# From *Ulva lactuca* to Fermentable Sugars and Later Up-grade

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Thesis to obtain the Master of Science Degree in

**Biotechnology**

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## Declaration

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

## Preface

The work presented in this thesis was performed at the Institute for Bioengineering and Biosciences of Instituto Superior Técnico (Lisbon, Portugal), during the period February-November 2020, under the supervision of Prof. Maria Teresa Ferreira Cesário Smolders and Prof. Pedro Carlos de Barros Fernandes

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Huge thanks to my family and friends, for all the support, encouragement and motivation.

## Abstract

Biobased plastics synthesized from renewable resources, such as polyhydroxyalkanoates (PHAs), have gained attention, due to the search for more sustainable and environmentally friendly approaches. PHAs can be synthesized by a wide range of bacteria but most species produce PHA under conditions of excess of carbon and a limiting nutrient. This study addresses the hydrolysis, in different conditions, of the polysaccharides of the green algae *Ulva lactuca* to monosaccharides for further production of PHAs by *Halomonas elongata*. The results show the inability of the enzymatic cocktail alone to release rhamnose from ulvan, since its release in a significant amount requires an acid pre-treatment with at least 1.0% (w/v) of sulfuric acid ($H_2SO_4$) or with 2 M trifluoroacetic acid (TFA). It was also demonstrated that the enzymatic treatment with the highest monosaccharides release, from using a biomass concentration of 43.2 g/l, involved the combination of four enzymes namely cellulase, β-glucosidase, glucoamylase and α-amylase where the highest concentration was 9.6 g/l after a chemical pre-treatment with TFA 2 M. However, no growth was observed using the TFA hydrolysate due to the presence of 5-hydroxymethylfurfural (HMF) and furfural produced during acid pre-treatment. The hydrolysate prepared with 1.0% $H_2SO_4$ allowed cell growth since furfural was not detected and the HMF levels were lower. However, no poly-3-hydroxybutyrate (PHB) accumulation was observed since C/N ratio was too low due to the high nitrogen content in the *U. lactuca* hydrolysates.

**Keywords:** *Ulva lactuca*, *Halomonas elongata*, Acid Pre-treatment, Enzymatic Hydrolysis, Bioplastic, Polyhydroxyalkanoates

## Resumo

Os polihidroxialcanoatos (PHAs), bioplásticos produzidos a partir de fontes renováveis, têm sido alvo de uma maior atenção, devido à procura por processos mais sustentáveis e ecológicos. Os PHAs podem ser produzidos por uma grande variedade de bactérias mas na maioria das espécies a produção ocorre sob condições de stress por falta de um nutriente e em excesso de carbono. Neste trabalho são abordadas diferentes condições para a hidrólise dos polissacáridos da alga verde *Ulva lactuca* em monossacáridos para a produção de PHAs pela *Halomonas elongata*. Os resultados demonstram que o cocktail enzimático não é capaz de clivar ulvano libertando ramnose, pois para se obter uma quantidade significativa é necessário um pré-tratamento químico com pelo menos 1,0% (m/v) de $H_2SO_4$ ou 2 M de ácido trifluoroacético (TFA). Também foi demonstrado que o tratamento enzimático com maior produção de monossacáridos, a partir de 43,2 g/l de *U. lactuca*, envolve a combinação de quatro enzimas: cellulase, β-glucosidase, glucoamilase e α-amilase, sendo o hidrolisado com maior concentração de carbohidratos (9,6 g/l) obtido após pré-tratamento químico com 2 M de TFA. Contudo, não foi observado crescimento usando este hidrolisado devido à presença de furfural e 5-hidroximetilfurfural (HMF) produzidos durante o pré-tratamento. Por outro lado, quando usado o hidrolisado preparado com 1,0% (m/v) de $H_2SO_4$, onde não foi detectado furfural e os níveis de HMF eram menores, foi observado crescimento. Contudo, não existiu acumulação de poli-3-hidroxibutirato (PHB) devido à baixa razão C/N provocada pelo alto teor em azoto do hidrolisado da *U. lactuca*.

**Palavras-chave:** *Ulva lactuca*, *Halomonas elongata*, Pré-tratamento ácido, Hidrólise Enzimática, Bioplástico, Polihidroxialcanoatos

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## Abbreviations

| | |
|---|---|
| CBP | Consolidated bioprocess |
| CDW | Cell dry weight |
| DNS | 3,5-Dinitrosalicylic acid |
| DTT | Dithiothreitol |
| dw | Dry weight |
| GC | Gas chromatography |
| HMF | 5-Hydroxymethylfurfural |
| HPLC | High-performance liquid chromatography |
| $OD_{600}$ | Optical density at 600 nm |
| PBS | Poly(butylene succinate) |
| PHA | Polyhydroxyalkanoate |
| PHB or P(3HB) | Poly-3-hydroxybutyrate |
| PLA | Polylactic acid |
| PSSSF or PSSF | Pre-saccharification and simultaneous saccharification and fermentation |
| PUFA | Polyunsaturated fatty acids |
| SDS | Sodium dodecyl sulphate |
| SHF | Separate hydrolysis and fermentation |
| SSF | Simultaneous saccharification and fermentation |
| TFA | Trifluoroacetic acid |

# 1. Introduction

## 1.1. Petrochemical industry

Nowadays the petrochemical industry is responsible for providing not only gasoline but a huge range of useful products, including plastics, synthetic rubber, solvents, fertilisers, pharmaceuticals, additives, explosives and adhesives. These materials have important applications in almost all areas of modern society, such as in transport, packaging, household goods, medical equipment, paints, clothing and building material [1].

### 1.1.1. Petroleum-based plastics

Petroleum-based plastics have replaced many materials like glass, wood, fibres and metal in their former applications [2, 3]. Plastic became attractive due to its properties: it is versatile and easily moulded, they can be transparent, lightweight, strong, durable and cost-effective. Moreover, plastics have a lower production cost than alternative materials [3, 4].

This makes them an ideal material for single-use disposable devices [3]. As a result of the widespread use and consumption of plastic products, the world plastic production almost reached 350 million tonnes in 2017 [5], about 50% of this volume is used in disposable applications [3].

Its disposal becomes problematic since most of the petroleum-based plastics are durable and resistant to microbial degradation, hence they persist in the environment for a very long time [2,6]. Although the amount of plastic that is recycled has been increasing, there is still much going to landfill or incineration [5]. Incineration returns some of the energy from plastic production but is known to produce negative environmental and health effect and it is a huge source of air pollution [3]. From incineration results the release of carbon dioxide, a greenhouse gas which contributes to climate change, and of other air pollutants, including carcinogenic polycyclic aromatic hydrocarbons (s) and dioxins, furans, mercury and polychlorinated biphenyls into the atmosphere [3, 6]. Additionally, plastic waste in landfills can contaminate the groundwater by releasing hazardous chemicals [2].

Moreover, there is a portion of plastic waste that is non-collected, 60-80% of the waste found on beaches or floating on the ocean is plastic [6]. On marine ecosystems have been shown to pose an even more serious impact not only due to entangled from its ingestion but also due to plastic capacity to concentrate persistent organic pollutants (POPs), that are often hydrophobic compounds with a high affinity to microplastics. Microplastics are partially degraded plastic debris of less than 5 mm in diameter, that are more likely to infiltrate in food webs causing injuries, stress, contaminant bioaccumulation and tumour formation [2, 6, 7].

Besides the environmental issues, a comparison between the estimates of oil production and utilization clearly shows that its use is unfeasible it in the long-term. Its production, from biomass through geological processes, is slow compared with its consumption making into an imbalanced cycle. As result, it is necessary to drive the industry to more sustainable and environmentally friendly approaches to produce goods and energy. Plant biomass, using the biorefinery concept, offers an attractive

alternative that bypasses the need of fossil resources and mitigates greenhouse gas emission by balancing the carbon cycle, represented on figure 1, between the time constants of feedstock utilization and its production by carbon dioxide fixation [8].

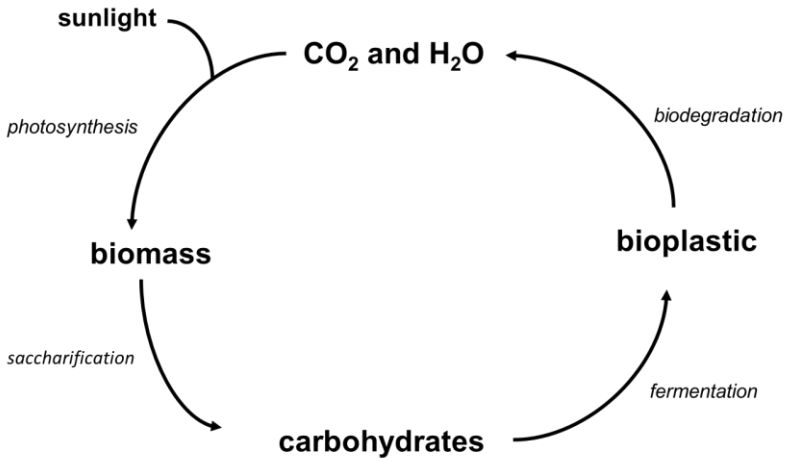



**Figure 1.** The carbon cycle of bioplastic, adapted from [9].

## 1.2. Biorefinery

The biorefinery is a facility that combines various biomass conversion processes and equipment to produce biofuels, energy and high-value products. This approach has risen as a promising and sustained solution to the increasing consumption and production demands, that relies on renewable rather than petrochemical resources, where the accumulated environmental impact of industrial processes diminish [10, 11]. The biorefinery was proposed to maximize the utilization of biomass from different feedstocks to efficiently produce high-value products while minimizing resource consumption and waste generation with a reduction of environmental impacts [11]. In figure 2 is shown a schematic overview of a potential biorefinery.

In biorefinery, a wide range of technologies are able to separate almost all the types of biomass feedstocks into their components (carbohydrates, proteins, triglycerides...) which can be converted to value-added products, biofuels and biochemicals through conversion technologies [12].

Several challenges have been identified for implementation of successful future biorefineries. The operation of scaling up a successful small-scale operation to a large scale biorefinery requires a significant capital investment. Moreover, investors also have a low return on investment and face an unstable future situation, as the laws regarding biofuels and biochemicals are not yet long-term stable. Other important challenges to overcome include biomass availability during the year or methodologies that enable to run more than one raw material, which constitutes a tremendous challenge when a large-scale production facility is considered. So, one of the major challenges that the biorefinery concept faces to become successful is to find suitable raw materials [13].

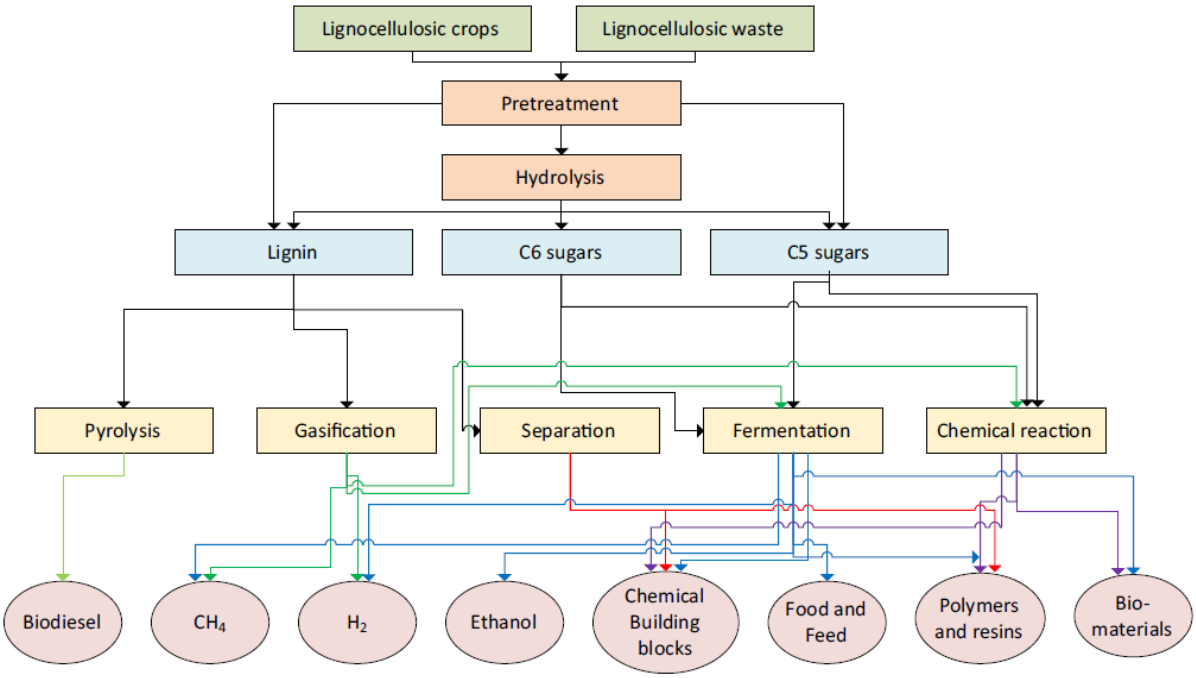



**Figure 2.** A schematic overview, from [13], of a potential biorefinery for the production of energy carriers, biochemical and polymers from lignocellulosic crops or waste.

In this context, it is important to find a feedstock that does not compete for land and water like terrestrial crops (first-generation), such as sugarcane [14]. As result, lignocellulosic materials (second-generation), such as agro-industrial by-products and wood residues from forest pruning and woodwork activities, have been suggested as sustainable alternative carbon sources for the production of biofuels and biomaterials through biological processes, moreover, these sources are inexpensive [15]. However, its valorisation to commercially relevant fuels and chemicals has been relatively challenging due to lignin's high degree of polymerization, diverse spectrum of chemical moieties, and complex structure [10]. The high degree of polymerization of lignocellulose constituents and their complexity implies higher costs for pre-treatment and usually, the generation of toxic by-products that can inhibit subsequent microbial fermentation and consequently yield of the product of interest is lower [10, 14, 16].

Therefore, algal biomass (third-generation) has been considered as biomass feedstocks. Due to the lack of lignin, algal biomass is easier to process by hydrolysis than lignocellulosic materials and is advantageous for the extraction of sensitive bioactive components (antioxidants, vitamins and proteins) [16]. Besides this alga biomass presents other benefits as it does not use arable land and potable water to grow. Instead, it can assimilate nutrients directly from seawater or wastewater, so agricultural land and freshwater are not needed. Moreover, biomass productivity and yield are much higher when compared with lignocellulosic biomass [11, 14, 16].

## 1.3. Bioplastic

All the mentioned problems with petroleum-based plastics, drive the industry to more sustainable and environmentally friendly approaches to decreasing the world's dependence on petrochemicals and petrol fuels. So, using algae biomass as the feedstock for monosaccharides enables the production of bioplastic while bypassing the need for fossil resources.

Plastic is a polymer of repetitive monomers that can assume a linear, branched or cross-linked structure. It can be defined according to its material source and its biodegradability. Biobased plastic is synthesized from renewable resources, namely biomass, while petroleum-based plastics are synthesized from petroleum resources, a fossil fuel. Biodegradable plastics are broken down into natural substances (water, carbon dioxide and compost) by microorganisms available in the environment without artificial additives. According to European Bioplastics, the concept of bioplastic covers the whole list of materials that are bio-based, biodegradable or both, as illustrated in figure 3 [17,18].

