TÉCNICO LISBOA

From Macroalgae to Bioplastic

Seaweed Hydrolysates for Polyhydroxyalkanoate Production by Marine Bacteria

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Summary

Polyhydroxyalkanoates (PHAs) are biodegradable and biocompatible polyesters produced by various bacterial strains as reserve materials. They present a broad range of properties, finding thus applications from agriculture to medicine as plastic substitutes. Despite their versatility, the commercialization of PHAs still has limited success and one of the major reasons is the cost of the substrate. This work aims to present macroalgae hydrolysates as a solution for this setback. Macroalgae are a rich source of carbohydrates and present many benefits compared to terrestrial biomass: production yields of algae per unit area are higher and are easier to process due to lack of lignin. The green seaweed *Ulva lactuca* and residues of the red seaweed *Gelidium sesquipedale* obtained after agar-agar extraction were tested as sugar sources for the production of poly-3-hydroxybutyrate P(3HB) by two different marine bacterial strains. U. *lactuca* whole biomass and the G. *sesquipedale* residues were found to have, respectively, 37% and 48% (w/w) of carbohydrates. Both the whole *Ulva* biomass and the *Gelidium* residues underwent hydrolysis to yield a sugar-rich algal hydrolysate. Enzymatic hydrolysis and pretreatment assays were performed with the aim of scaling up the hydrolysate production for bacterial usage. Using 43.2 g/L algal substrate concentration and phosphate buffer, the combined enzymatic hydrolysis yielded between 80 to 90% of total carbohydrate for both algae. Halophilic bacterial strains able to consume the main sugars present in each algal hydrolysate were selected from literature. H. *boliviensis* shown to grow effectively on glucose and galactose was chosen for growth on *Gelidium* hydrolysates attaining 10.4 g/L CDW and 23% (w/w) PHB accumulation on shake flasks. H. *elongata* demonstrated an ability to grow effectively on glucose, rhamnose and xylose and was therefore chosen for growth on U. *lactuca* hydrolysates reaching 3.9 g/L CDW and a 26% (w/w) PHB content. Even though these findings are still in embryonic development, it can be perceived that U. *lactuca* and G. *sesquipedale* have high potential to be used as a carbon sources, and therefore should be integrated in biorefinery contexts, which can be extrapolated to macroalgae biomass. Also, both *H. boliviensis* and *H. elongata* can produce PHB from a variety of sugars derived from macroalgae biomass and thus these halophilic bacteria have an auspicious future as hosts for producing useful compounds from biomass resources.

Keywords: Polyhydroxyalkanoates, PHB Production, Macroalgae Hydrolysis, Bioplastics, *Halomonas boliviensis*, *Halomonas elongata*, Marine Biorefinery, Halophilic Bacteria

1. Introduction

Plastic is a relatively economical, durable and adaptable material. These properties have led to the creation of thousands of products, which have brought benefits to society in terms of economic activity and quality of life [1]. However, the broad use of synthetic, petroleum-derived plastics has created a generally recognized environmental problem since these materials take several decades to degrade and accumulate in the environment with noxious effects. The necessity to replace oil-based plastics with biodegradable plastics is urgent and widely acknowledged. The EU Waste Framework Directive states that 10% of the plastic market should be bioplastics by 2020 [2].

Polyhydroxyalkanoates (PHAs) are bacterial produced bioplastics that represent an alternative to a wide range of petroplastics such as poly-propylene, poly-styrene and poly-ethylene and can be processed into packaging medical devices, cups, compostable bags and other disposable items. [3]. PHA's are both bio-based and biodegradable, comprising multiple environmental benefits. They can quickly become one of the major players in the bioplastic world. Yet, PHAs are not commercialized as bulk plastics due to the high price of the carbon source, reaching 30% of total cost, and the low productivity of the bioprocesses [4], [5]. Furthermore, the production of PHAs is currently based on human food sources, competing with edible feedstocks and leading to ethical concerns. Hence, in order to achieve sustainable and efficient production of PHAs, the first obstacle to overcome is finding a more appropriate carbon source. Residual industrial and agricultural streams are currently being tested [6] and lignocellulosic biomass is considered a desirable raw material, since it yields a sugar-rich hydrolysate, but the lignin fraction hinders the easy

release of the monosaccharides upon treatment. Acid hydrolysis is successfully applied but often leads to unwanted by-products which are mostly toxic to microorganisms and need to be removed, adding a detoxification step and increasing the bioprocess costs [7]. Macroalgae are a promising but underexploited raw material, that contain 25-60% (w/w) of carbohydrates. Compared to lignocellulosic biomass, hydrolysis of macroalgae occurs under milder conditions since lignin is absent. Furthermore, macroalgae hydrolysates are pentose-poor, increasing the number of strains able to use the released sugars, and do not compete for land and fresh water since they grow on seawater [8]. Thus, a sustainable process for PHA production may be attained by using macroalgal hydrolysates as carbon source for PHA production by bacterial cells within an integrated biorefinery approach. For an even more competitive approach halophilic bacteria were chosen since their cultivation is "self-aseptic", consequently requiring less energy (less strict disinfection conditions), as well as being less prone to contamination.

