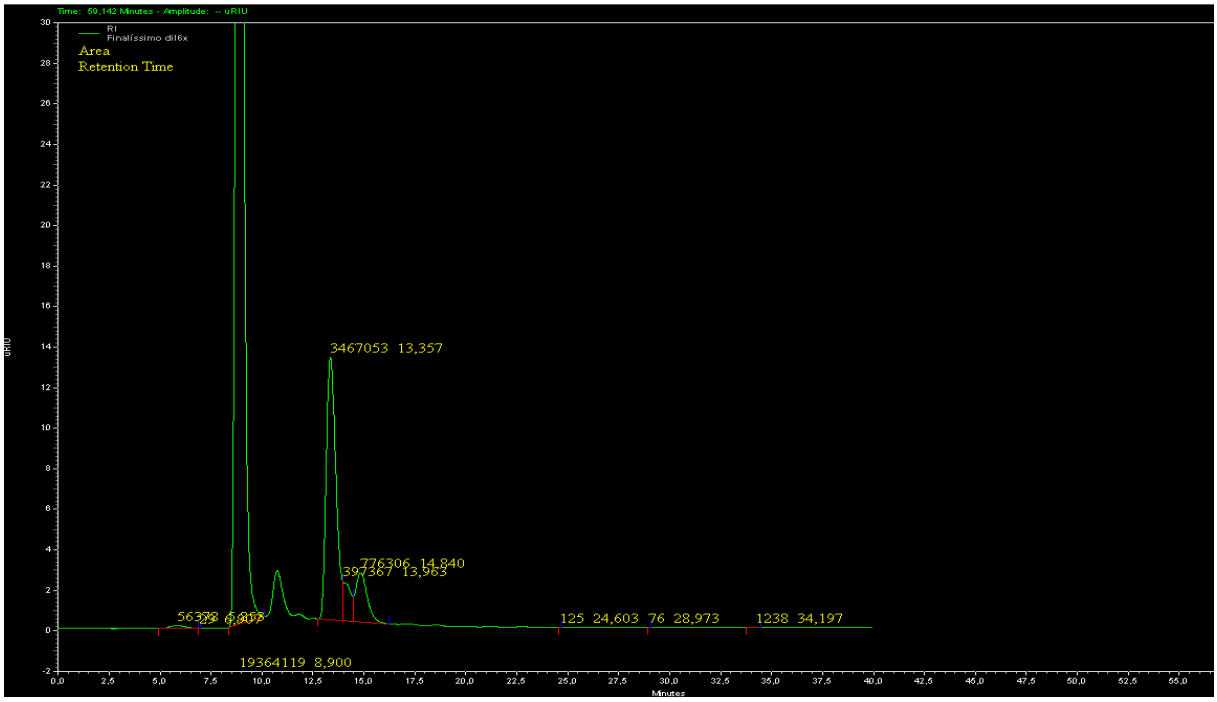

Figure B.5: Chromatogram (RI) of a 6-fold diluted sample of hydrolysate ran in the HPLC after the concentration step. The peaks with retention times of 13.357 min, 13,963 min and 14.840 min correspond to glucose, xylose and rhamnose, respectively.

B.2.4 UV-range chromatogram after the concentration step

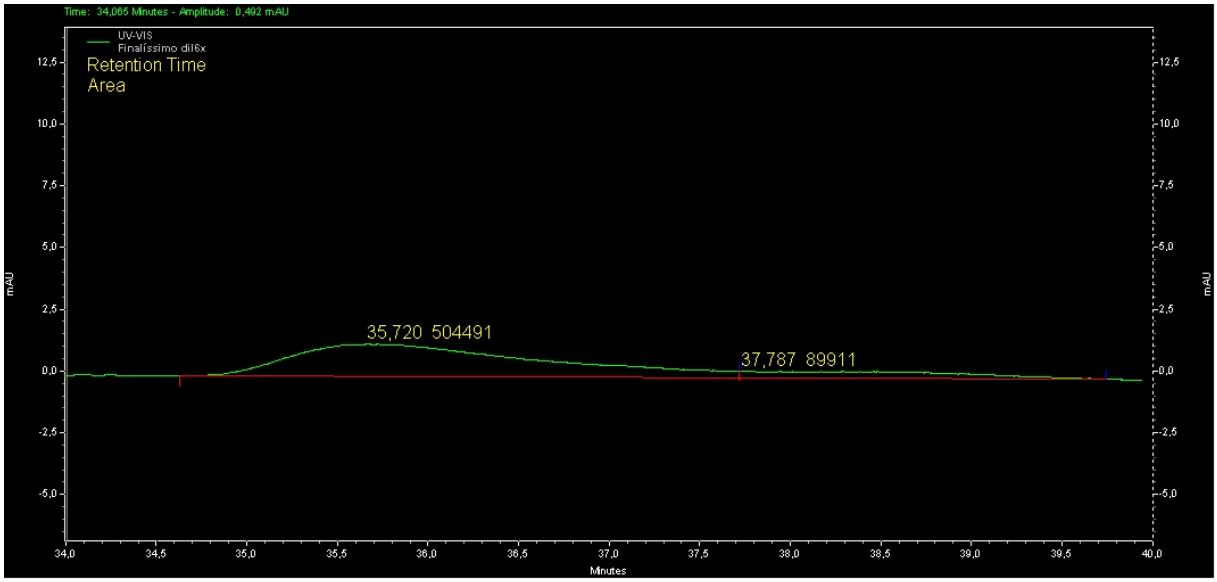

Figure B.6: Chromatogram (UV-VIS) of a 6-fold diluted sample of hydrolysate ran in the HPLC after the concentration step where, after redimensioning the X-axis to the range of retention times between 34 min and 40 min, 2 peaks are observed. A peak at around 36.5 min corresponding to HFM is no longer observed, however, the area from the peak at 35.7 min has increased significantly.