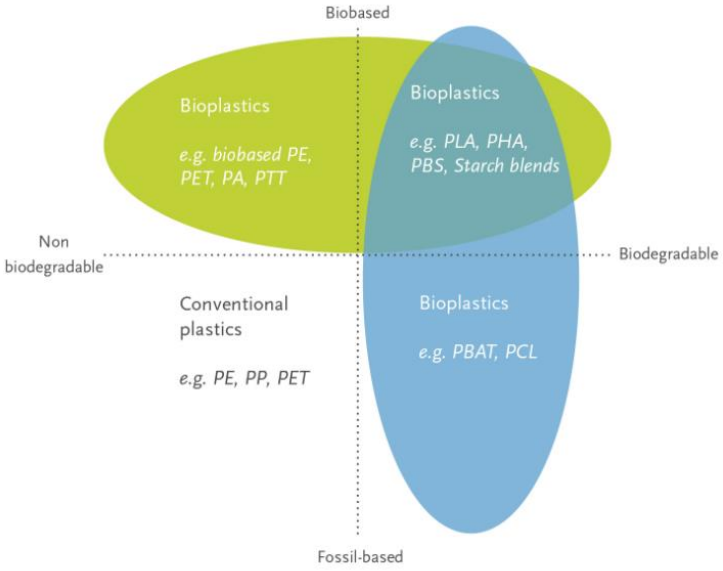
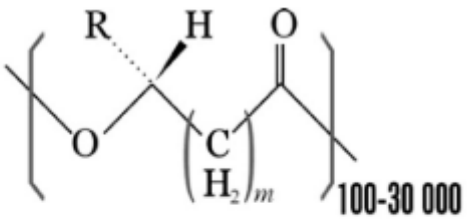



**Figure 3.** Classification of plastic according to its biodegradability and material source, from [18]. PE: polyethylene, PET: polyethylene terephthalate, PA: polyamide, PTT: polytrimethylene terephthalate, PP: polypropylene, PLA: polylactic Acid, PHA: polyhydroxyalkanoates, PBS: Polybutylene succinate, PBAT: polybutylene adipate terephthalate, PCL: polycaprolactone.

Microorganisms responsible for the biodegradation and catabolism of bioplastics can be found extensively in soil or compost materials. These microorganisms include aerobes, anaerobes, photosynthetic bacteria, archaebacterial and lower eukaryotic which synthesize enzymes, intracellular or extracellular, responsible for enzymatic degradation of bioplastics [19]. These microorganisms use the monomers and oligomers resulting from bioplastic degradation as a carbon source. Isolated fungal have been shown to allow for faster polymers degradation when compared to bacteria, due to their versatile depolymerase activities [20].

Bioplastics have a strong potential to become good substitutes for the conventional petroleum-based plastics in various applications. Mainly because they could assume different properties according to their composition and structure. The most commonly used bioplastics are polylactic acid (PLA), poly(butylene succinate) (PBS), starch-based plastics and polyhydroxyalkanoates (PHAs, polyesters of hydroxyalkanoates). PHAs attract more attention because they are bio-produced from a wide array of feedstocks, they are biocompatible, 100% biodegradable to carbon dioxide and water under aerobic conditions while under anaerobic conditions carbon dioxide and methane are produced, and have similar properties to conventional plastics [21, 22, 23]. PHAs have an advantage over other biodegradable polymers, they are hydrolysable to soluble monomers without interference from organisms. The resulting monomers are natural metabolites, for example, 3-hydroxybutyrate (3HB) is a common human blood constituent [24]. So they are immunologically inert, having promising future applications, particularly in medical-related fields, like heart valves and scaffolds [25]. PHAs can assume different properties according to polymer molecular weight (50–1000 kDa), composition and structure which is determined by the synthesizing microorganism, substrate, cultivation mode (batch, fed-batch or continuous), conditions and the downstream process of recovery [21, 22].

These polymers can be synthesized by a wide variety of Gram-positive and Gram-negative bacteria. Most species produce PHAs under excess of carbon source and limitation of one essential nutrient (nitrogen, phosphorus, sulphur, magnesium or oxygen). PHAs are thermoplastic or elastomeric polymers, formed by R-hydroxyalkanoic acid (HA) monomers (figure 4) bound to each other that are accumulated in cytoplasmic granules, easily identified with Sudan black or Nile blue. Once the supply of the limiting nutrient is restored, intracellular depolymerases can degrade PHA to serve as carbon, energy or reducing-power source accessible if required [9, 25]. The number per cell and size of the cytoplasmic granules (accumulation capacity) can vary among the different species. On table 1 are given some examples of bacteria able to produce and accumulate PHA. The selection of the appropriate microorganism for PHA production should be done based not only in maximum extent of polymer accumulation but also on cell's ability to utilize an inexpensive carbon source within a mixture of different monomers, growth rate and polymer synthesis rate [26].




| | | | |
|---|---|---|---|
| m=1 | R=H | Poly(3-hydroxypropionate) | P(3HP) |
| | $R=CH_3$ | Poly(3-hydroxybutyrate) | P(3HB) |
| | $R=C_2H_5$ | Poly(3-hydroxyvalerate) | P(3HV) |
| | $R=C_3H_7$ | Poly(3-hydroxyhexanoate) | P(3HX) |
| m=2 | R=H | Poly(4-hydroxybutyrate) | P(4HB) |
| m=3 | R=H | Poly(5-hydroxyvalerate) | P(5HV) |

**Figure 4.** General structure of polyhydroxyalkanoates, where m represents the number of carbon atoms in the linear polyester structure (m=1-4) for each monomer, x ranges from 100 to 30000 and R the alkyl groups (C1-C13). Polymer designation depends on the number of C in the monomeric unit and on the alkyl side chain R [27].

**Table 1.** Examples of PHA production bacteria and its capacity to accumulate it expressed in percentage of cell dry weight (CDW).

| Microorganisms | PHA content (% of DCW) | Ref. |
|---|---|---|
| *B. licheniformis* PHA 007 | 68.8 | 28 |
| *B. subtilis* PHA12 | 7.37 | |
| *Bacillus* sp. PHA 013 | 13.06 | |
| *Bacillus* sp. PHA 023 | 60.66 | |
| *Pseudomonas* sp. PHA045 | 39.25 | |
| *Aeromonas* sp. PHA046 | 21.1 | |
| *Bacillus thuriengensis* | 31.8 | 29 |
| *Bacillus* sp B58 | 35.6 | |
| *Pseudomonas* sp B68 | 25.5 | |
| *Burkholderia sp* B64 | 26.5 | |
| *Halomonas boliviensis* LC1 | 56 | 30 |
| *Halomonas elongata* | 40-50 | 31,32 |

PHAs are classified as homopolymers or heteropolymers depending on the type of monomeric units that compose the polymer chain. The final composition of the polymer chain is determined by the carbon source available and the substrate specificity of PHA synthase of the host organism. The PHA synthase only accepts the monomers with a certain number of carbon atoms in the main chain making possible to divide into three classes [26]. Polymers with monomers of 1-5 carbon atoms are classified as short chain length PHAs (scl-PHAs), predominantly used to produce food packaging and disposable items. If the monomers contain 6-14 carbon atoms, they are classified as medium chain length (mcl-PHAs) which are appropriate for high value-added applications, such as surgical stitching, implants, and drug delivery

systems. Finally, polymers composed of monomers with more than 14 carbon atoms are long chain length (lcl-PHAs) [4, 24, 25]. The later are naturally scarce and with low impact within the development of bioplastics [33].

### 1.3.1. Halophilic Microorganisms for PHB Production

Halophilic microorganisms usually inhabit on hypersaline environments that offer several applications in various fields of biotechnology. These bacteria have shown potential to produce polyhydroxyalkanoates (PHA), compatible solutes and enzymes as therapeutic agents since are naturally tolerant to osmolarity in physiological conditions[34,35].

Halophiles require salt for growth and are present in the tree life domains *Archaea*, *Bacteria* and *Eukarya*. Based on optimal salt concentration halophiles can be divided into two groups, moderate that grows in salt concentrations of 3-15% (w/v) and extreme in concentrations of 15-30% (w/v). In order to cope with high salinities, these microorganisms have two adaptation mechanisms. Halophiles can be grown in high pH and high NaCl concentration, making contamination-free fermentation processes possible. One mechanism, mainly used by aerobic and extremely halophilic archaea and some anaerobic halophilic bacteria, is the accumulation of inorganic ions by pumping out intracellular potassium in the accumulation of exchange for sodium to balance osmotic pressure. The other osmoregulatory mechanism consists in accumulate water-soluble organic compounds, named compatible solutes, which is mainly used by most halophilic bacteria and eukarya [34,36].

The use of *Halomonas* bacteria for PHA production has some advantages. The high osmotic pressure resulted from high salt concentration prevents and reduces the risk of contamination by non-halophiles, reducing the costs, energy and process complexity for sterilization. Besides it also allow continuous fermentation letting to higher efficiency when compared to a batch one [34].

### 1.3.2. Biosynthesis of P(3HB)

From around 150 diverse PHA structures recognized, the most well-known and better characterized is the homopolymer poly-3-hydroxybutyrate (P(3HB)), which attracted more attention than the other types because of its physical, mechanical, and immunologically properties making it a strong candidate for use in various applications in agriculture, food, and medical fields [37]. However, P(3HB) is a very hard and brittle material which strongly limit its processing by conventional methodologies [26]. In order to improve the polymer properties and eliminate or decrease the brittleness and thermal instability of polyhydroxybutyrate, copolymers, such as poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (P(3HB-*co*-3HV)) and poly(3-hydroxybutyrate-co-4-hydroxybutyrate) (P(3HB-co-4HB) [27] have been successfully produced [22, 25]. For instance, the increase of 3-hydroxyvalerate fraction, decreases the melting temperature, increasing the melt stability, without affecting the degradation temperature, as in P3HB these two temperatures are close [26]. So, the polymer properties can be controlled by adjusting the fraction in copolymers during the fermentation.

The polymer of PHB is formed, as shown in figure 5, by the polymerization of (R)-3-hydroxybutyryl-CoA in the growing chain, which is catalysed by PHB synthase that establishes ester bonds between the carboxyl group and an available hydroxyl group of the next monomer. The molecule of (R)-3-hydroxybutyryl-CoA is synthesized by two sequential enzymatic reactions, first two acetyl-CoA are condensed to acetoacetyl-CoA by acetyl-CoA-acetyltransferase (β-ketothiolase) and then an NADPH-dependent reductase, acetoacetyl-CoA-reductase, catalyses its reduction to (R)-3-hydroxybutyryl-CoA [37, 38].

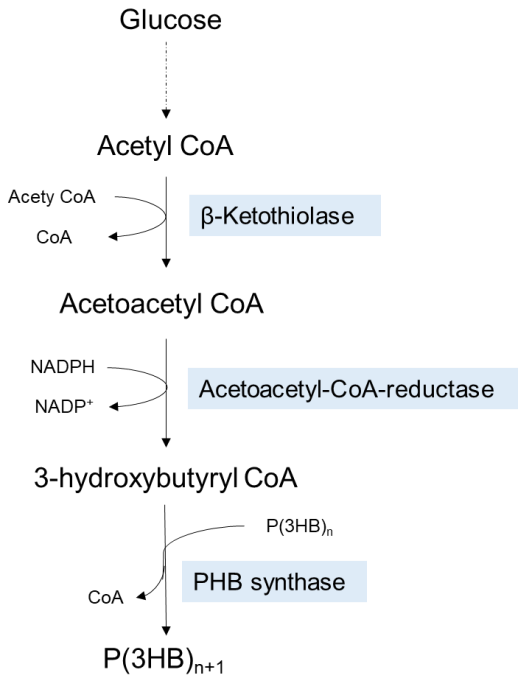



**Figure 5.** Biosynthesis pathway of the homopolymer poly-3-hydroxybutyrate (P(3HB)), adapted from [9].

To determine their functional groups and chemical structure, the biopolymers are characterized by Fourier-transform infrared spectroscopy and nuclear magnetic resonance (NMR), respectively. Gas chromatography (GC) can be used to determine the purity of PHA samples, defined as the percentage of PHA amount in the total dry matter after recovery, and the monomer composition in PHA polymer [37, 39].

### 1.3.3. Recovery of P(3HB)

Recovery of PHA significantly affects the overall production cost, making these polymers much more expensive than petroleum-based plastic. Significant efforts have thus been done to develop/improve a cheap and safe process towards the recovery of PHA, which will have a significant impact on industrial production of this biopolymer.

After fermentation, the cells containing PHAs are separated by conventional procedures such as centrifugation or filtration and then disrupted in order to recover the polymers. One of the first methods

developed to do it was the solvent extraction where the solvent, for instance, chloroform, methylene chloride or propylene carbonate, modifies the cell membrane permeability and then solves PHAs granules. The separation of PHA from the solvent can be performed by solvent evaporation or by precipitation of PHA in a non-solvent [26, 27]. This strategy has been widely used to obtain a high level of purity but does not lead to very high recovery. So, cell pre-treatment steps, such as grinding and spray-drying, are required to improve the solvent extractability, contributing to make this process economically unfavourable. Moreover, and even considering recycling, large volumes of solvent are required. Another downside is the toxic and/or volatile nature of the solvents used, rendering its application environmentally hazardous. [27, 38].

Another recovery method is digestion by sodium hypochlorite based on differential digestion of non-PHA cellular materials. This method is simple and effective, allowing high purity levels of PHA, however sodium hypochlorite, as a strong oxidant, degrades P(3HB) resulting in a 50% reduction in molecular weight. To reduce this degradation the use of sodium hypochlorite can be combined with solvent extraction (chloroform) to dissolve the polymer isolated by the action of hypochlorite and protect P(3HB) from degradation. However, this approach increases costs and as mentioned before has an environmental hazard associated [26, 27, 38, 40].

Digestion by surfactants has also been implemented. Surfactants, such as the anionic sodium dodecyl sulphate (SDS), are incorporated in the cell membrane. Its addition will eventually break the membrane to produce micelles of surfactant and membrane phospholipids. This leads the cellular content, including P(3HB) granules, to be released into the solution. Then the granules can be recovered, while other non-PHA cellular materials are solubilized by the surfactant. The use of surfactant alone will not end up with a high PHA recovery but can be combined with other agents such as hypochlorite and sodium hydroxide. This method has a low operating cost and the surfactants do not degrade polymer granules with cell lysis, but a high surfactant dose increases the recovery cost and causes pollution problems resulting in increased wastewater treatment costs [27, 38].

The enzymatic digestion method comes up as a 100% biological alternative to solvent extraction, to separate PHA from other constituents of cells, for example, proteases have high activity on the dissolution of proteins and little effects on PHA degradation. A typical process starts with a thermal pre-treatment of biomass containing P(3HB) followed by enzymatic digestion and washing with a surfactant that will solubilize non-PHA cellular materials (proteins and lipids). The thermal treatment has the objective of rupturing cell, making the intercellular compounds accessible. It is advantageous because it also degrades the polynucleic acids, which prevents a subsequent viscosity problem, denatures protein, making these more susceptible to a subsequent protease treatment and inactivates PHA depolymerase, thus ensuring the integrity of PHA. Enzymatic digestion leads to good recovery levels with a relatively high purity but it is relatively expensive due to enzyme related high costs [27, 38, 41].

The cell disruption to release and recover the PHA granules can also be done by mechanical methods, such as bead mill, high-pressure homogenizer and ultrasonication or with supercritical fluids. Most of the mentioned methods require a large amount of energy and toxic or pollutant compounds. Supercritical fluids have unique physicochemical properties that make them suitable as extraction

solvents. The most widely used is $CO_2$, because of its low toxicity and reactivity, availability, nonflammabilty, low cost and moderate temperature and pressure for critical point (31°C and 73 atm) [27, 42].

In order to get a better recovery a pre-treatment step, such as heat, alkaline or salt pre-treatment, could be added and a higher purity with a purification step (hydrogen peroxide or ozone treatment) [27].

## 1.4. Feedstock

Despite the current use of bioplastics in different areas such as agriculture, medicine and packaging, they still represent a small share in the global plastic market [21]. The historic low price of fossil feedstock together with optimised production processes and high production costs of PHAs have restricted commercial production of these bio-based products [13, 23]. So, the high production cost is a limiting factor for their commercialization at large scale. In addition to the recovery costs mentioned previously, another factor that contributes significantly is the price of the carbon source, which contributes up to 30-45% of the total production cost, as typically refined substrates such as pure glucose are used. In order to reduce raw materials costs for bioplastic production, inexpensive and sustainable carbon sources can be used as an alternative such as industrial by-products/wastes, which reduce the amount of waste in the environment contribute to the circular economy. These by-products and wastes include agricultural residues, forest and garden waste, as well as food and paper waste [21, 43]. These materials have great potential as carbohydrates platform because they consist mainly of cellulose, hemicellulose and lignin. Cellulose and hemicellulose constitute an excellent source of carbon to be used in different biological processes after hydrolysis to monomeric sugars [23].