The core purpose of this work was poly-3-hydroxybutyrate (PHB) production by marine bacteria using sugar-rich seaweed hydrolysates as carbon source. Preliminary studies were performed in order to achieve this goal which can be divided into two main groups: a) Improvement and scale-up of the enzymatic deconstruction of macroalgae residues and whole macroalgae biomass and b) Growth and PHB accumulation by halophilic bacteria using the digested carbohydrate fraction as carbon source.

2 Materials and Methods

2.1. Raw Materials

Gelidium sesquipedale residues from agar extraction process courtesy Iberagar SA, Portugal) were maintained at -4ºC after reception, being afterwards washed several times with distilled water to remove sands and other residues and dried at 50ºC in a Memmert oven (Model 400). Subsequently, in order to remove all remaining humidity, the residues were lyophilized, being firstly freezed at -80ºC and then placed under vacuum at -43ºC, for 48 hours. *Ulva lactuca* was cultivated in 1 m^3 tanks containing filtered sea water in a greenhouse with artificial illumination and home temperature control, property of Wageningen University and Research (Nergena, Wageningen – The Netherlands). Samples was harvested in the period June-August 2016 After biomass collection, the excess water was removed and the samples were freeze dried in a sublimator 2x3x3 (Zirbus Technology GmbH, Germany) for 72 hours.

Dried samples were stored in sealed bags and maintained in the dark, at room temperature, until further use. Lyophilized *Gelidium* and *Ulva* were milled separately in a kitchen coffee grinder for 3 to 5 minutes, to obtain a fine and homogeneous powder that was used as raw material during the assays. The samples were stored in plastic bags at room temperature.

Enzymatic hydrolysis was performed using specific cocktails of commercial enzymes, namely, cellulase complex (NS 22086), β-glucosidase (NS 22118) glucoamylase (NS 22035) and xylanase (NS 22083), from Novozymes® (Bagsvaerd, Denmark).

2.2. Algal Hydrolysate Preparation

Total carbohydrate quantification was determined, according to NREL (National Renewable Energy Laboratory, USA) specific method for algal biomass [9].

Enzymatic hydrolysis was carried out at 50ºC and pH 4.8 with continuous stirring (660 rpm) in a thermostatically controlled incubator chamber (JP Selected). The enzyme cocktail, 1:100 (v/v), used for *Gelidium* residues was 100 µL of cellulase with 75 µL β-glucosidase and 100 µL of cellulase, 75 µL glucosidase, 75 µL glucoamylase, 75 µL of xylanase, for *Ulva lactuca*. Buffer relevance was studied, comparing the carbohydrate yield of enzymatic hydrolysis on 0.05M acetate buffer; 0.1M Sørensen's phosphate, chosen for its buffering range from pH 5.0 to 8.0.

Different types of chemical pretreatment were tested before the enzymatic hydrolysis namely: mild acid hydrolysis with 0.5 % and 1% H_2SO_4 and mild alkaline hydrolysis with 1M NaOH. These assays were performed in 50mL flasks with 20mL total volume with a substrate concentration of 43.2 g/L, or 0.864 g of grinded lyophilized algae, in duplicate. Samples were placed in the autoclave at 121ºC, 1 bar for 30 min and sugar quantification after being removed from the autoclave.

The chosen final substrate concentration for scale up was of 43.2 g/L allowing for a minimum 20 g/L total sugar concentration in the shake flask. Scale up was performed in 250 mL Schott® flasks with 4.32 g grinded lyophilized algal material. Pretreatment was performed on 75 mL 0.5% and 1% H_2SO_4 in the autoclave at 121ºC, 1 bar for 30 min, neutralized using NaOH 6M, concentrated buffer (1:10 v/v) and the enzyme cocktails defined previously in the same 1:100 (v/v) proportion. Mili-Q® water was also added in order to make up to 100 mL of total volume. 200 µL samples were taken before the enzymatic cocktail was added, after the enzyme addition and during enzymatic hydrolysis reaction. After 24h of enzymatic hydrolysis samples were neutralized to pH 7.2 or 8.0 according to the bacteria used, sterilized in the autoclave for 20 minutes at 121ºC, 1 bar and centrifuged for 10 000 rpm for 15 minutes (1-15 P microcentrifuge, Sigma) in aseptic conditions. Supernatant was collected, also in aseptic conditions, and stored in the freezer at -20ºC.

2.3. Cultivation Conditions

All procedures were carried out in a laminar flow chamber (BIOAIR Instruments aura 2000 M.A.C.) and all the material used was sterilized previously n the autoclave at 121ºC, 1 bar for 20 minutes, to guarantee aseptic conditions.

Culture Storage

Both bacteria were maintained at 4ºC on petri dishes with solid HM medium. Cultures were monthly refreshed.