As mentioned previously, algal biomass is more advantageous when compared with lignocellulosic biomass, because it is easier to process by hydrolysis due to the lack of lignin. For this reason, this type of biomass was selected as carbon platform for polyhydoxyalkanoates production. Inexpensive algae biomass can be obtained from algal blooms that cause eutrophication of the ecosystems and may cause nuisance when washed by the tides in beaches in touristic coastal areas. Another source of inexpensive biomass is the seaweed that is not adequate for applications in the food and feed area, such as macroalgae that have been used to treat polluted waters with heavy metals due to their high biosorption capacity [14, 44]. For these reasons, algae are treated as wastes, despite their content in valuable compounds, with costs associated with their disposal [45]. Also, by-products after the extraction of biomolecules (bioactive compounds, proteins, gel polymeric materials, pigments) can be used as an inexpensive biomass source, because the cellulose-rich fraction remains [14]. Algal biomass can be categorized into two main categories macroalgae and microalgae biomass, based on morphology.

## 1.5. Macroalgae

Macroalgae, also known as seaweeds, are multicellular aquatic photosynthetic organisms, which are abundant in the oceans, particularly in coastal areas, where they may attach to rocks and other solid

surfaces or exist as free-living forms. They are classified as green, brown and red algae and their colours are derived from natural pigments and chlorophylls, some examples of representative species of these different classes are provided in table 2. By absorbing specific light wavelengths, the different pigments enable seaweeds vertical distribution in marine ecosystems, for example, red algae can be found in deep sea [14]. Seaweeds composition varies not only with species but also with habitat and harvest time (growth stage and season) [46]. Unlike microalgae, macroalgae have low protein (7-15% dw) and lipid contents (1-5% dw) but has high carbohydrates contents (25-60% dw). This confers an enormous potential as a carbon source for biofuels, biochemical, building blocks and biomaterials using biotechnology processes. As referred previously, macroalgae do not contain lignin, which facilitates saccharification. As result, the biorefinery using wastes (non-edible feedstocks and biogenic wastes) has emerged as an alternative for the production of biobased products such as biopolymers, biofuels, and biochemicals. The valorisation of the wastes is important to circular economy [11].

**Table 2.** Examples of species belonging to the three macroalgae groups.

| Seaweed Group | Species |
|---|---|
| Green | *Ulva lactuca, Ulva pertusa, Acrosiphonia centralis, Cladophora rupestris, Monostroma grevillei, Codium fragile* |
| Brown | *Alaria marginata, Cymathere triplicata, Laminaria hyperborea, Fucus vesiculosus, Macrocystis pyrifera* |
| Red | *Iridaea cordata, Asparagopsis taxiformis, Delesseria sanguinea, Gelidium sesquipedale, Chondrus crispus, Gigartina papillata* |

### 1.5.1. Green Macroalgae

Green macroalgae, which have the same ratio of chlorophyll a to b as land plants, are present mostly in shallow waters and are common in bays and estuaries. These seaweeds have small quantities of lipids (0-6%) and have starch as reserve polysaccharides (1-4%) and ulvan and cellulose as structural polysaccharides (38-52% dw). Ulvan (8-29% dw) contributes to the strength of the cell and gives flexibility. This polysaccharide, soluble in acidic aqueous solutions, constituting of a disaccharide repeating units, ulvanobiouronic acid, composed of uronic acids, namely glucuronic acid and iduronic acid, and of sulphated L-rhamnose, xylose and glucose [14, 47]. Cellulose is a linear chain homopolysaccharide and consists of recurring units of D-glucose linked by β-1-4 glycosidic bonds, in contrast to the α-1-4 bonds of amylose and starch [48].

### 1.5.2. Brown Macroalgae

The principal photosynthetic pigments in brown macroalgae are chlorophyll a and c, β-carotene, fucoxanthin and other xanthophylls. In these macroalgae is laminarin (β-1,3-glucans; up to 35% dw) the main storage polysaccharide and alginate (40% dw) the major structural polysaccharide. Laminarin is

water-soluble and it is composed of 20-25 glucose units, a β-(1, 3) glucan chain with small amounts of β-(1, 6) branches, with mannitol linked to the reducing end. Alginate is made up of two different types of uronic acids: mannuronic (M) and guluronic (G) acids. It has different properties depending on the M/G ratio and has been used mostly in the textile (50%) and food (30%) industries. Another polysaccharide present is fucoidan, that is composed of sulphated fucose and also small amounts of xylose, galactose, mannose and glucuronic acid [14,16, 47].

### 1.5.3. Red Macroalgae

The red colour in these macroalgae is a result of the presence of chlorophyll a, phycoerythrin and phycocyanin pigments. In red macroalgae, the typical reserve polysaccharides are floridean starch (up to 80% of the cell volume), an α-1,4-glucosidic linked glucose homopolymer, and floridoside also structurally similar to starch with glucose units. While the structural polysaccharides present are cellulose and agar (up to 52% dw), composed of β-D-galactose and α-L-galactose with scarce sulfatations, or carrageenan (up to 75% dw) which is composed of repeating D-galactose unit and anhydrogalactose, that may or may not be sulfated [14, 47].

### 1.5.4. Macroalgae in Biorefinery

As mentioned, seaweeds have structural differences, having different polysaccharides and monosaccharides which are summarised on table 3.

**Table 3.** Polysaccharides in the three macroalgae groups and monosaccharides resulting from their hydrolysis [49].

| Seaweed type | Polysaccharides | Monosaccharides |
|---|---|---|
| Green | Ulvan, starch, xylopyranose, glucopyranose, xyloglucan, glucuronan, cellulose, hemicellulose | Glucose, xylose, uronic acids, rhamnose, galactose |
| Brown | Fucoidan, laminaran, alginates, cellulose | Mannitol, glucose, guluronate, mannuronate, glucuronate, sulphated fucose |
| Red | Agar, carrageenan, agaropectin, cellulose, xylans, mannans | D-galactose, D-fructose, 3,6-anhydro-D-galactose, glucose |

Seaweeds can be used as a whole or can be fractionated in their different constituents as carbohydrates, proteins, lipids, minerals (ash), pigments, vitamins and antioxidants, as illustrated in figure 6. The whole macroalgae have been used for human food, animal feed, fertilizers, cosmetic ingredients, therapeutic materials and energy production. Besides these uses, macroalgae can also be used to produce diverse biomaterials and bioproducts in various industries, following a biorefinery approach with sequential extraction. The lipid fraction could be used for food and in the pharmaceutic industry. Despite the low lipid content, the polyunsaturated fatty acids (PUFA) have a most relevant presence in macroalgae than in terrestrial vegetables. PUFA are known to exhibit anti-

hypercholesterolemic, antioxidant, anticancer, antidiabetic, antihypertensive and anti-inflammatory activities [14]. The protein content is low in seaweeds (higher in red macroalgae) but protein digestibility is 86%, indicating its suitability for use in food supplements and source of peptides, amino acids and nitrogen [47]. The pigments present in seaweeds are a good substituent of synthetic ones, very useful in biomedicine, food, cosmetics and pharmaceutical industries. The minerals fraction present, such as iodine, potash and phosphorus, can be used as a food supplement for some essential minerals or fertilizer. In seaweeds, the most significant fraction is the carbohydrate fraction with applications on food technology, biotechnology, microbiology and medicine due to their bioactivity [14, 47]. To be applied as C-source in biological processes, algae polysaccharides need first to undergo saccharification into their constituent monomeric sugars, such as glucose, mannose and galactose, then the hydrolysate can be used to produce bioethanol, biochemicals (pyruvate, lactic acid, citric acid, propanediol), building blocks and biomaterials, such as PHAs by means of microbial fermentation. The hydrolysate is already successfully used for the production of acetone, butanol, ethanol and 1,2-propanediol [50].

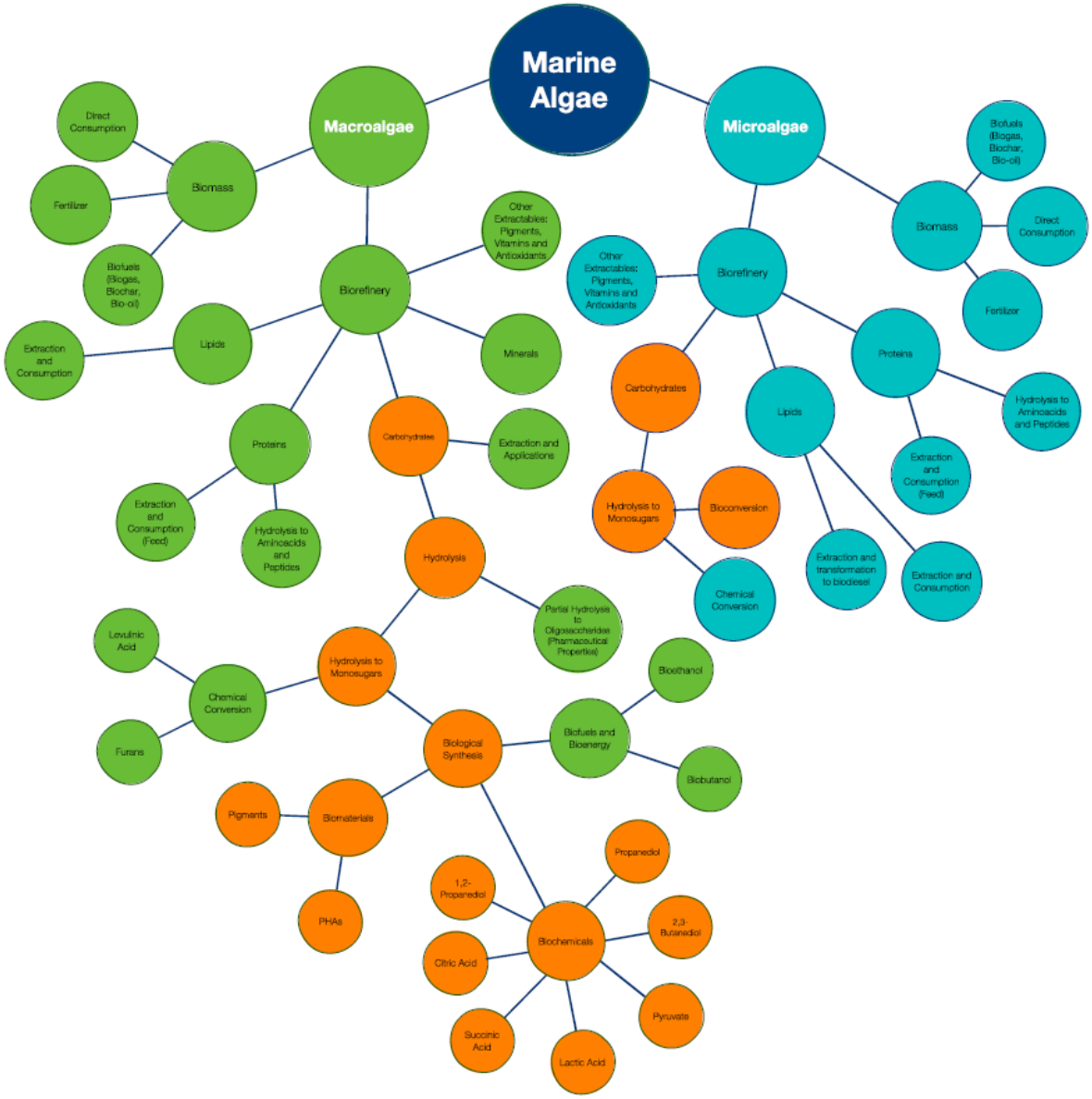



**Figure 6.** Applications of marine algae as a whole or after extraction of proteins, lipids, sugars, antioxidants, pigments and nutraceuticals [14]. In orange are represented the possible biochemicals and biomaterials produced after the saccharification of algae polysaccharides into their monomeric sugars.

## 1.6. Enzymatic Hydrolysis

In order to produce this hydrolysate, the selection of an appropriate mixture of enzymes is vital to obtain the expected output. Optimization of hydrolysis conditions (temperature, pH, incubation time, the proportion between substrate and enzymes concentration and agitation) to maximize reducing sugars production is the required [51].

A common structural polysaccharide in all seaweeds' cell wall is cellulose, which can be hydrolysed by cellulases, a preferred approach since no inhibitors to the subsequent fermentation are formed as by-products of hydrolysis. Cellulases are also useful in a wide range of applications in food, animal feed, textile, fuel, chemical industries, paper industries, waste management [52]. There are three types of

cellulases for hydrolysis of intermolecular β-1-4-glycosidic bonds and release of glucose, endo-1,4-β-glucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21). The endo-1,4-β-glucanase randomly cleaves the internal β-1,4-glycosidic bonds present in the amorphous region of cellulose. Exo-1,4-β-glucanase cleaves from the reducing or non-reducing end of the polymer forming cellobiose. Finally, β-glucosidase cleaves cellobiose into two glucose molecules [53].

The hydrolysis of starch, a reserve polysaccharide, is the responsibility of α-amylase, isoamylase, pullulanase, β-amylase, and glucoamylase. Isoamylases (E.C.3.2.1.68) and pullulanases (EC 3.2.1.41) debranch amylopectin into amylose via α-(1→6) glycosidic bond cleavage. α-Amylase (EC 3.2.1.1), a calcium metalloenzyme, plays a dominant role in carbohydrate metabolism catalysing the cleavage of α-D-(1→4) glycosidic bonds randomly into shorter oligosaccharides. Then β–amylases (EC 3.2.1.2), an exo-type carbohydrase enzyme, hydrolyse amylose from the non-reducing ends to produce predominantly maltose, which is cleaved into two monosaccharides of glucose by glucoamylases (EC 3.2.1.3) [54,55].

Aiming at the complete hydrolysis of the typical algal polysaccharides such as ulvan, agar and alginate in green, red and brown algae, respectively, specific enzymes must be used. In the case of ulvan in green algae, ulvan lyase (EC 4.2.2.-) should be used. Ulvan lyase cleaves the β(1→4) linkage between rhamnose and the glucuronic acid producing oligosaccharides that have unsaturated uronic acid at the non-reducing end [56]. Recently other enzymes were identified that act on ulvan oligomers, such as β-D-glucuronidase (EC 3.2.1.31) that allowed the hydrolysis of residual ulvanobiouronic acid in rhamnose and glucuronic acid [57]. Glucuronan lyase (EC 4.2.2.14), that cleaves (1→4)-β-D-glucuronans, has also been reported to partially digest ulvan due to the presence of deviant repeating structures in ulvan similar to glucuronan structure [58].

Complete hydrolysis of agar in red seaweeds involves the action of endo-type agarases which cleave agarose releasing oligosaccharides, while exo-agarases play an important role in releasing disaccharide units of D-galactose and 3,6-Anhydro-L-galactose. According to their cleavage pattern they are classified as β-agarases (EC 3.2.1.81) which hydrolyse β-(1,4) glycosidic bonds while α-agarases (EC 3.2.1.158) cleave α-(1,3) glycosidic linkages. The activity of β-agarases results in the production of neoagaro-oligosaccharides and neoagarobiose that can be further hydrolyzed by α-neoagarobiose hydrolase into D-galactose and 3,6-anhydro-L-galactose [59].

In brown macroalgae, alginate can be degraded by alginate lyases, mannuronate lyases (EC 4.2.2.3) and guluronate lyases (EC 4.2.2.11) that has endolytic alginate activity producing oligosaccharides. Exolytic alginate lyase further degrades oligomeric alginate into unsaturated manuronic acid [60].

The yield of enzymatic hydrolysis depends on a variety of factors, such as type of substrate pre-treatment, inhibition of enzymatic activity by the end-products, thermostability of enzymes, their concentration and adsorption on the substrate, duration of the hydrolysis, medium pH, substrate concentration in the medium and hydrodynamics. Therefore, in order to achieve a high yield in the saccharification processes is necessary to optimize the hydrolysis conditions [61]. In the case of cellulose,

the typical operating temperature for its hydrolysis ranges between 40°C and 55 °C while optimum pH ranges from 4.5 to 5.5 [62].