Solid HM medium: (medium composition for bacterial growth and storage): The medium used for isolation and maintenance of the bacteria was described by [10]. The medium contained: NaCl 45.0 g/L; MgSO4.7H2O 2.5 mL/L of a 100 g/L MgSO4.7H2O solution; CaCl2.2H2O, 0,09 g/L; KCl, 0.5 g/L; NaBr, 0.06 g/L; Peptone (Difco), 5 g/L; yeast extract (Difco), 10 g/L; glucose 1g/L and granulated agar, 2.0%. The pH was adjusted to 7.5 by using 1 M NaOH and sterilized in the autoclave at 121ºC, 1 bar for 20 minutes.

Inoculum preparation

Both bacteria were grown in 100 mL of seed culture medium, described below, supplemented with 20g/L of glucose in 500 mL flasks in an orbital incubator at 170 rpm, 30°C, till the exponential phase was reached. When the cultures achieved an O.D.600nm of 1.50 (approximately 14h for H. *boliviensis* and 24h for H. *elongata*), culture samples were then used as inoculum 0.05% (v/v) in the production media.

Defined medium composition for growth of *Halomonas boliviensis* (seed culture medium), as defined by [11], [12] was composed of 45 g/L NaCl; 25 mL/L of a 100 g/L MgSO4.7H2O solution; 0.55 g/L K2HPO4; 2.3 g/L NH4Cl, 15 g/L Tris; 3 g/L Monosodium glutamate (MSG) and 0.005 g/L FeSO4.7H2O. The pH of the medium was adjusted to 7.5 using concentrated HCl and sterilized in the autoclave at 121ºC, 1bar for 20 min. For simplicity reasons this medium was also used as seed for H. *elongata*.

Culture Media for PHB Production

The assays were performed in 500 mL shake flasks, using the production medium supplemented with 20 g/L sugar or algal hydrolysate to a total volume of 100 mL. The assays were performed at 30ºC and 170 rpm in an orbital shaker (Infors HT) for at least 140 hours.

For the growth on the hydrolysates, a 20-fold concentrated PHB production medium specific for each strain was prepared and 5 mL were added to the 75 mL of previously prepared hydrolysate, alongside with 15 mL of 300 g/L NaCl concentrated solution, 5 mL of 100 g/L MgSO4.7H2O in the assays with H. *boliviensis* and 500 µL in the assays with H. *elongata* and 5 mL of the bacterial inoculum. In the H. *elongata* case 4.5 mL of Mili-Q® was also added to make up to 100 mL of total volume.

Halomonas boliviensis

H. *boliviensis* medium composition for PHB production was 45 g/L NaCl; 50 mL/L of a 100 g/L MgSO4.7H2O solution; 2.2 g/L K2HPO4; 0.005 g/L FeSO4.7H2O.

Variable concentrations of the following constituents were tested, namely, NH4Cl: 1.0 g/L and 4.0 g/L; Tris: 0 g/L and 15 g/L; Monosodium glutamate (MSG): 2g/L and 20g/L. For each one of these media, the pH was adjusted to 7.5 using concentrated HCl and sterilized in the autoclave at 121ºC, 1 bar for 20 minutes.

Halomonas elongata

H. *elongata* medium composition for PHB production was 45 g/L NaCl; 3.0 g/L K2HPO4; 1.0 g/L NH4Cl; 12.8 g/L Na2HPO4.7H2O; 0.15 g/L CaCl2.2H2O; 1.0 g/L Yeast extract and 1 mL/L of trace element solution [13] that contained 10 mL/L of a 25% HCl solution; FeCl2.4H20, 1.5 g/L; CoCl2.6H2O, 190 mg/L; MnCl2.4H2O 100 mg/L; ZnCl2, 70 mg/L; H3BO3, 62 mg/L; Na2MoO4.2H2O, 36 mg/L; NiCl2.6H2O, 24 mg/L; CuCl2.2H2O, 17 mg/L; at first the ferrous chloride was dissolved in the hydrochloric acid, while the other components were added after addition of distilled water. The pH of the medium was adjusted to 8.0 using 1M NaOH and sterilized in the autoclave at 121ºC, 1 bar for 20 minutes.

2.4. Analytical Procedures

Cellular growth was monitored by measuring optical density (O.D.) at 600 nm in a double beam spectrophotometer (Hitachi U-2000), using ultrapure water as reference sample. Towards the determination of the $O.D._{600nm}$ an aliquot of the culture sample was diluted with Mili-Q® water in order to obtain an absorbance value in a range between 0.05 and 0.6. The 3 mL glass cuvettes used had an optical path length of 1 cm.

For dry weight measurements, 1.2 mL samples were collected in previously dried and weighted 1.5 mL eppendorf tubes and centrifuged in a Sigma 1-15 P microcentrifuge at 12 000 rpm for 3 minutes. The pellet was washed with Mili-Q® water, re-suspended and centrifuged again following the same protocol twice. The supernatant was eliminated and the eppendorf containing the washed pellet was dried at 60 ºC in a Memmert oven (Model 400) until constant weight. The weight difference between the eppendorf with the dried pellet and the same previously dried eppendorf was used to calculate the total dry weight of the culture.