The enzymatic activity of cellulases can be inhibited by the accumulation of cellobiose, glucose or xylose. Cellobiose has been found to be a stronger cellulase inhibitor than glucose, especially when the quantity of β-glucosidase, that cleaves cellobiose into two glucose molecules, is insufficient leading to accumulation of cellobiose. Consequently, a decrease in the efficiency of the saccharification process is observed as well as a lower final yield. The inhibition of hydrolysis by end products can also be prevented by performing simultaneous saccharification and fermentation (SSF). In this case, the monosaccharides can be directly used without accumulation [61, 62].

In addition to the carbon source, the enzymatic process contributes significantly to the cost of production. Therefore, intensive research is being carried out on the enhancement of cellulases synthesis by microorganisms to reduce the cellulase production costs, optimize growth conditions or modifying the enzyme source, using genetic engineering [61]. The challenge of the costs of enzymes in the production of biofuels and bioplastics can be overcome through enzyme recycling by readsorption but this has been shown unsuitable for β-glucosidase [62].

## 1.7. Chemical Pre-treatment

Optimization of the saccharification process can also be attained with a pre-treatment of biomass. Many pre-treatments have been developed, which can be mechanical (size reduction, beating and washing), thermal (microwave, steam explosion) chemical (alkali or acidic treatment and peroxide treatment) or biological [49]. The objective is to disrupt the cellular matrix to make the cellulose more accessible to enzymes [62]. The chemical treatment is the one that appears to be close to near-term commercial application [61].

At low pH, algal polysaccharides are hydrolysed and if high acid concentrations are used, cellulose is also hydrolysed into oligo- or monosaccharides leaving a porous structure of primarily cellulose and also lignin, in case of lignocellulosic biomass, more accessible to enzymatic action. In this process, sulfuric acid or other strong acids are added to the feedstock, and the mixture is heated to about 140-160°C for 5-20 min [13, 62]. However, depending on the severity, some non-sugar compounds are also released, among them furfural and 5-hydroxymethylfurfural (HMF) that are degradation products of pentose and hexose sugars, respectively, known to decrease subsequent fermentation yield for acting as inhibitors of several enzymes [63, 64].

At high pH, using NaOH, lime or $Na_2CO_3$, can result in the dissolution of the lignin fraction while most of the cellulose and hemicelluloses is still in its solid-state [13].

## 1.8. Separate Hydrolysis and Fermentation vs. the Simultaneous Saccharification and Fermentation

The operations of polysaccharides hydrolysis and fermentation of the resulting monosaccharides can be carried out consecutively, in a process called separate hydrolysis and fermentation (SHF). In order to find a more efficient process, different process configurations have been studied. One alternative scheme is the simultaneous saccharification and fermentation (SSF) that combines both operations in a single step and vessel [14]. SSF has been used to avoid the inhibition of hydrolytic enzymes by negative feedback, caused by the end product (monosaccharides), increasing the saccharification rate and yield since the monosaccharides can be directly used without accumulate [16, 65]. SFF has other advantages when comparing to SHF, the global processing time is reduced, it has reduced investment costs (involve less equipment), it is simpler to operate and it has a lower risk of contamination by glucose-dependent organisms because the glucose is consumed continuously as it is released [16,65–68,69].

On the other hand, the main advantage of SHF is that both operations occur in their favourable conditions (temperature, pH, nutrient composition, solid loading) while SSF mode is operated at ideal conditions for the fermenting microorganisms which temperature is lower than that optimum for hydrolysis inducing a lower sugars yield [16,65.] The optimal temperature for saccharification with cellulolytic enzymes is around 50 °C, while most fermenting microorganisms have an optimum temperature between 28 °C and 37 °C. In practice, it would be difficult to lower the optimum temperature of cellulases through protein engineering. Alternatively, thermotolerant yeast strains, capable of producing at temperatures more favourable for saccharification, have been screened to overcome this problem and improve SSF efficiency [67].

## 1.9. Pre-Saccharification and Simultaneous Saccharification and Fermentation

One way to eliminate the problem caused by the discrepancy in the optimal temperature for enzymes and microorganisms, or at least reduce it, is to employ the pre-saccharification and simultaneous saccharification and fermentation (PSSSF or PSSF) strategy, that consist in a pre-saccharification before SSF process [65]. The biomass is firstly pre-hydrolysed with cellulases at the optimum temperature (45–55 °C depending on the enzyme mixture) then, after a certain time, cooled to the ideal fermentation temperature, the same that SSF is operated, and immediately inoculated without inactivating the enzymes, but it is not industrially feasible yet [65,70]. Therefore, there is an increase in the rate of cellulose hydrolysis and of the product production rate in the early stages, once glucose is already assessable when it is inoculated [70].

Since this process is between SHF and SSF processes, it combines the advantages of both SHF and SSF. It is expected that PSSSF presents higher sugars yield than SSF process and higher fermentation productivity than SHF process, if a suitable pre-hydrolytic time is selected [65]. The duration of pre-hydrolysis is an important factor that will affect the yield, productivity and product concentration, and thus consequently the final evaluation of the process. This factor is rarely optimized and there are contradictions between some studies about if PSSSF improves the yield of SHF and SSF [70].

## 1.10. Consolidated Bioprocessing

The last operating scheme is the consolidated bioprocessing (CBP) defined as a one-step process in which a feedstock is directly converted into the desired product by a microorganism without requiring pre-treatment of the feedstock [70]. It permits avoiding the costs associated with enzyme production and also simplifies the operation [67].

The most challenging task with CBP is selection or design of a suitable microorganism that must not only be able to hydrolyse the feedstock's polysaccharides and to consume the originated mixture of sugars with efficiency but also have to be resistant to fermentation inhibitors and too stressful environments [14, 70].

## 1.11. *Ulva lactuca* and *Halomonas elongata*

This work addresses the enzymatic hydrolysis of *Ulva lactuca* macroalga to monosaccharides which are foreseen to be used as C source by *Halomonas elongata* to produce and accumulate PHB.

As mentioned above, algal biomass has several advantages compared to lignocellulosic biomass as it has a higher photosynthetic rate, does not need arable land or potable water to grow, lacks lignin and most importantly it has a high polysaccharide content. For this reason, this marine biomass was chosen as carbon platform for the production of polyhydroxyalkanoates by biological processes. The green macroalga *Ulva lactuca* was chosen as model seaweed because of its worldwide distribution, living in littoral and sublittoral zones, for example, the Mediterranean Sea and Baltic Sea, and it is an inexpensive raw material commonly found in green tides that annually hit the shore [71]. Moreover, it can be obtained from wastewater treatment, which can not be used in food and feed aplications[46]. *Ulva lactuca* is a common marine green macroalga that belongs to the phylum *Chlorophyta* and can grow attached, sessile or free floating and it is able to rapidly proliferate by sexual reproduction or by fragmentation, rarely observed. *U. lactuca* is a polymorphic species with morphologies dependent on the degree of water salinity or symbiosis with bacteria. The different phenotypes, tubular or a sheet-like tail, observed in *Ulva lactuca* led to the proposal that different species may exist, such as *Ulva armoricana*, *U. rigida*, *U. prolifera*, *U. pertusa*, *U. fasciata* or *U. rotundata*. However, genetic analysis revealed that the different phenotypes observed were not based on genetic variations that would justify the existence of different species other than *Ulva lactuca* [50].

*Halomonas elongata*, an aerobic and halophilic gram-negative bacterium that belongs to the class γ-proteobacteria [72], was chosen as PHA producer because it can grow in a variety of carbon sources glucose, rhamnose and xylose the main sugars released from *Ulva* polysaccharides namely cellulose, starch and ulvan. As a moderate halophile *H. elongata* has a broad salt tolerance of approximately 0.1 to 4 M NaCl producing ectoine as a compatible solute, synthetized from aspartate [72,73]. Its ability to accumulate 40-55% of PHB (dry weight basis) under N-limiting conditions has been shown [31,32].

## 2. Materials and Methods

### 2.1. Materials

*Ulva lactuca*, batch V100618H, was supplied by the company AlgaPlus. The seaweed was washed with seawater and grounded in flakes of 1.5-4 mm after dehydration. The commercial enzymes used in this work were cellulase complex (NS 22086), β-glucosidase (NS 22118), glucoamylase (NS 22035), xylanase (NS22083), all from Novozymes, α-Amylase from *Bacillus amyloliquefaciens* and β-glucuronidase from *Helix pomatia* from Sigma-Aldrich, Bradford reagent (batch number TG267988) was from Thermo Scientific. *Halomonas elongata* DSMZ 2581 was selected because of its ability to produce PHB from *Ulva lactuca* sugars. All remaining reagents were from different suppliers with p.a. grade.

### 2.2. Methods

#### 2.2.1. Determination of Total Carbohydrates in Biomass

Total carbohydrates of *U. lactuca* and extracted ulvan were determined based on the method provided by the National Renewable Energy Laboratory ("NREL"), which consists of a two-step acid hydrolysis. This procedure was performed in duplicate. Firstly, 25 mg of *Ulva lactuca* (or ulvan) were weighed into a glass pressure tube. Then 250 µl of 72% (w/w) $H_2SO_4$ were added and the suspension was incubated at 30°C for 60 minutes under shaking. For the second step, the acid was diluted to 4% (w/w) by adding 7 ml of Milli-Q water. To eliminate phase separation the tubes were mixed by inversion. The tubes were incubated in an autoclave at 121°C for one hour and then cooled to room temperature. The resulting hydrolysate was collected from the settled biomass and was neutralized using calcium carbonate to a pH of 5-6. Samples of the neutralized hydrolysate were collected, centrifuged at 9167 g for 5 minutes at room temperature and then the sugar content of the supernatant was determined by HPLC after a 20-fold dilution in $H_2SO_4$ 50 mM.

#### 2.2.2. Ulvan Extraction

Ulvan extraction was carried out from 6.0 g of *Ulva lactuca* in 100 ml of 0.01 M HCl at 80°C under stirring. After extraction, the suspension was filtered through gauze and then was allowed to cool at room temperature. The filtrate was centrifuged at 10°C for 20 min at 10000 rpm. The obtained supernatant was filtered again using Whatman filter paper N. 1 to remove the impurities and the pH was adjusted to 3.5 with 1 M NaOH. Precipitation of ulvan was performed as isolation method by adding three volumes of 96% (w/w) ethanol to one volume of the filtered extract. The precipitate was recovered by centrifugation at 5000 rpm for 20 min at 10°C. Then, the alcohol precipitate was washed three times with 50%, 75% and 96% ethanol and centrifuged at 5000 rpm for 10 min at 10°C. Finally, it was dried at 40°C to a constant weight and then grounded.

#### 2.2.3. Enzymatic Hydrolysis of *U. lactuca* Polysaccharides

*U. lactuca*, in a concentration of 43.2 g/l, was subjected to enzymatic hydrolysis at 50°C under magnetic stirring, for about 24 h. This hydrolysis was carried out in either 5 ml of sodium acetate buffer (100 mM) at pH 5.0 or in 5 or 10 ml of distilled water with pH adjusted to 4.8 in the presence of 0.220 $mg_{protein}$/ml of cellulase, 0.054 $mg_{protein}$/ml of β-glucosidase, 0.047 $mg_{protein}$/ml of glucoamylase and 0.017 $mg_{protein}$/ml of α-amylase or 0.075 $mg_{protein}$/ml of xylanase, except if stated otherwise. Samples were

collected periodically for reducing sugars quantification by 3,5-dinitrosalicylic acid (DNS) method and for the quantification of the individual monosaccharides by high-performance liquid chromatography (HPLC). From each sample, the sugars contribution from the enzyme preparations were subtracted. The release yield of each monosaccharide was determined as the mass ratio of each monosaccharide to the total carbohydrates of *U. lactuca* previously determined. All trials were performed in duplicate.

2.2.4. Combined Hydrolysis

Prior to enzymatic hydrolysis, a chemical pre-treatment with either 0.25%, 0.5% or 1.0% (w/v) of $H_2SO_4$ or 2 M trifluoroacetic acid (TFA) was performed. In a 100 ml Schott flask, 1.728 g of *U. lactuca* were weighed and 20 ml of acid were added, resulting in 86.4 g/l of biomass. The flasks were incubated in the autoclave at 121°C for 30 minutes for treatment with $H_2SO_4$ or 45 minutes for treatment with TFA. After that, the pH was adjusted to 4.8 with 1 M or 5 M NaOH, distilled water was added to make up an *U. lactuca* concentration of 43.2 g/l. After collecting a 300 µl sample the enzymatic hydrolysis was carried out as described before (2.2.3. Enzymatic Hydrolysis of *Ulva lactuca*). All trials were performed in duplicate.

2.2.5. Strain Storage

Cultures of *H. elongata* were stored at -80°C in 2 ml cryovials containing 900 µl aliquots of actively growing culture samples in mid-exponential phase and 900 µl of a previously sterilized glycerol solution (30%). The cultures were grown in 500 ml Erlenmeyer flasks with 100 ml of HM medium. This medium had the pH adjusted to 7.5 using 1M KOH and the following composition (g/l): NaCl 45.0; $MgSO_4 \cdot 7H_2O$ 0.25; KCl 0.5; peptone 5.0; yeast extract 1.0 and $CaCl_2 \cdot 2H_2O$ 0.09. Incubation was performed at 35°C and 200 rpm orbital shaking.

2.2.6. Inoculum Preparation

The inoculation medium prepared had the following composition (g/l): Tris 15.0; NaCl 45.0; $K_2PO_4$ 3.0; $NH_4Cl$ 4.0, monosodium glutamate 8.9 and trace elements 1.0. The pH of this medium was adjusted to 7.5 and then sterilized at 121°C for 20 minutes. Trace elements solution[74] had the following composition in 1l: 25% HCl, 10 ml; $FeCl_2 \cdot 4H_2O$ 1.5g; $CoCl_2 \cdot 6H_2O$, 0.19g; $MnCl_2 \cdot 4H_2O$, 0.1g; $ZnCl_2$, 0.07g; $H_3BO_3$, 0.062g; $NaMoO_4 \cdot 2H_2O$, 0.036g; $NiCl_2 \cdot 6H_2O$, 0.024g;$CuCl_2 \cdot 2H_2O$, 0.017g.

The inoculum culture was prepared in Erlenmeyer flasks with medium supplemented with $MgSO_4 \cdot 7H_2O$ and glucose to a final composition of 2.5 g/l and 20.0 g/l, respectively, to a final volume of 65 ml. The $MgSO_4 \cdot 7H_2O$ solution was sterilized separately to avoid precipitation. After addition of the content of two cryovials of *H. elongata*, the culture was incubated at 35°C and 200 rpm orbital shaking overnight.

2.2.7. Culture for PHB Production

A 20-fold concentrated PHB production medium composed by: 300.0 g/l of Tris, 76.0 g/l of $K_2PO_4$ and 20 g/l of trace elements was used. The hydrolysate used as C and N source for cell growth and polymer production was obtained from 100 g/l of *U. lactuca* in a total volume of 200 ml after an acid pre-treatment with 1.0% (w/v) of $H_2SO_4$ or TFA 2 M and 24 hours of enzymatic hydrolysis with 0.220 $mg_{protein}$/ml cellulase, 0.054 $mg_{protein}$/ml of β-glucosidase, 0.047 $mg_{protein}$/ml of glucoamylase and 0.017

$mg_{protein}$/ml of α-amylase. Then, for one assay, the TFA hydrolysate was treated with 10 g/l of activated carbon overnight, centrifuged at 13 000 g for 20 minutes, sterilized at 121°C for 20 min and collected a sample for HPLC analysis.

The assays were performed in Erlenmeyer flasks of 500 ml with 2.5 ml of the concentrated medium supplemented with 7.5 ml of 300 g/l NaCl (to a final concentration of 45.0 g/l) and 34.9 ml of the prepared hydrolysate and 1.25 ml of 100 g/l $MgSO_4 \cdot 7H_2O$ (to a final concentration of 2.5 g/l). Before inoculating with 2.6 ml of the inoculum culture the pH was adjusted to 8.2, then the culture with a final volume of 50 ml was incubated at 35°C and 200 rpm orbital shaking. Aliquotes were collected periodically to monitor pH, optical density at 600 nm ($OD_{600}$), cell dry weight (CDW), PHB production and monosaccharides uptake, the two later for analysis by GC and HPLC. All trials were performed in duplicate.

## 2.3. Analytical Methods

### 2.3.1. Quantification of Protein with Bradford Assay

The Bradford[75] assay was performed according to micro microplate protocol provided by Thermo Fisher Scientific with a working range of 1-25 µg/ml. Briefly, 150 µl of adequately diluted enzyme preparation were pipetted to the microplate and 150 µl of Bradford reagent were added. This mixture was incubated at room temperature for 10 minutes. Then the absorbance was measured at 595 nm by a microplate reader and the concentration of protein was calculated based on a calibration curve of bovine serum albumin (BSA).

### 2.3.2. Protein Analysis by SDS-Page

The enzymes used were analysed by SDS-page, the enzymes samples were prepared by adding 25 µl of Bio-Rad loading buffer and 5 µl of dithiothreitol (DTT) 1 M to 20 µl of the enzyme sample, previously diluted 1/50 with distilled water. These samples were denatured in reducing conditions (DTT 100 mM) at 100°C for 5 minutes. After that, the samples were load and run at 90 mV in SDS-page gel with 12% T resolving gel and 4% T stacking gel. The gel was stained with Coomassie Phast Gel (Pharmacia AB Laboratory Separations®). The molecular markers used were Precision Plus Protein Standards Dual Colour from Bio-Rad.

### 2.3.3. Determination of the Reducing Sugars by 3,5-Dinitrosalicylic Acid Method

The collected samples were centrifuged at 9167 g for 5 minutes and the supernatant was recovered. The reaction of DNS[76,77] with reducing sugars of the supernatants was made in triplicate, in 96-deep well microplates. For that, 100 µl of DNS was added to 20 µl of the supernatant, diluted with 80 µl of distilled water. The covered microplate was incubated at 100°C for 5 minutes. After cooling it down to room temperature, 500 µl of distilled water were added to each well and 200 µl of the resulting mixture were transferred to a 96-shallow well reading microplate. The absorbance was measured at 540 nm using a microplate reader and the concentration of reducing sugars was calculated based on a calibration curve of glucose.

### 2.3.4. Total Nitrogen Quantification in Hydrolysates

Total Nitrogen content in the hydrolysates was determined spectrophotometrically (DR2800, Hach Lange) by using standard test kits (LCK 338 LATON; manufacturer: Hach Lange, Düsseldorf).

2.3.5. Quantifications by High-Performance Liquid Chromatography

Glucose, rhamnose and xylose, as well as 5-hydroxymethylfurfural (HMF) concentrations, were determined. The HPLC is equipped with a Rezex ROA-Organic acid H+ 8% (300 mm x 7.8 mm) column, an HPLC pump (Hitachi LaChrome Elite L-2130), an autosampler (Hitachi LaChrome Elite L-2200), a Hitachi L-2420 UV-Vis detector for organic acids and a Hitachi L-2490 refraction index (RI) detector for sugars and phosphate. For heating purposes, it was connected externally to the HPLC system a column heater for large columns (Croco-CIL 100-040-220P, 40 cm _ 8 cm _ 8 cm, 30-99°C). The column was kept at 65 °C under a pressure of 26 bar, and the pump operated at a flow rate of 0.5 $mL.min^{-1}$. The injection volume was 20 µL and elution was achieved using a 5 mM solution of $H_2SO_4$ as the mobile phase. The samples were prepared by diluting 100 µl of the supernatant (obtained after centrifugation of the sample at 9167 g for 5 minutes) with 100 µl of $H_2SO_4$ 50 mM. This solution, after vortexing, was centrifuged again in the same conditions. Finally, the vials for HPLC were prepared by adding 900 µl of $H_2SO_4$ 50 mM with 100 µl of the second supernatant, which makes a final dilution of 20-fold. The concentration of sugar in the samples was determined using calibration curves prepared previously using the same procedure.

2.3.6. Biomass Quantification

Cellular growth was monitored by measuring the optical density at 600 nm ($OD_{600}$) in a spectrophotometer Hitachi U-2000. The cell dry weight (CDW) was followed collecting 1.2 ml of the culture to a previously dried and weighed Eppendorf. These samples were centrifuged at 9167 g for 5 minutes, the pellet was washed with distilled water and centrifuged in the same conditions. The washed pellet was dried at 60°C for at least 48 hours.

2.3.7. Quantifications by Gas Chromatography

Cultures samples of 1.2 ml were centrifuged at 9167 g for 5 minutes. Then the pellet was washed with distilled water and centrifuged at the same conditions. Next, the polymer in the pellet was converted into stable and volatile hydroxycarboxylic acid methyl esters, through acidic methanolysis. For that, 1 ml of chloroform was added, the pellet was resuspended and transferred to Pyrex hermetic tubes with Teflon cases. Then 1 ml of an acidic methanol solution was added to each tube. This solution is composed of 97 ml of methanol, 3 ml of $H_2SO_4$ (96%) and 330 µl of hexanoic acid to a final volume of 100 ml. After vortexing, the tubes were incubated for 5 hours at 100°C, then after cooling the samples were neutralized to stop the reaction with 1 ml of $Na_2CO_3$. Finally, the samples, after vortexing, were centrifuged at 4500 rpm for 5 minutes and 200 µl of the organic phase were transferred to GC vials and stored at -20°C until GC analysis.

Cultures samples for quantification of PHB produced were analysed by GC (Hewlett Packard 5890 series II) equipped with a FID detector and 7683B injector. The oven, injector and detector were kept tat constant temperatures of 60°C, 120°C and 150°C, respectively. The capillarity column was a HP-5 from Agilent J&W Scientific, with 30 m length and 0.32 mm of internal diameter. Data acquisition and integration were performed by a Shimadzu CBM-102 communication Bus Module and a Shimadzu GC solution software (version 2.3). The concentration of PHB was achieved using 3-methylhydroxybutyrate (Sigma-Aldrich) as an internal standard.

## 3. Results and Discussion

### 3.1. Protein Content in the Enzyme Preparations and SDS-Page Analysis

Firstly, the protein concentration of the preparations from Novozymes and Sigma-Aldrich was determined by Bradford assay for all the enzymes used in this work. The obtained results are present in table 4.

**Table 4.** Protein concentrations of the enzyme preparations from Novozymes and Sigma-Aldrich, determined by Bradford assay.

| | Cellulase | β-glucosidase | Xylanase | Glucoamylase | α-amylase |
|---|---|---|---|---|---|
| [Protein] (mg/ml) | 194.9±12.4 | 45.1±4.9 | 100.6±8.1 | 63.2±5.5 | 28,9±4.7 |

These enzymes preparations were also analysed by SDS-page electrophoresis (figure 7). The results show a higher purity for α-amylase from Sigma-Aldrich (lane 5) with 60 KDa, as expected. The calculated molecular weight has an error of 8.8% in relation to the value given by the supplier, 55 KDa. In the same lane, it is possible to observe two lighter bands, probably originated by α-amylase degradation, since the sum of their weight is closed to 60 KDa (38 and 24 KDa).

The purity in the Novozymes preparations is lower. There are some bands in common for cellulase complex and β-glucosidase (lane 1 and 2, respectively), suggesting that the cellulase complex has some β-glucosidase activity.

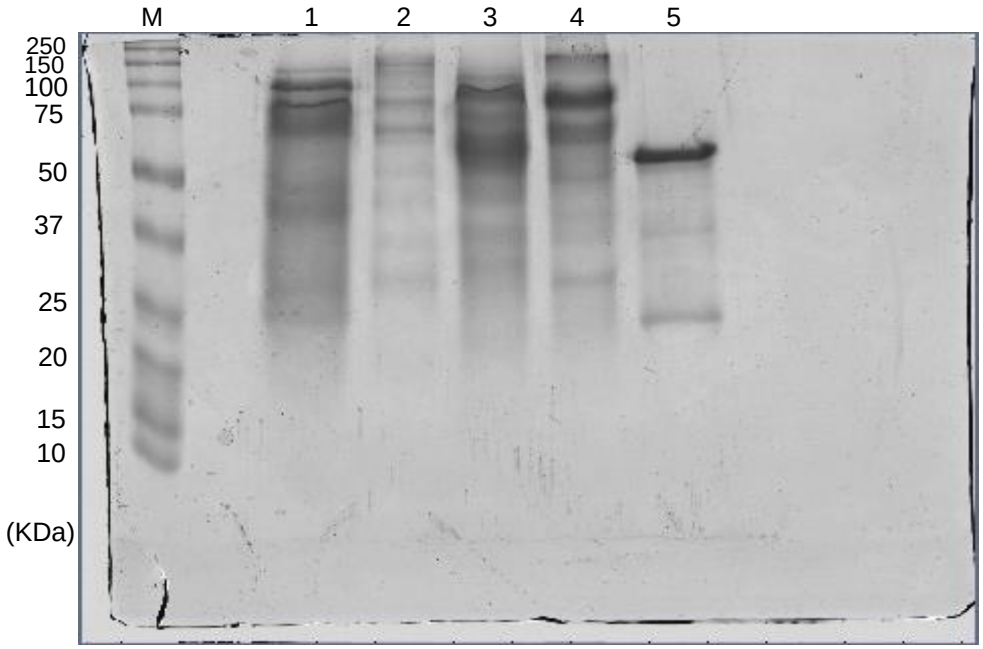



**Figure 7.** Enzyme preparations analysis by SDS-page. Lane M: molecular weight markers from Bio-Rad, lane 1: cellulase complex (NS 22086), lane 2: β-glucosidase (NS 22118), lane 3: xylanase (NS22083), lane 4: glucoamylase (NS 22035) and lane 5: α-Amylase from *Bacillus amyloliquefaciens*.

## 3.2. Determination of Total Carbohydrates in *U. lactuca*

The total carbohydrates content of *U. lactuca* was determined. From the literature, it is known that green macroalga has ulvan and cellulose as structural polysaccharides representing 38-52% of dry weight [14].

Ulvan consists of disaccharide repeating units composed of uronic acids, namely glucuronic acid and iduronic acid, and of sulphated L-rhamnose, xylose and glucose [78]. Cellulose is a linear chain homopolysaccharide consisting of recurring units of D-glucose linked by β-1-4 glycosidic bonds, in contrast to the α-1-4 bonds of amylose and starch.

As expected from the complete hydrolysis of *U. lactuca* glucose, xylose and rhamnose were obtained (table 5). Among them, glucose is the most abundant, representing 11.1% ± 0.1, then rhamnose 10.6% ± 0.3 and xylose 3.6% ± 0.1 of dry weight (dw), which make a total of 25.3% of reducing sugars based on the total biomass dry weight. These values are in agreement with previous results (11.3% of glucose, 9.0% of rhamnose and 2.9% of xylose) [46], having in mind that seaweeds composition has large variations according to growth location and harvest time (growth stage and season).

**Table 5.** The total carbohydrates content of *U. lactuca* in percentage of dry weight, determined by HPLC after a two-step acid hydrolysis.

| *U. lactuca* | Glucose (% dw) | Xylose (% dw) | Rhamnose (% dw) | Total (% dw) |
|---|---|---|---|---|
| Total Carbohydrates | 11.1 ± 0.1 | 3.6 ± 0.1 | 10.6 ± 0.3 | 25.3 |

## 3.3. Addition of Glucoamylase and Xylanase to the Cellulase Enzymatic Cocktail and its Effect on Total Sugar Release

Enzymatic hydrolysis of *U. lactuca* (43.2 g/l) was tentatively performed in buffered medium with cellulase and β-glucosidase, to obtain a sugar-rich hydrolysate. Cellulase was used for hydrolysis of intermolecular β-1-4-glycosidic bonds and β-glucosidase was used to cleave cellobiose, a known cellulase inhibitor, into two glucose molecules [53]. The time course of reducing sugars obtained by DNS method is given in figure 8 (●). After 23.8 hours of incubation 4.3 ± 0.2 g/l of reducing sugars were obtained, which correspond to a conversion yield close to 39.2%, (the maximum being 10.9 g/l (=25,3 %* 43.2 g/l) if the hydrolysis was complete).

Knowing that *U. lactuca* does not contain only cellulose as polysaccharide, two more enzymes were added (glucoamylase and xylanase), expecting better results. Glucoamylase (exoamylases) is extensively used to hydrolyse starch by tearing-off glucose units from the non-reduced end of the polysaccharide chain and xylanase hydrolyses β-1,4-glycosidic bond of xylan. The additional enzymes allowed a 1.2-fold increase in reducing sugars concentration, from 4.3 ± 0.2 to 5.1 ± 0.1 g/l, which correspond to a 46.3% of yield, as shown in figure 8 (■).

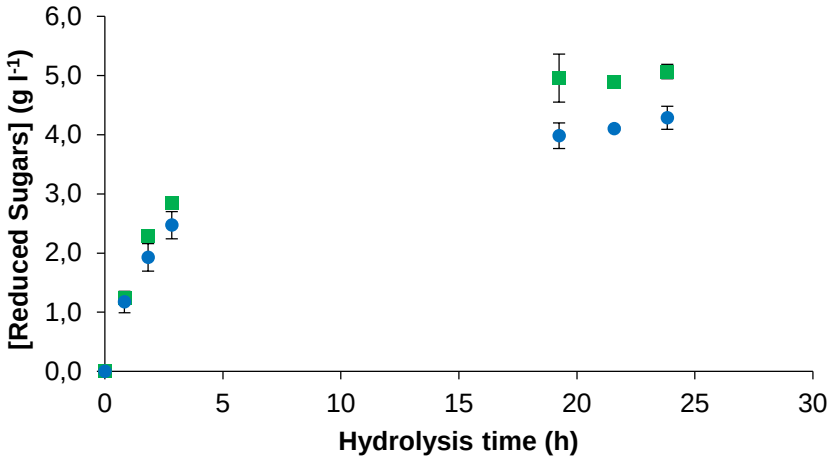



**Figure 8.** The concentration of released reducing sugars (g/l), determined by DNS method, over the enzymatic hydrolysis of *U. lactuca* at 43.2 g/l. The hydrolysis was performed in 5 ml of sodium acetate buffer 100 mM pH 5.0 and at 50°C under stirring with 50 µl of cellulase solution and 37.5 µl of β-glucosidase solution (●) or with these two enzymes and 37.5 µl of glucoamylase and xylanase solutions (■). The enzymes solutions added were 10 fold dilutions of commercial enzyme preparations.

### 3.4. Effect of Buffer *vs* Distilled Water

Acetate buffer is not the most adequate for the fermentation downstream where the reducing sugars produced upon *Ulva* hydrolysis will be used. Hence, the feasibility of using water rather than acetate buffer in the reaction medium was assessed. Hydrolysis runs under the previous conditions (either 2- or 4-enzyme cocktail) was made but in 5 ml of distilled water with pH adjusted to 4.8 rather than in buffer. The final pH was about 5.8-6.0 which is still in within the pH range where xylanase (4.5-6.0) and β-glucosidase (2.5-6.5) are most active, while cellulase and glucoamylase are most active at pH of 5.0-5.5 and 4.5-5.5, respectively.

The concentrations of reducing sugars obtained over the hydrolysis time are represented in figure 9. It is shown that after 25.8 hours of incubation, with 2-enzyme cocktail (figure 9 (●)), 3.7 ± 0.04 g/l of reducing sugars were obtained, which corresponds to a yield of 33.6%, while with 4-enzyme cocktail (figure 9 (■)) 4.6 ± 0.02 g/l (41.9%) were obtained. Once again, the use of four enzymes is advantageous increasing the concentration of the reducing sugar 1.2 times. However, comparing with the results using acetate buffer (figure 8) the maximum reducing sugars decreased, using 4-enzyme cocktail, from 5.1 ± 0.1 g/l to 4.6± 0.02 g/l. Nevertheless, the use of water simplifies the process and ensures the compatibility of the hydrolysate with the fermentation microorganism, to be carried out afterwards.