The identification of monosaccharides in the hydrolysates and in the cell culture media was

determined offline in a High-Performance Liquid Chromatography (HPLC) apparatus (Hitachi LaChrom Elite) equipped with a Rezex ROA.Organic acid H+ 8% (30 mm x 7.8 mm) column, an autosampler (Hitachi LaChrom Elite L-2200), an HPLC pump (Hitachi LaChrom Elite L-2130) and a Hitachi L-2490 refraction index detector. A column heater for long columns (Croco-CIL 100-040-220P, 40 cm x 8 cm x 8 cm, 30-99ºC) was connected externally to the HPLC system. The injection volume was 20 µL and elution was achieved using 5mM H_2SO_4 solution as mobile phase. The column was kept at 65ºC under a pressure of 26 bar, and the pump operated at a flow rate of 0.5 mL/min.Vials for HPLC analysis were prepared by mixing 200 µL of sample with 200 µL of a 50 mM H_2SO_4 solution in an microtube. After vortexing, these solutions were centrifuged (1-15 P microcentrifuge, Sigma) at 12 000 rpm for 3 minutes. Samples for injection consisted in 100 µL of the previous dilution plus 900 µL of the 50mM H_2SO_4 solution, resulting in a final dilution of 1:20. Calibration curves for glucose and xylose determinations were obtained for working ranges of 0.2 to 80 g/L and calibration curves for rhamnose and galactose were obtained for working ranges of 0.05 to 50 g/L.

In order to perform the PHB quantification, 1.2 mL aliquots of the culture sample were centrifuged in a Sigma 1-15 P microcentrifuge. The pellet was frozen after being washed with distilled water and then subjected to acidic methanolysis. The cells were re-suspended on 1mL of chloroform and transferred to Pyrex hermetic test tubes where a 1mL of a solution A containing the internal standard (97 mL of methanol, 3 mL of H_2SO_4 96% and 330 µL of hexanoic acid) was added. After using the vortex for 1 minute, the preparation was incubated for 5 h at 100 °C in a Memmert GmbH oven (Model 200). In order to neutralize the sample, after cooling a 60 g/L solution of Na_2CO_3 was added and the samples were vortexed for 1 min. These preparations were then centrifuged at 2000 rpm for 5 minutes and 200 µL of the organic phase were collected in GC vials and kept at -20°C for GC analysis. The polyhydroxyalkanoates quantification was carried out by gas chromatography (GC). Samples of the organic phase were analyzed in a gas chromatograph (Agilent Technologies 5890 series II) equipped with a FID detector and a 7683B injector. The oven, injector and detector temperatures were kept constant at 60 ºC, 120 ºC and 150 ºC, respectively. The capillary column was a HP-5 from Agilent J&W Scientific, 30 m in length and 0.32 mm of internal diameter. The data acquisition and integration were performed by a Shimadzu CBM-102 communication Bus Module and Shimadzu GC Solution software (Version 2.3), respectively. Peak identification was achieved using as standard 3-methyl hydroxybutyrate (Sigma).

3. Results and Discussion

3.1. Optimization of Enzymatic Deconstruction of Macroalgae for Bacterial Usage

Total Carbohydrate Quantification

Following the NREL protocol for [9] total carbohydrate quantification and subsequent HPLC analysis it was possible to verify that the *Gelidium* residues had a total of 48.11 ± 0.06 % of its dry weight in carbohydrates, with 36.05 ± 0.05% d.w. in glucose and 12.06 ± 0.01% d.w. in galactose. These results are coincident with the 51% described in the literature [15]. The fact that even after agar extraction the residues still possess c.a. 50% of carbohydrates illustrates that they are very good candidates for biorefinery integration.

The *Ulva* residues, as expected for green algae, consisted mainly of glucose, xylose and rhamnose, with the latter being obtain by hydrolysis of the polysaccharide *ulva*n, present in *Ulva* cell wall [16]. The total carbohydrate content was 37.40 ± 0.03% d.w., with 23.60 ± 0.02% d.w. of glucose, 11.12 ± 0.01% d.w. of rhamnose and 2.68 ± 0.02% d.w. of xylose. These results are coincident with the results obtained in the literature, that described a total carbohydrate content between 20 to 28% (w/w), with 8 to 17% (w/w) glucose, 7 to 9% rhamnose and 1 to 5% (w/w) xylose [17]–[19] by acid hydrolysis in terms of relative sugar quantity although there are small differences regarding total quantity since *Ulva lactuca* composition is known to change seasonally [20].

It is important to refer that the results for both algae may be an underestimation since some expected sugars, such as arabinose and mannose are not found, since they are reportedly present in small concentrations [19], [21] and therefore undetectable in the used HPLC apparatus.