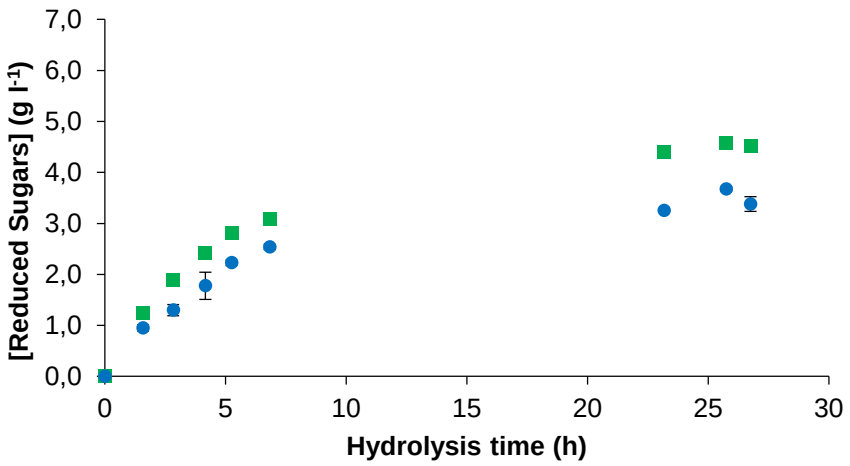



**Figure 9.** Concentration of released reducing sugars (g/l), determined by DNS method, over the enzymatic hydrolysis of *U. lactuca* at 43.2 g/l. The hydrolysis was performed in 5 ml of distilled water with pH adjusted to 4.8 and at 50°C under stirring with 50 µl of cellulase solution and 37.5 µl of β-glucosidase (●) or with these two enzymes and 37.5 µl of glucoamylase and xylanase solutions (■). The enzymes solutions added were diluted 10 fold dilutions of commercial enzyme preparations.

### 3.5. Comparison between Enzymatic Cocktails

Once established the feasibility of performing the enzymatic hydrolysis in water, further experiments were performed aiming to gain insight on the nature of the monosaccharides produced and to increase their titre. Reactions were thus performed in 10 ml volume with two different enzymatic cocktails already tested previously. Parallel hydrolysis runs were performed under *U. lactuca* concentration of 43.2 g/l, using the following cocktails: 0.195 $mg_{protein}$/ml of cellulase, 0.034 $mg_{protein}$/ml of β-glucosidase, 0.075 $mg_{protein}$/ml of xylanase and 0.047 $mg_{protein}$/ml of glucoamylase (figure 10 (●)) or 0.220 $mg_{protein}$/ml of cellulase, 0.054 $mg_{protein}$/ml of β-glucosidase, 0.017 $mg_{protein}$/ml of α-amylase and 0.047 $mg_{protein}$/ml of glucoamylase (figure 10 (■)). The second cocktail includes α-amylase that catalyses the cleavage of starch α-D-(1-4) glycosidic bonds into shorter oligosaccharides. Then glucoamylase, an exo-type carbohydrase enzyme, hydrolyses these oligosaccharides into monosaccharides of glucose. The use of the second cocktail allowed a higher reducing sugars concentration, 5.2 ± 0.3 g/l (47.2%), suggesting that the enzymes and enzyme/biomass ratio used in this cocktail is more adequate.

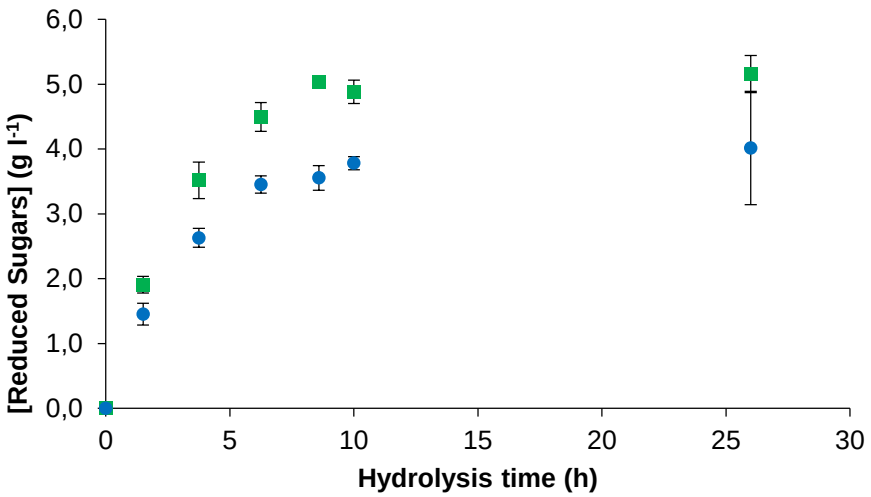



**Figure 10.** The concentration of released reducing sugars (g/l), determined by the DNS method, over the enzymatic hydrolysis of *U. lactuca* at 43.2 g/l. The hydrolysis was performed in 10 ml of distilled water with pH adjusted to 4.8 and at 50°C under agitation with 100 µl of cellulase and 75 µl of β-glucosidase, xylanase and glucoamylase, all diluted 10 fold (●), or with 11.3 µl of cellulase, 12 µl of β-glucosidase, 6 µl of α-amylase and 7.5 µl of glucoamylase (■).

The samples collected during the process of hydrolysis were also analysed by HPLC, allowing to establish the time course of glucose and xylose production present in figure 11. It is important to mention that the sugar concentration determined by DNS method differs with HPLC results, there is an overestimation, around 1.3 g/l, on the sugar concentration in the hydrolysates analysed by DNS method. When using the second cocktail the glucose release yield was 67.1% ± 1.1 and 31.4% ± 2.3 for xylose, while using the first cocktail was 47.8% ± 1.1 and 25.6% ± 1.5, respectively, after 10 hours of hydrolysis, further demonstrating the advantage in using the second enzymatic cocktail. Besides, the obtained concentration of xylose was higher even without xylanase. The decrease in the reducing sugars formed (figure 10 and 11), consequently an increase in standard error from duplicates, after 10 hours of incubation was due to microbial contamination.

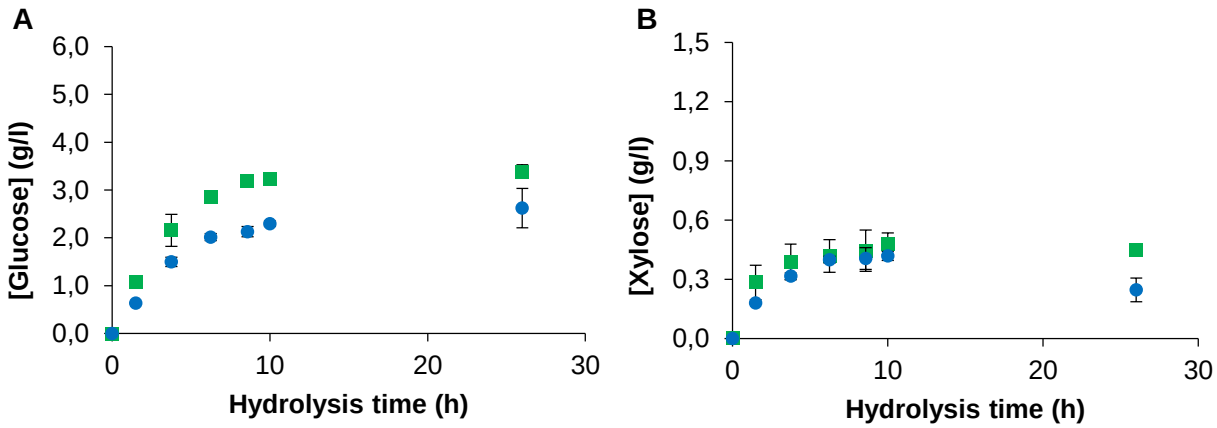



**Figure 11.** Concentration of released glucose (**A**) and xylose (**B**) (g/l), analysed by HPLC, over the enzymatic hydrolysis of *U. lactuca* at 43.2 g/l. The hydrolysis was performed in 10 ml of distilled water with pH adjusted to 4.8 and at 50°C under stirring with 100 µl of cellulase and 75 µl of β-glucosidase, xylanase and glucoamylase, all diluted 10 fold (●), or with 11.3 µl of cellulase, 12 µl of β-glucosidase, 6 µl of α-amylase and 7.5 µl of glucoamylase (■).

### 3.6. Enzymatic Hydrolysis of *U. lactuca* after Biomass Sterilization

With the purpose to avoid microbial contamination of the hydrolysates, *U. lactuca* was sterilized at 121°C for 20 minutes before the enzymatic hydrolysis. After sterilization, a sample was analysed by HPLC where it was possible to identify a peak corresponding to rhamnose. However, its release was residual and not quantifiable with the calibration curve used and remained constant during the enzymatic hydrolysis. In the enzymatic hydrolysis, using the second enzymatic cocktail that shown to be more adequate, it was possible to release 106.4% ± 6.3 of glucose and 76.9% ± 6.5 of xylose. The glucose release yield greater than 100% is due to errors associated with glucose concentration determination. The increase of carbohydrates concentration, when sterilized *U. lactuca* was used, maybe due to greater accessibility of enzymes to the *U. lactuca* polysaccharides. Besides the increase in carbohydrates release the reaction plateau was reached in less time, as seen in figure 12 (▲).

The same procedure was performed with some differences in the enzyme cocktail, first, it was used only cellulase and β-glucosidase (figure 12 (●)) and then α-amylase was substituted by xylanase (figure 12 (■)). These results demonstrate the advantage of using four enzymes and it is more favourable the presence of α-amylase in the enzyme cocktail instead of xylanase. The results obtained suggest a synergistic action of cellulolytic and amylolytic enzymes for the hydrolysis of the carbohydrates in *U. lactuca*, a pattern also reported by Shokrkar and Ebrahimi (2018) while addressing the hydrolysis of microalgal carbohydrates. More specifically, these authors suggested that the simultaneous use of the four enzymes prevented the inhibitory effect of cellobiose on cellulase activity and the inhibitory effects of oligosaccharides, maltose and cellulose on α-amylase activity [79].

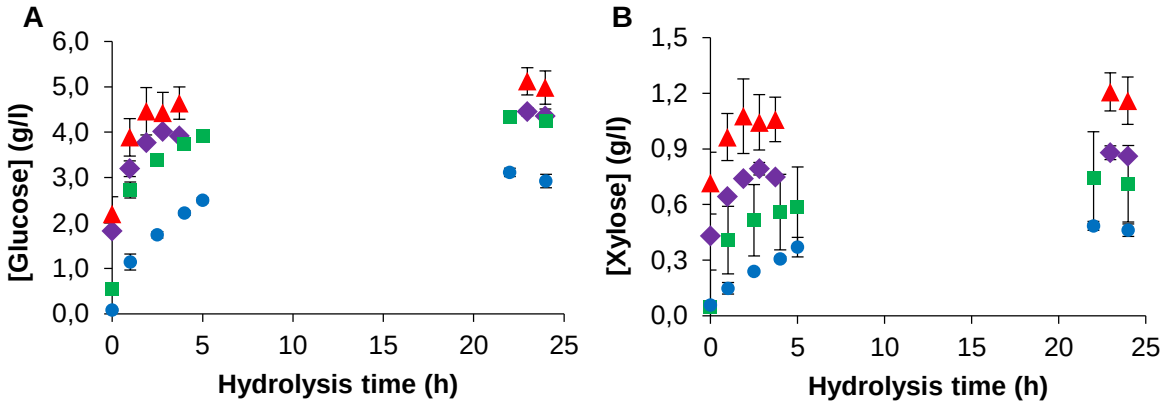



**Figure 12.** The concentration of released glucose (**A**) and xylose (**B**) (g/l), analysed by HPLC, over the enzymatic hydrolysis of *U. lactuca* at 43.2 g/l, after sterilization at 121°C for 20 minutes. The hydrolysis was performed in 10 ml of distilled water with pH adjusted to 4.8 and at 50°C under agitation with some differences in the enzymatic cocktail. 11.3 µl of cellulase and 12 µl of β-glucosidase (●).11.3 µl of cellulase and 12 µl of β-glucosidase, 7.5 µl of xylanase and glucoamylase (■). 11.3 µl of cellulase, 12 µl of β-glucosidase, 6 µl of α-amylase and 7.5 µl of glucoamylase (▲) and these enzymes plus glucuronidase at 0.5% (w/v) (♦).

In an effort to increase rhamnose concentration, 0.05% (w/v) glucuronidase was added to the cocktail (figure 12(♦)). Glucuronidase has been described as a catalyst to ulvanobiouronic acid hydrolysis, the main constituent of ulvan, releasing rhamnose and glucuronic acid [57]. However, rhamnose release

during enzymatic hydrolysis was not observed (its values remaining below the calibration curve limit), may be due to low accessibility of this enzyme to ulvan due to the stability of the glycosidic linkages of aldobiouronic acids [57]. The enzymes' accessibility can eventually be increased with a suitable pre-treatment. Moreover, the addition of this enzyme to the cocktail had a negative effect on the yield of glucose and xylose formation.

### 3.7. Combined Hydrolysis of *U. lactuca* using $H_2SO_4$ Pre-treatment

Despite sterilization had released some rhamnose, its concentration was very lower than expected since it represents the second more abundant monosaccharide in *U. lactuca* (10.6% dw). So an acid pre-treatment was carried out aiming at the release of rhamnose from *U. lactuca* [14].

Among the different pre-treatments carried out, the maximum glucose and xylose released yield was obtained when *U. lactuca* was pre-treated with 1.0% (w/v) of $H_2SO_4$ at 121°C for 30 minutes before enzymatic hydrolysis. There was no significant difference observed between treatment with 0.25% or 0.5% (w/v) of $H_2SO_4$. The calculated yields of monosaccharides released for combined hydrolysis are in table 6 and the time course for each monosaccharide during enzymatic hydrolysis in figure 13. Only after the pre-treatment with 1.0% (w/v) of $H_2SO_4$ rhamnose was present in an amount that remained unaltered during the enzymatic hydrolysis, as seen in figure 13-C, further highlighting the inability of the enzymatic cocktail to release rhamnose. The absence of rhamnose when 0.25% or 0.5% (w/v) of $H_2SO_4$ were used is due to little aggressive conditions in the pre-treatment. The presence of HMF in these hydrolysates was also detected in HPLC runs (data not shown), at concentrations between 0.01 g/l and 0.04 g/l, upon acid pre-treatment with 0.25% (w/v) and 1.0% (w/v), respectively. These concentrations remained unaltered during enzymatic hydrolysis. The presence of HMF in the hydrolysates is a factor to take into account and evaluate its possible inhibitory effect on microbial cultivations using *Ulva* hydrolysates as a C-source. A concentration above 0.1 g/l of HMF has shown to have an inhibitory effect on *H. boliviensis* growth [80].

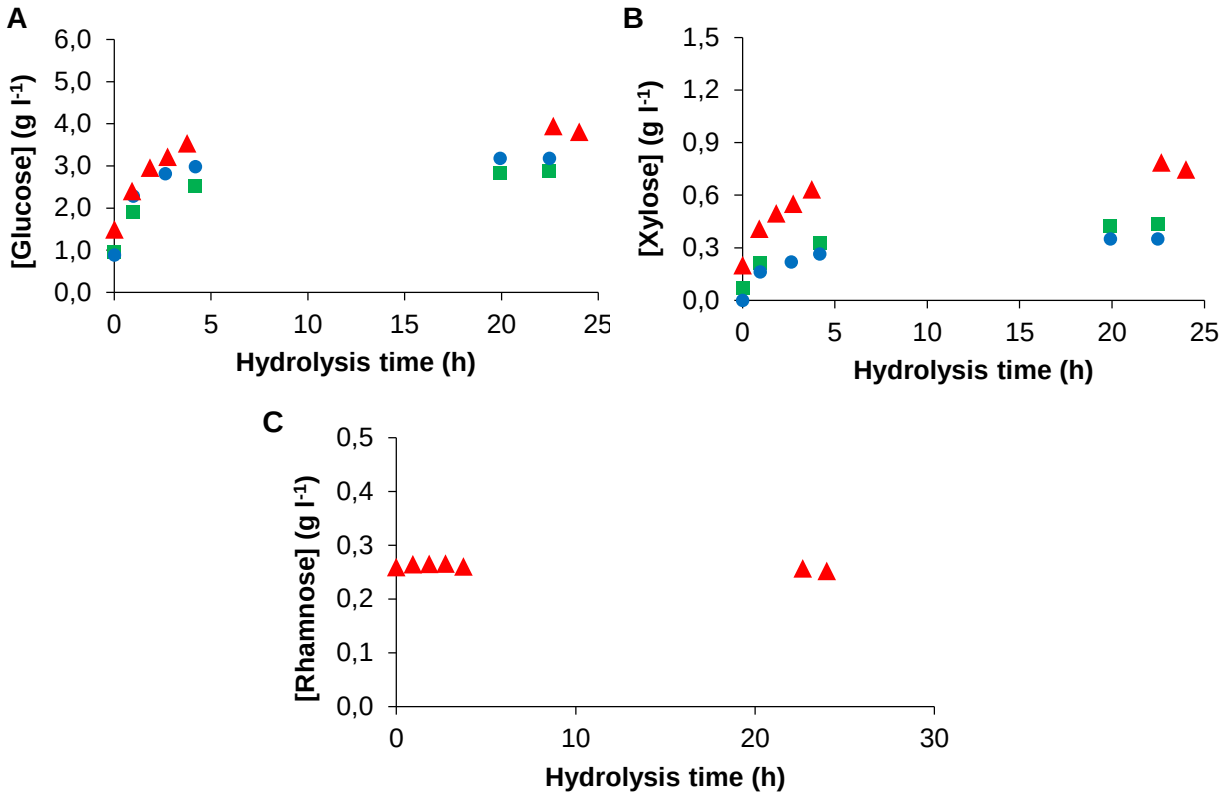



**Figure 13.** The concentration of released glucose (**A**), xylose (**B**) and rhamnose (**C**) (g/l), analysed by HPLC, over the enzymatic hydrolysis of *U. lactuca* at 43.2 g/l, after a chemical pre-treatment with 0.25% (●), 0.5% (■) or 1.0% (▲) (w/V) of $H_2SO_4$ at 121°C for 30 minutes. The hydrolysis was performed in a total volume of 40 ml and at 50°C under agitation with 45.2 µl of cellulase and 48 µl of β-glucosidase, 24 µl of α-amylase and 30 µl of glucoamylase.