Enzymatic Deconstruction of Macroalgae

Enzymatic deconstruction assays were performed based on the results of [14] and [22] In order to perform scale-up of the enzymatic deconstruction, it was necessary to calculate a substrate concentration that allowed for both algae to have at least 20 g/L of reducing sugars in the hydrolysate, including the sugars already present in the enzyme cocktail, that were quantified via HPLC to 8.0 ± 1.0 g/L, therefore expecting a minimum 30% conversion rate and applying a 10% excess margin to this result was attained the desired substrate concentration of 43.2 g/L. The increase of the substrate concentration as a scale up method was performed in order to avoid having to concentrate the hydrolysate for usage due to the inherent concerns of concentrating high

viscosity fluids such as sugar-rich solutions. In order to allow direct hydrolysate usage by bacteria the buffer effect was also studied. Since acetate buffer has a pH range of 3.6 to 5.6 and the bacteria chosen had an optimal grow above pH 7 [10], [23] it was not ideal for scale up. Sorenson's phosphate buffer was also studied since it had a pH range of 5.0 to 8.2, but the excess of phosphate ions could inhibit bacterial growth or enzyme performance. Accordingly, in order to test a more economical approach that couldn't hinder bacterial growth, enzymatic hydrolysis was also tested using only water with adjusted pH to 4.8 using HCl 1M. Nevertheless, when measuring pH after enzyme addition to pure water it was noticed that pH was already at approximately 4.8, meaning that the Novozymes® enzyme commercial solution has integrated pH control.

As pointed out in [14] the yield of released sugars using enzymatic hydrolysis decreases significantly when a concentrated substrate is used, although this happens more predominantly on *Gelidium*. This can be explained by the fact that even when heavily grinded the *Gelidium* residues particles are bigger than the powder created when grinding *Ulva*, meaning that is the substrate molecules are less accessible to the enzyme. This has a significant impact in the scale up process since it would be required to add a concentration step in the process in order to maintain the yield of enzymatic hydrolysis, leading to higher process costs. It's also possible to verify that altering from acetate buffer to phosphate buffer has a diminishing effect in the hydrolysis yield of *Gelidium* and a very small augmenting effect on the hydrolysis of *Ulva*.

The use of phosphate buffer instead of acetate buffer has the consequence of having the reaction pH adjusted to 5.0 instead of 4.8 due to the fact that the latter is below its buffering range. Since the cocktail used for *U. lactuca* is more complex and is not yet optimized, it is possible that the shift in pH has caused an improvement in enzyme activity since the Novozymes® enzymes have different optimal pH between themselves. Using no added buffer for the reaction had, for both algae, a diminishing effect in yield although, the algae still released a considerable amount of sugars.

Nevertheless, it is important to state that the Novozymes® enzymes used are not capable of releasing the rhamnose fraction of *U. lactuca* onto the medium. The rhamnose fraction is c.a. 12% of dry weight therefore it would be compulsory to add another enzyme to the cocktail that can release the rhamnose fraction.

Chemical Pretreatment

Again, it is possible to see that overall *Ulva* has better results than *Gelidium*. Since there is no agitation in autoclave and, as previously stated, the grinded green seaweed has a smaller particle size, the acid may have more capability to perform the hydrolysis in *Ulva*, adding up to the conclusion that the substrate molecules of *Gelidium* are less accessible.

Using 1% sulfuric acid proves to be more effective in both algae although there is a concern of toxic byproduct formation since Maillard reaction products can inhibit bacterial growth [24] and this reaction is favoured by acidic conditions [25], meaning that using alkaline pretreatment could be an interesting idea, but since it achieves so low yields and does not release the most difficult carbohydrates to obtain in enzymatic hydrolysis (galactose in the red seaweed and rhamnose in the green seaweed) there no relevant advantage of using this type of pretreatment.

Table 1 - Sugar content (%dw) of *Ulva lactuca* and *Gelidium* residues and monosugars (%dw) released by hydrolytic treatments using 43.2 g/L of substrate and phosphate buffer.

	Ulva lactuca				*Gelidium sesquipedale* residues		
	Glucose	Rhamnose	Xylose	Total	Glucose	Galactose	Total
Acid Hydrolysis	23	11	3	**37**	36	12	**48**
Enzymatic Hydrolysis (43.2 g/L Phosphate Buffer)	23	0	3	**26**	17	0	**17**
Pretreatment 1% H_2SO_4	11	4	1	**16**	0	7	**7**
Pretreatment 0.5% H_2SO_4	3	1	0	**4**	0	2	**2**
Combined Hydrolysis 1% H_2SO_4	23	4	1	**33**	36	11	**47**
Combined Hydrolysis 0.5% H_2SO_4	23	1	0	**24**	36	3	**39**

The acidic pretreatment seems to be the best option and pre-treating the algal material creates a possibility of an easier access of enzymes to the substrate molecules and therefore a potential improvement in yield when combined.

Combined Hydrolysis: Pretreatment and Enzymatic Deconstruction

According to the previous assays combined hydrolysis was performed. Firstly using 50 mL volume and acetate buffer and with samples only taken at the end of the process, to make sure no biomass or sugars were lost, and later using 100 mL of final volume for the enzymatic hydrolysis using phosphate buffer. The final results between the two assays were coherent, meaning that when taking samples, the final concentration of sugars was not significantly affected and there was minor biomass loss.

The results for the green seaweed showed only significant difference in the amount of rhamnose being released to the medium, with glucose being totally released in both cases. The xylose fraction was more noticeable with 1% sulfuric acid pretreatment, albeit since it is less that 3% of dry weight of the algae it can be under estimated in quantification, or conjoined with glucose, due to HPLC apparatus limitations, nevertheless since it is c.a. 7% of all possible releasable sugars it has a meaningful decreasing effect in yield.