**Table 6.** Calculated yields of glucose, xylose and rhamnose released after combined hydrolysis with different acid concentrations in the chemical pre-treatment. The pre-treatment was performed at 121°C for 30 minutes with 0.25%, 0.5% or 1.0% (w/v) of $H_2SO_4$. Then the enzymatic hydrolysis was performed in a total volume of 40 ml and at 50°C under agitation with 45.2 µl of cellulase and 48 µl of β-glucosidase, 24 µl of α-amylase and 30 µl of glucoamylase.

| Acid pre-treatment | Glucose yield (%) | Xylose yield (%) | Rhamnose yield (%) |
|---|---|---|---|
| 0.25% (w/v) of $H_2SO_4$ | 66.1 | 22.4 | ---- |
| 0.50% (w/v) of $H_2SO_4$ | 59.7 | 27.6 | ---- |
| 1.00% (w/v) of $H_2SO_4$ | 82.1 | 50.1 | 5.9 |

In order to make a comparison between enzymatic hydrolysis and combined hydrolysis, the same enzyme cocktails used for the experimental run described in figure 12 were used on the hydrolysate from acid pre-treatment with 1.0% (w/v) of $H_2SO_4$ (figure 14).

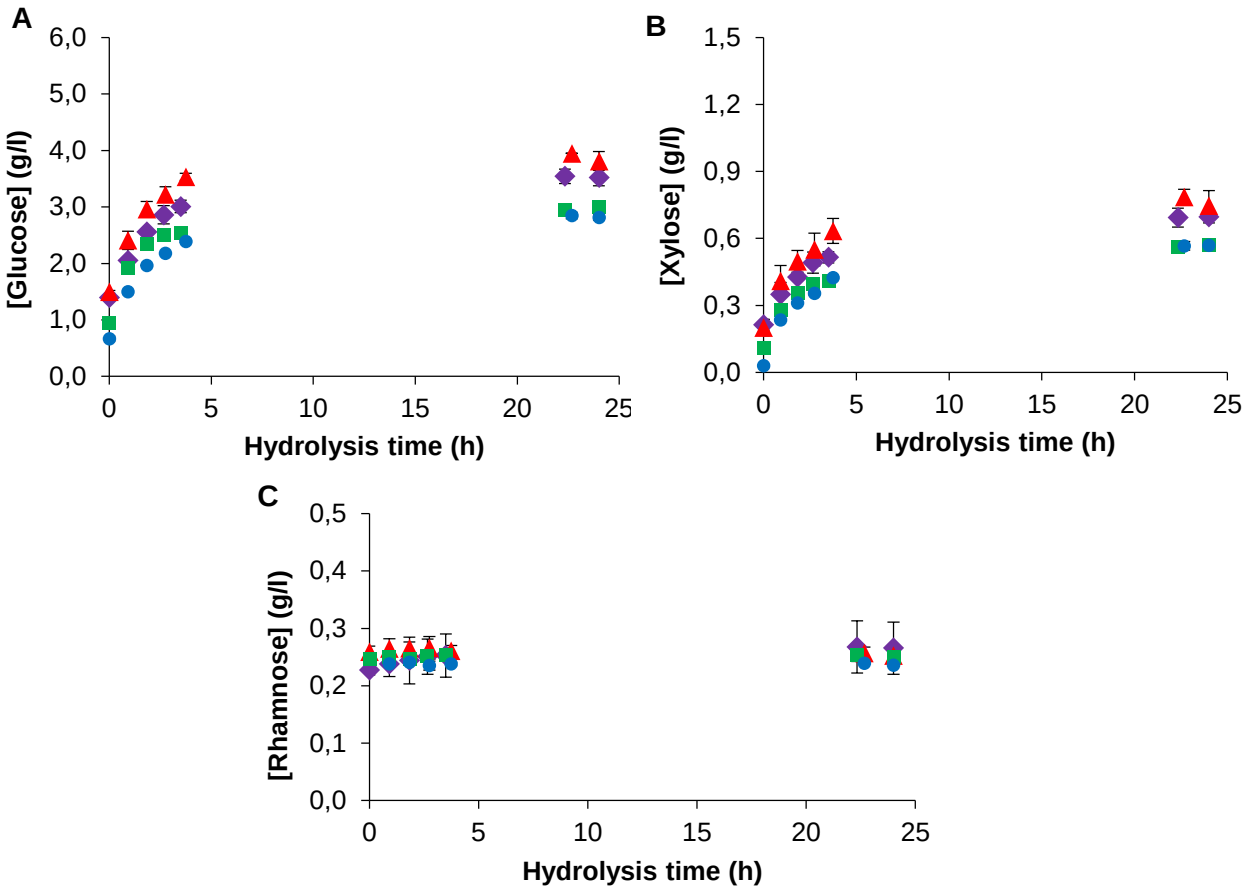



**Figure 14.** The concentration of released glucose (**A**), xylose (**B**) and rhamnose (**C**) (g/l), analysed by HPLC, over the enzymatic hydrolysis of *U. lactuca* at 43.2 g/l, after a chemical pre-treatment with 1.0% (w/V) of $H_2SO_4$ at 121°C for 30 minutes. The hydrolysis was performed in a total volume of 40 ml and at 50°C under agitation with some differences in the enzymatic cocktail. 11.3 µl of cellulase and 12 µl of β-glucosidase (●).11.3 µl of cellulase and 12 µl of β-glucosidase, 7.5 µl of xylanase and glucoamylase (■). 11.3 µl of cellulase, 12 µl of β-glucosidase, 6 µl of α-amylase and 7.5 µl of glucoamylase (▲) and these enzymes plus glucuronidase at 0.5% (w/v) (♦).

In general, the glucose and xylose yields of released monosaccharides were lower in combined hydrolysis when compared with enzymatic hydrolysis after biomass sterilization. This could be a result of the presence of some enzymes inhibitors formed during acid pre-treatment. As before the use of a four-enzyme cocktail with α-amylase is favourable and the addition of glucuronidase did not increase the release of rhamnose and affected negatively the yield of glucose and xylose. In combined hydrolysis, the pH had small variation when compared with enzymatic hydrolysis, since the final pH is about 4.5-4.6, which is still in within the pH range for optimal activity of the used enzymes mentioned previously.

### 3.8. Ulvan Extraction and Glucuronidase Activity Assay

In an effort to evaluate the glucuronidase activity an assay was performed using ulvan as substrate. As mention before, some authors reported the hydrolytic activity of β-glucuronidase on ulvanobiouronic acid from ulvan originating rhamnose and glucuronic acid [57].

Firstly, ulvan was extracted from *U. lactuca* by enhancing its solubility through incubation at high temperatures and at low pH to disperse ulvan aggregates, facilitating its extraction. The ulvan extracted correspond to 14.4% of *U. lactuca* dry weight. This value is within the expected since ulvan represents 9-36% of *Ulva* dry weight [81]. Other authors using the same extraction method report the release of 21.97% of ulvan [82]. The obtained ulvan is composed of 19.5% ± 0.2 of rhamnose, 5.6% ± 0.1 of glucose and 3.5% ± 0.1 of xylose. As expected ulvan is rich in rhamnose (16-29%) [57] and these values are in agreement with the composition of the ulvan extracted in the same conditions from *Ulva lactuca* (14.27%, 3.25% and 2.63% of rhamnose, glucose and xylose, respectively) [82].

The efficiency of ulvan extraction can be expressed as the percentage recovery of the initial rhamnose content in *Ulva lactuca*, since this is the main constituent in ulvan. The calculated efficiency of ulvan extraction, 26.7%, when compared with the literature under the same conditions, 49.20% [82], was lower. The low efficiency can be explained due to some difficulties to maintain the temperature in the extraction. The temperature is important to solubilise ulvan enhancing its solubility [81] and it was demonstrated that the efficiency increase with the temperature (80-90°C) for all the pH and time extractions tested [82].

The evaluation of glucuronidase activity was performed combining acid-hydrolysis using TFA 2 M and enzymatic hydrolysis using β-glucuronidase as described in the literature by Costa C. et al. (2012) [83]. As happened before the rhamnose concentration does not change during enzymatic hydrolysis (data not shown). However, the acid treatment with TFA 2 M allowed the released of almost 85% of rhamnose from ulvan.

## 3.9. Influence of Chemical Pre-treatment with TFA on the Release of Rhamnose in the Combined Hydrolysis of *U. lactuca*

TFA 2 M was used instead of $H_2SO_4$ 1.0% (w/v) to increase rhamnose concentration in the hydrolysate. This pre-treatment allowed the released of 64-72% of rhamnose and the maximum glucose and xylose were obtained also when using the four-enzyme cocktail with α-amylase (figure 15). As in combined hydrolysis with $H_2SO_4$ the pH decreases during the enzymatic hydrolysis but with a more marked change, since the final pH is lower (4.2-4.3) and more distant from the ideal pH for the enzymes. Despite the increase in rhamnose, the obtained glucose and xylose concentrations were lower than when $H_2SO_4$ 1.0% (w/v) was used as well as without any chemical pre-treatment. Rhamnose is the second monosaccharide more abundant in *U. lactuca*, its release allowed a higher final concentration of carbohydrates, 9.3 g/l in the hydrolysate. However, higher HMF concentrations, compared to the treatment with 1.0% (w/v) $H_2SO_4$, namely 0.06 g/l, have to be taken into account. Additionally, furfural, another possible inhibitor, was also detected by HPLC analysis upon hydrolysis with TFA. Still, this hydrolysate was used for further studies involving microbial cultivation since it is the one with higher total carbons and rhamnose concentration and where HMF concentration was still lower than the inhibition level indicated previously for *H. boliviensis*.

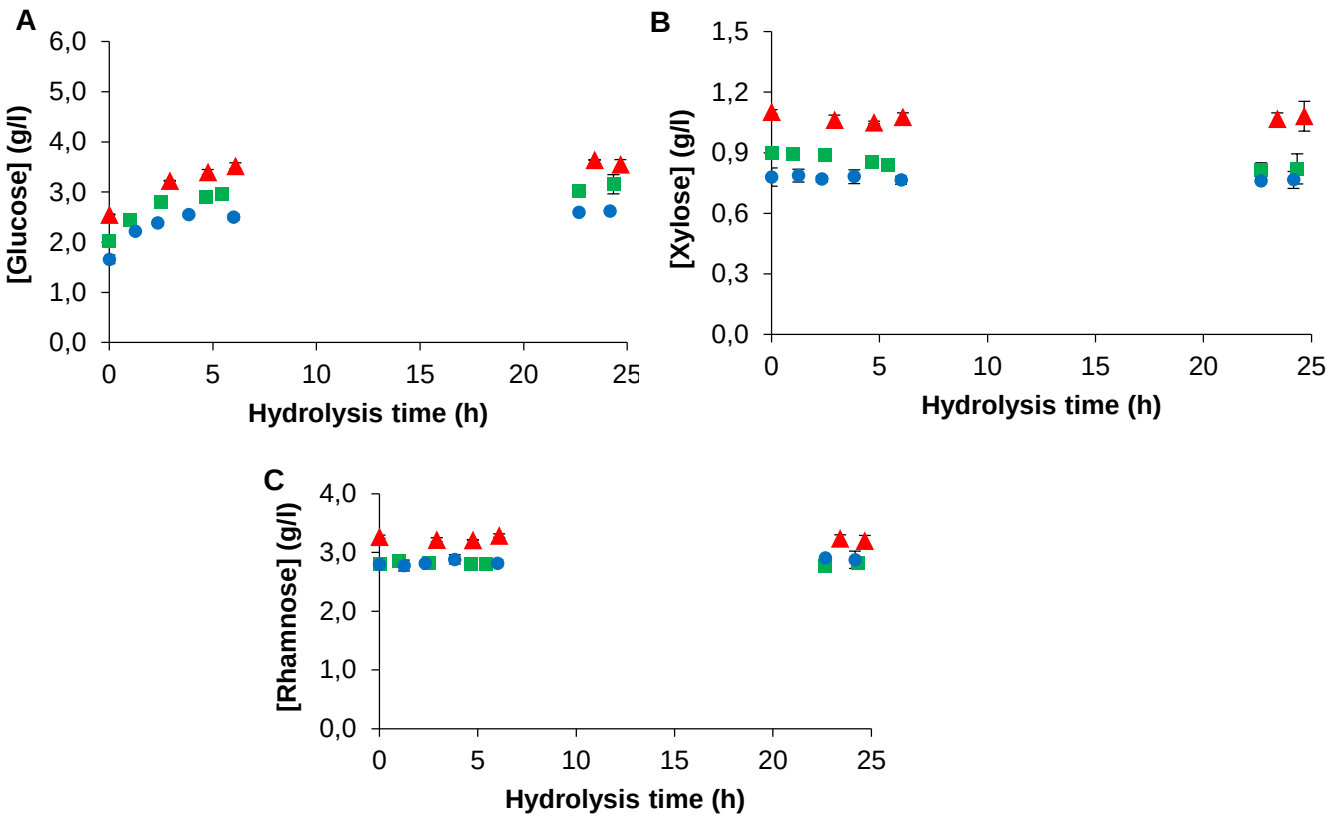



**Figure 15.** The concentration of released glucose (**A**), xylose (**B**) and rhamnose (**C**) (g/l), analysed by HPLC, over the enzymatic hydrolysis of *U. lactuca* at 43.2 g/l, after a chemical pre-treatment with TFA 2 M at 121°C for 45 minutes. The hydrolysis was performed in a total volume of 40 ml and at 50°C under agitation with some differences in the enzymatic cocktail. 11.3 µl of cellulase and 12 µl of β-glucosidase (●).11.3 µl of cellulase and 12 µl of β-glucosidase, 7.5 µl of xylanase and glucoamylase (■). 11.3 µl of cellulase, 12 µl of β-glucosidase, 6 µl of α-amylase and 7.5 µl of glucoamylase (▲).

The monosaccharides released yield after all described hydrolytic treatments for 43.2 g/l of *U. lactuca* are present in table 7.