Finally, as expected, the pretreatment with 1% sulfuric acid released less than 4% dry weight of rhamnose, and less than 1% dry weight for the 0.5% sulfuric acid pretreatment which poses the most relevant contribution to the lower yield since it's total 11% dry weight represents 30% of possible releasable sugars, and therefore only being achieved a maximum of 90% yield with the 1% sulfuric acid pretreatment and 80% with 0.5% sulfuric acid pretreatment.

The results for *G. sesquipedale* were also in line with the knowledge gained from previous assays. Galactose is mainly released in the mild acid pretreatment and more glucose release is achieved by the use of the enzyme cocktail, meaning that the pretreatment made the substrate molecules more accessible to the enzymes, with c.a. 100% yield of possible released sugars for 1% sulfuric acid. Regrettably, when using 0.5% sulfuric acid as pretreatment, the maximum obtained yield is only 80%.

Since the phosphate buffer significantly decreases enzyme effectiveness, meaning that the 0.5% did not made the substrate accessible enough to fill this effectiveness gap. Nevertheless, both algae results are promising for scale up, with effect on bacteria and possible by-products being discussed posteriorly.

3.2. Halomonas boliviensis Growth and PHB Accumulation at Shake Flask Scale

Within the deeper analysis of *H. boliviensis* growth and PHB production on glucose and galactose over time, the maximal growth and PHB production can be perceived at c.a. 90 hours of fermentation attaining a 10.8 ± 1.5 g/L of cell dry weight with a PHB production of 5.0 ± 0.2 g/L, standing for 46.7 ± 6.8% PHB content in cells, when growing on glucose.

These results are coincidental with the results of [26] in shake flasks, and therefore can be improved significantly when pH and oxygen transfer is effectively controlled in a fermenter or even more using fed batch techniques.

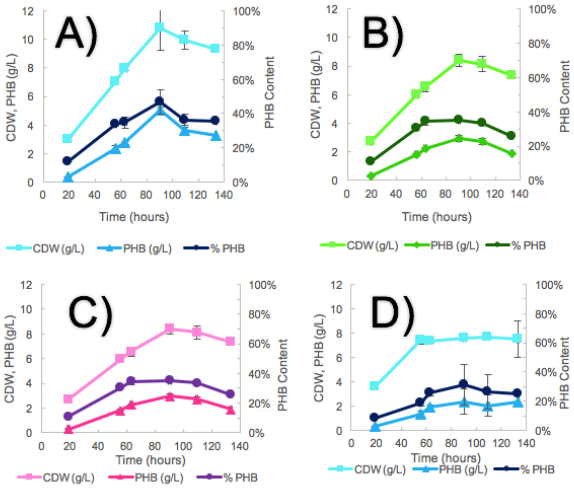

Figure 1 - *H. boliviensis* growth and PHB production on A) 20g/L glucose, B) 20 g/L galactose, C) 20g/L glucose (75%) and galactose (25%) mixture simulating the *Gelidium* hydrolysate and D) 20g/L glucose, with phosphate buffer.

The growth on galactose showed also great results and relatively close to the ones obtained with glucose, achieving at c.a. 90 hours of fermentation time 8.4 ± 0.4 g/L of cell dry weight with a PHB production of 3.0 ± 0.2 g/L, standing for 35.1 ± 0.7% PHB content in cells, which according to the improvement tendency described by [27] when scaling up, can be greatly enhanced. Before taking into consideration the growth of *H. boliviensis* on the *Gelidium* hydrolysates is still necessary to report the bacterium growth in the glucose and galactose mixture simulating the algal material making up to 20 g/L sugars concentration composed by 75% glucose and 25% galactose, which was an estimation based on the composition of the algae, but came across slight different since the galactose fraction

was not totally released, especially when using the 0.5% sulfuric acid pretreatment, i.e., in reality the hydrolysate glucose fraction was larger than 75% of total sugars. The results obtained for the mixture were 10.1 ± 1.1 of cell dry weight with a PHB production of 4.7 ± 0.5 g/L, standing for 46.7 ± 0.7% PHB accumulation in cells. These results are decidedly motivating, since there is no noteworthy decrease for using part of galactose in the mixture instead of pure glucose. When adding the magnesium solution to the medium, supplemented with the phosphate buffer as used on the algal hydrolysate, it was observed turbidity in the medium, indicating a reaction between the magnesium and the phosphate compounds. When following the reaction through time, it was noticed that even though there was a decrease, there was still growth, meaning that there was still potential for using the hydrolysate on phosphate buffer, since there was no growth in the acetate buffer. The results for PHB production had noteworthy variability between the duplicates, meaning that the effect still needs to be further studied for conclusive evaluation. It was achieved 7.6 ± 0.1 of cell dry weight with a PHB production of 2.4 ± 1.1 g/L, standing for 31.1 ± 13.8% PHB accumulation in cells. The outcomes of this assay were rather unexpected, for the hydrolysate with 0.5% sulfuric acid pretreatment it was obtained 10.4 ± 1.0 g/L of cell dry weight and a PHB production of 2.4 ± 0.2 g/L, corresponding to a 22.8 ± 0.2% PHB accumulation in cells, in contrast with all the assays performed before, the maximum growth was at c.a. 110 hours of fermentation, with an extended lag phase up to 60 hours, which required for an analysis for further than the usual 120 hours. For the hydrolysate with 1% sulfuric acid pretreatment the results were a bit more as predicted, with maximal growth at around 90 hours, it was obtained 8.9 ± 1.6 g/L of cell dry weight and a PHB production of 3.0 ± 0.2 g/L, corresponding to a 33.7% ± 6.9% PHB