**Table 7.**Calculated yields of glucose, xylose and rhamnose released after enzymatic hydrolysis with sterilized biomass and combined hydrolysis with 1.0 % (w/v) of $H_2SO_4$ and 2 M TFA chemical pre-treatment. The pre-treatment was performed at 121°C for 30 minutes with 1.0% (w/v) of $H_2SO_4$ and for 45 minutes with 2 M TFA. **Enzymatic cocktail 1:** 0.220 $mg_{protein}$/ml of cellulase and 0.054 $mg_{protein}$/ml of β-glucosidase. **Enzyme cocktail 2:** 0.220 $mg_{protein}$/ml of cellulase, 0.054 $mg_{protein}$/ml of β-glucosidase, 0.075 $mg_{protein}$/ml of xylanase and 0.075 $mg_{protein}$/ml of glucoamylase. **Enzyme cocktail 3:** 0.220 $mg_{protein}$/ml of cellulase, 0.054 $mg_{protein}$/ml of β-glucosidase, 0.017 $mg_{protein}$/ml of α-amylase and 0.047 $mg_{protein}$/ml of glucoamylase. **Enzyme cocktail 4:** enzyme cocktail 3 more 0.5 % (w/v) of glucuronidase.

| Hydrolysis Conditions | Enzymatic Cocktail | Glucose yield (%) | Xylose yield (%) | Rhamnose yield (%) |
|---|---|---|---|---|
| Enzymatic Hydrolysis | 1 | 64.7±1.9 | 31.5±0.7 | ---- |
| | 2 | 90.6±1.2 | 47.5±6.7 | ---- |
| | 3 | 106.4±6.3 | 76.9±6.5 | ---- |
| | 4 | 92.8±0.9 | 56.3±2.0 | ---- |
| Combined Hydrolysis with 1.0 % (w/v) of $H_2SO_4$ | 1 | 59.2±1.2 | 36.3±1.0 | 5.3±0.1 |
| | 2 | 62.4±0.6 | 36.4±1.3 | 5.7±0.01 |
| | 3 | 82.1±0.1 | 50.1±2.2 | 5.9±0.4 |
| | 4 | 73.6±2.7 | 44.6±2.1 | 5.9±1.0 |
| Combined Hydrolysis with TFA 2 M | 1 | 54.5±0.3 | 51.6±0.1 | 64.9±0.6 |
| | 2 | 65.6±4.0 | 57.4±0.8 | 63.7±1.5 |
| | 3 | 75.7±0.1 | 71.0±1.8 | 72.4±0.3 |

## 3.10. *Halomonas elongata* Cultivation

For the selection of the strain, besides the production of PHB, it is important its ability to uptake the mixture of different monosaccharides present in the hydrolysate. The reported substrate utilization spectrum of *Halomonas elongata* includes not only glucose and xylose but also rhamnose, making a good candidate for cultivation using *U. lactuca* hydrolysate [73]. *H. elongata* is a halophilic gram-negative bacteria capable of accumulating PHB of 55% (w/w) of the cell dry weight[73].

As mentioned above, for PHB production *H. elongata* has to grow with an excess of carbon source under stressed conditions, due to limitation of one essential nutrient. In the assays aiming at PHB production, the cultivation medium was designed to attain N limitation as no N source was added except the one that might be present in the *Ulva* hydrolysate. The first hydrolysate tested was the one prepared using the combined hydrolysis with TFA 2 M as it contained higher rhamnose and total carbohydrates concentration.

*Ulva* hydrolysates were prepared by treating 100.0 g/l *U. lactuca* instead of 43.2 g/l in a total volume of 200 ml. The increase of algae biomass concentration decreased the releasing yields (57.8%, 63.1% and 57.5% for glucose, rhamnose and xylose, respectively) but allowed to obtain a hydrolysate with higher sugar concentration namely 16.2 g/l of carbohydrates (7.6 g/l, 6.0 g/l and 2.6 g/l of glucose, rhamnose and xylose, respectively), after pre-treatment with TFA 2 M and enzymatic hydrolysis with four-enzyme cocktail with α-amylase. However, the HMF formed by the degradation of hexose monosaccharides also increased to 0.11 g/l, which after addition of the concentrated mineral medium to attain the final PHB production medium is diluted to 0.07 g/l.

The results showed that *H. elongata* was not able to grow in the culture containing the obtained hydrolysate, since cell ($OD_{600}$ and CDW) and carbohydrates concentrations did not change. The culture maintained the initial 3.25 ± 0.12 g/l of CDW mainly due to residues present in the hydrolysate.

In order to evaluate if TFA had an inhibitory effect on cell growth, the complete pre-inoculum medium supplemented with glucose to a final concentration of 20 g/l was used and TFA added in the same concentration as in the assay described above. The growth was monitored and compared with a control without TFA in the medium. The uptake of glucose and *H. elongata* growth is represented in figure 16.

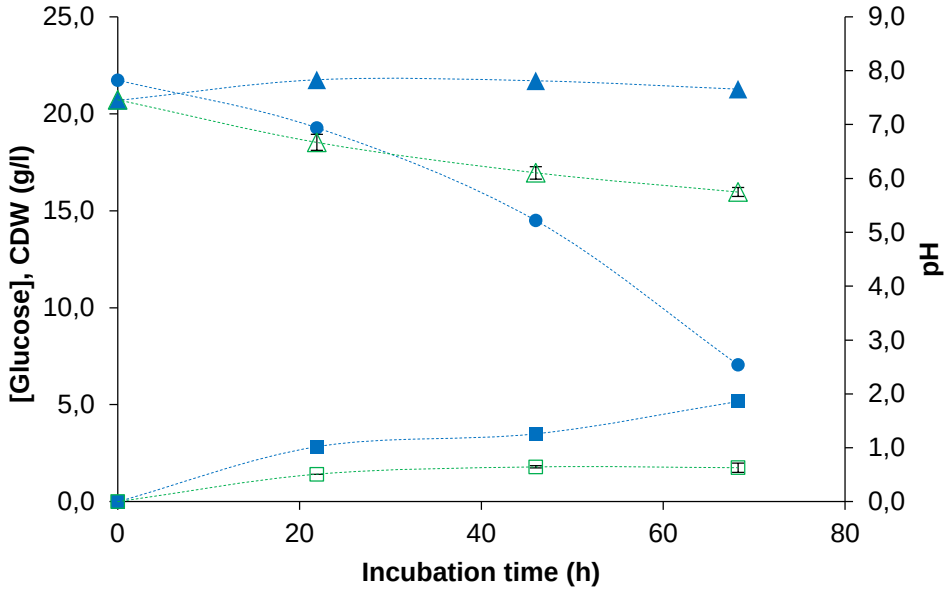



**Figure 16.** Glucose concentration (g/l), obtained by HPLC analysis, CDW (g/l) and pH during incubation time of *H. elongata* cultures at 35°C and 200 rpm orbital shaking, represented with circles (● and ○) squares (■ and □) and triangles (▲ and Δ), respectively. The cultures were inoculated in the pre-inoculum medium with 20 g/l of glucose and TFA 0.70 M represented by the open circles, squares and triangles (○, □ and Δ) or without TFA represented by the filled ones (●, ■ and ▲).

Up to 46 hours of incubation *H. elongata* had similar consumption of glucose in both assays, in the control and in the presence of TFA, even when the biomass concentration in the control was 2.0 times higher. In the next 24 hours of incubation, the control continued to grow and consume glucose reaching a CDW 3.0 times higher than in the presence of TFA, which kept the same values of $OD_{600}$, CDW and

glucose concentration in the culture. The difference between the attained biomass in the control culture and in the presence of TFA may be due to pH since with TFA the pH culture decreased faster. After 22 hours of incubation, the pH was 6.7 while in the control was 7.8. The interruption in growth after 46 hours of incubation may be due to the low pH, 6.1 that dropped to 5.7 after 68 hours, while in the control culture was 7.66. *H. elongata* has a pH range between 5.0 and 9.0 and an optimal pH of 8[84, 85]. The collected samples were analysed by GC to determine PHB concentration. As expected, the biomass of these assays did not accumulate PHB, since the nitrogen concentration in the inoculum medium used is high.

These results demonstrated that TFA with a concentration of 0.70 M in *H. elongata* cultures affects negatively the growth but does not inhibit it. As TFA-produced hydrolysates revealed a higher HMF and furfural concentrations (the latter was not quantified due to the non-availability of the standard at the time) the hydrolysate was treated with activated carbon, the most effectively used adsorbent that has been shown to remove between 0.1 and 2 g/l of furfural[86]. The hydrolysate was processed with 10 g/L activated carbon prior to sterilization. This treatment was able to remove all the HMF present and reduce 3.6 fold the furfural concentration, i.e the area corresponding to furfural. However, after the sterilization of the obtained hydrolysate, HMF concentration increased to 0.05 g/l, (0.03 g/l after dilution in the culture medium), and furfural concentration increased 2.8 times, almost the initial concentration before activated carbon treatment. Besides, after sterilization, the total carbohydrates concentration decreased to 14.3 g/l (6.4 g/l of glucose, 2.0 of xylose and 5.9 g/l of rhamnose).

The activated carbon-treated hydrolysate was tested as C source for growth and polymer production. The results showed that *H. elongata* also was not able to grow in the culture containing the treated hydrolysate. This might be due to the high concentrations of HMF and specially furfural that increase after sterilization even after activated carbon treatment.

The hydrolysate produced with the combined treatment with 1.0% (w/v) sulphuric acid followed by enzymatic hydrolysis was also tested as C source for the growth and polymer production by *H. elongata*. Aiming at this, 200 ml of hydrolysate were prepared using 100 g/l of *U. lactuca*. The hydrolysate obtained contained 10.9 g/l of carbohydrates (9.0 g/l of glucose, 1.7 g/l of xylose and 0.2 g/l rhamnose). The total carbohydrates concentration is lower than in the hydrolysates produced with TFA treatment, despite a higher glucose titre. Like before the increase in *U. lactuca* concentration from 43.2 g/l to 100 g/l reduce the releasing yields of each monosaccharide (72.2%, 36.1% and 2.3% of releasing yields for glucose, xylose and rhamnose, respectively). HMF concentration was 0.05 g/l and furfural was not detected by HPLC.

From the results given in figure 17, it is observed that *H. elongata* was able to grow in the culture containing the obtained hydrolysate.

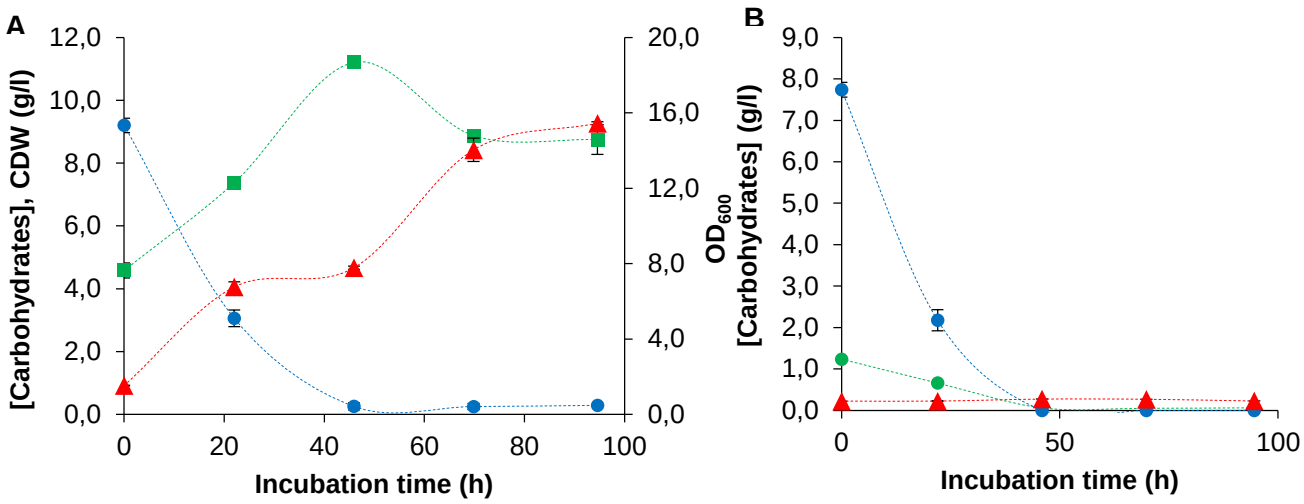



**Figure 17. A)** Total carbohydrates concentration (●), obtained by HPLC analysis, CDW (■) (g/l) and $OD_{600}$ (▲) during incubation time of *H. elongata* cultures at 35°C and 200 rpm orbital shaking. **B)** Glucose (●), xylose (■) and rhamnose (▲) concentration during incubation time of *H. elongata* cultures at 35°C and 200 rpm orbital shaking. The cultures were inoculated in the medium for PHB production with hydrolysate from enzymatic hydrolysis of *U. lactuca* after chemical pre-treatment with 1.00% (w/v) of $H_2SO_4$.

Glucose was fully depleted after 48 hours of incubation and at this time xylose concentration is residual (below 0.1 g/l), remaining constant during the next days of incubation. *H. elongata* is thus able to uptake glucose and xylose in parallel. Rhamnose concentration although very low (0.2 g/l) did not change. This is unexpected as *H. elongata* has a high affinity for rhamnose. The difference between the profile of CDW and $OD_{600}$ is due to hydrolysate residues that contribute to CDW, since the cultures had an initial CDW of 4.58 ± 0.26 g/l from the hydrolysate residues. From $OD_{600}$ curve it is possible to observe a lag phase after 24 hours of incubation to *H. elongata*. This is probably an error in the $OD_{600}$ determination since at this time glucose was still available.

Finally, the biomass obtained was analysed to quantify the PHB production. Unfortunately, no accumulation of PHB was observed, probably due to low carbohydrates concentrations and high nitrogen concentration in *U. lactuca* hydrolysate that might be caused by the protein fraction of the seaweed. The nitrogen content on the hydrolysate was analysed and a value of 1125.3 mg N/l. Therefore, the C/N ratio is too far from the needed ratio of 20, that with this nitrogen concentration is needed 48.6 g/l of glucose. With this N value, the obtained C/N ratio of 3.8 is too far from the needed ratio of 20. To attain the needed C/N ratio, the initial glucose concentration in the cultivation medium should be approximately 50.0 g/l.

## 4. Conclusion

The historic low price of fossil feedstock together with optimised production processes and high production costs of PHAs have restricted commercial production of bio-based and biodegradable plastics, an efficient and environmentally friendly alternative. To reduce raw materials costs, inexpensive and sustainable carbon-rich biomass such as *U. lactuca* obtained from wastewater treatment or harvested from green tides at coastal areas can be used.

Different hydrolysis conditions to obtain monomeric sugars hydrolysate for further bioplastic production were evaluated. The enzymatic hydrolysis with a cellulase cocktail using water rather than acetate buffer delivered a released sugar yield of 41.87% and ensured the compatibility of the hydrolysate with the fermentation downstream process. The enzymatic treatment with the highest sugar release involved the combination of four enzymes namely cellulase, β-glucosidase, glucoamylase and α-amylase. The inability of the enzymatic cocktail alone to release rhamnose was demonstrated. The release of a significant amount of rhamnose from *U. lactuca* requires an acid pre-treatment with at least 1.0% (w/v) of $H_2SO_4$ or with TFA 2 M. A maximum reducing sugars concentration of 9.6 g/l using a biomass concentration of 43.2 g/l was achieved with a combined hydrolysis treatment using a chemical pre-treatment with TFA 2 M followed by enzymatic treatment with the four enzyme cocktail. However, HMF and furfural were produced after acidic pre-treatment; the highest titres found after TFA 2 M treatment. In the hydrolysate treated with 1.0% (w/v) of $H_2SO_4$, furfural was not detected and the HMF levels were lower. *H. elongata* cultivations were carried out using both hydrolysates as C source. While no growth was observed with the TFA hydrolysate, the hydrolysate prepared with 1% $H_2SO_4$ allowed cell growth. However, no PHB accumulation was observed since C/N ratio was too low due to the high nitrogen content of *U. lactuca* hydrolysates.

In future work, the TFA hydrolysate can be treated with activated carbon, for HMF and furfural removal, after TFA evaporation to avoid the new formation of these inhibitors during sterilization. Alternatively, in order to increase the C/N ratio and induce PHB production, either extra sugar should be supplemented to *U lactuca* hydrolysates or residues of *U. lactuca* after protein extraction should be used instead of the whole biomass. Also, the results of hydrolysis and subsequent *H. elongata* fermentation (SHF process) can be compared with *Saccharophagus degradans* fermentation. *S. degradans*, a gram-negative bacterium has been shown to degrade cellulose and several algal polysaccharides and accumulate PHAs (CBP process).

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