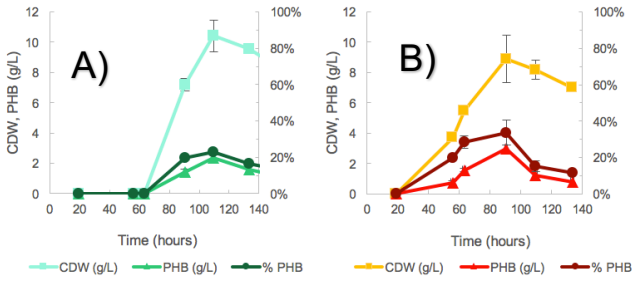

Figure 2 - *H. boliviensis* growth and PHB production on *G. sesquipedale* hydrolysate, obtained with A) 0.5% H_2SO_4 acid hydrolysis pretreatment and subsequent enzymatic hydrolysis on phosphate buffer B) obtained with a 1% H_2SO_4 acid hydrolysis pretreatment and subsequent enzymatic hydrolysis on phosphate buffer.

accumulation in cells. While very interesting these results show that the hydrolysate is still not perfected for bacterial usage.

The extended lag phase suggests that the cells needed to adapt to some toxic by-product present in the media, which may have been destructed when using a more aggressive acidic pretreatment. The cell dry weight was superior than when using the phosphate buffer directly on the medium, which suggests that the even the small dilution of this compound when mixing the hydrolysate with the salt and concentrated medium had a less negative effect.

3.3. *Halomonas elongata* Growth and PHB Accumulation at Shake Flask Scale

H. elongata although attains 5.0 ± 0.5 g/L CDW for glucose, which is coherent with the literature in shake flasks [28] and accumulates a PHB content of 46.0 ± 0.5% (w/w). Results for rhamnose are exceedingly good, with even more PHB production than on glucose, namely, 3.1 ± 0.2 g/L, corresponding to 49.3 ± 3.9% PHB content in cells. *H. elongata* growth on xylose wasn't as notable but still accumulated 30.3 ± 4.1%(w/w) PHB content in cells that corresponds to 0.8 ± 0.1 g/L of PHB for 2.7 ± 0.1 g/L CDW.

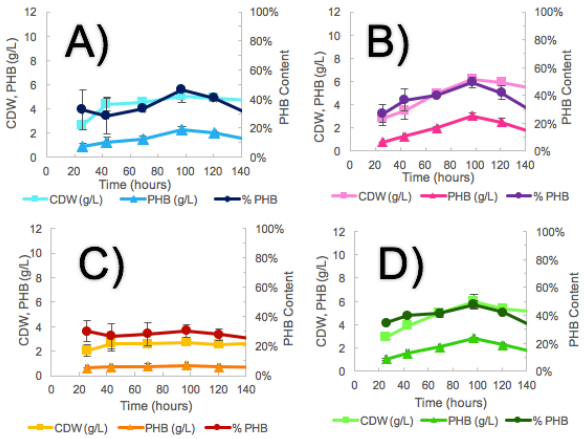

Figure 3 - *H. elongata* growth and PHB production on A) 20g/L glucose, B) 20g/L Rhamnose C) 20g/L Xylose and D) 20g/L glucose (71%) and rhamnose (15%) and xylose (14%) mixture simulating the *U. lactuca* hydrolysate.

The promising growth on single sugars created an expectation of similarly good results on the macroalgal simulated mixture. This was confirmed by the results that achieved 6.0 ± 0.6 g/L CDW with a PHB production of 2.9 ± 0.1 g/L, corresponding to 47.5 ± 2.7% (w/w) PHB content in cells.

Which means that the 15% rhamnose and 14% fraction improves the results in comparison to using solely glucose. This means that *H. elongata* is a very good candidate for using low cost carbon sources, such as *U. lactuca*.

Finally, the growth of *H. elongata* was performed on the previously obtained *U. lactuca* hydrolysates, on phosphate buffer with 0.5% and 1% sulfuric acid pretreatment. Since the phosphate buffer was the one used in the medium for all assays and recommended in the literature [23] there was no demand to study if it would hinder bacterial performance. These preliminary results were reasonable in terms of growth but PHB production was almost undetectable. Still, the for the hydrolysate with 0.5% sulfuric acid pretreatment it was obtained 3.9 ± 0.4 g/L of CDW and a PHB production of 1.0 ± 0.2 g/L, corresponding to a 25.6 ± 0.7% (w/w) PHB accumulation in cells.

For the hydrolysate with 1% sulfuric acid pretreatment the results were similar but a bit lower, with 3.5 ± 1.5 g/L of CDW and a PHB production of 0.8 ± 0.2 g/L, corresponding to a 22.9 ± 6.8% (w/w) PHB accumulation in cells.

In comparison with the results attained for the mixture the growth on the hydrolysates was considerably smaller, this indicates that there are by-products released during the chemical pretreatment that hinder bacterial growth, since results worsen even with more rhamnose concentration in the media obtained by the 1% sulfuric acid pretreatment, which caused a low PHB accumulation in cells.

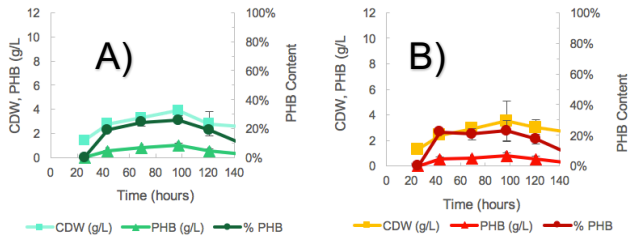

Figure 4 - *H. elongata* growth and PHB production on *U. lactuca* hydrolysate, obtained with A) 0.5% H_2SO_4 acid hydrolysis pretreatment and subsequent enzymatic hydrolysis on phosphate buffer and B) 1% H_2SO_4 acid hydrolysis pretreatment and subsequent enzymatic hydrolysis on phosphate buffer.

4. Conclusions and Future Work

The algae chosen had a high content of carbohydrates that can be used in bioprocessing. The *Gelidium sesquipedale* residues are composed of 48% of its dry weight in glucose and galactose and *Ulva lactuca* has 37% of its dry weight in glucose, rhamnose and xylose, which are an underestimation for their total carbohydrate content since they are known to contain trace amounts of other sugars that cannot be detected by limitations in the HPLC apparatus.

The macroalgal biomass is easier to process than the terrestrial biomass and enzymatic hydrolysis achieved almost 100% of sugar release, although the conditions that allow these results are not the most efficient for scale up. Economic assessment of using concentrated substrate and energy costs for pretreatment or using 5 g/L of substrate and energy costs of concentrating the solution to 20 g/L, which would be a requirement if fed batch strategies are used. The *U. lactuca* rhamnose fraction was not able to be released via enzymatic hydrolysis neither mild alkaline hydrolysis. The cocktail used still needs to be optimized in order to maximize yields and have another enzyme added that can in fact release the rhamnose fraction.

Using a mild acid pretreatment and phosphate buffer it was possible to achieve between 80 to 90% of sugar release, using a substrate concentration of 43.2 g/L, this allowed to obtain hydrolysates with at least 20 g/L of sugars, including the ones present in the commercial enzymatic cocktails.

H. boliviensis has shown effective growth in glucose and galactose and on the sugar mixtures simulating the algal hydrolysates with a PHB production of 47% (w/w) but only showing a maximum of 34% (w/w) PHB accumulation in cells, that can be due to the excess phosphate from the buffer, or to some toxic by-product formed in pretreatment, although higher acidity in the pretreatment had better PHB production results. Further studies need to be commenced to evaluate the trade backs of using water as a buffer, the loss in yield in the *Gelidium* sugar release and the possibility of improvement of PHB production. Scale up for pilot scale in fermenters should highly improve results since *Halomonas spp*. is highly sensitive to pH decrease below 5 and shake-flask scale does not allow efficient pH control, also more versatile production techniques can be tested and optimized in a fermenter scale, such as oxygen transfer and consider a fed-batch approach that had been showed by [11] to improve *H. boliviensis* results. *H. elongata* showed to consume effectively glucose, rhamnose, xylose and on the mixture simulating the *U. lactuca* hydrolysate, reaching up to 48% (w/w) of PHB accumulation. These results surpass the ones attained solely with glucose since it showed to produce more PHB in rhamnose than in glucose. There was no problem with the buffer used in the enzymatic hydrolysis since it is the one recommended for growing *H. elongata* in the literature. In spite of that, there was a huge decrease in PHB production on the hydrolysates that augmented with the acidity of pretreatment. The maximum achieved was 26% (w/w) PHB accumulation in cells. Therefore, it is necessary to have mind that the mild acid hydrolysis pretreatment may require a detoxification step, which is most noticeable in the green algae case,

although the particular compounds that are hindering fermentation still need to be identified. *H. elongata* studies for co-production of ectoine and PHB since it is a very well characterized producer of ectoines [29] and can further valorise the rhamnose fraction of *Ulva lactuca*. The scale up also should improve widely the results. For the bacterial growth in the hydrolysates, full hydrolysed residue use instead of the supernatant should be tested, since *Halomonas spp*. have been reported to secrete extracellular hydrolytic enzymes, such as amylases, lipases, proteases, xylanases and cellulases [30] that can improve the yield of carbohydrates released by macroalgae, although an economical balance should be made since it would difficult assays and downstream processing. In general, and even though this study was in an embryonic phase, to go from macroalgae to bioplastics seems to be a wholesome bet to invest in a better and more sustainable future.

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