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

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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* polysaccharides to monosaccharides for further production of PHAs by *Halomonas elongata*. The results show the inability of the enzymatic cocktail 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, without poly-3-hydroxybutyrate (PHB) accumulation since C/N ratio was too low due to the high N content in the *U. lactuca* hydrolysates.

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

1. Introduction

Petroleum-based plastics have replaced many materials, glass, wood, fibres and metal in their former applications [1]. Plastics became attractive due to their properties: they are versatile and easily moulded, they can be transparent, lightweight, strong, durable and cost-effective. Moreover, plastics have a lower production cost than alternative materials [1].

Besides the environmental issues concerning pollution and microplastics in the marine environment that can infiltrate in food webs [2], estimates of oil production and utilization clearly show that is unfeasible to use petroleum-based plastics in the long-term. As result, it is necessary to drive the industry to more sustainable and environmentally friendly approaches. Thus, biobased plastic, synthesized from renewable resources using biorefinery, has gained attention.

One of the most common used bioplastics is PHA, bio-produced from a wide array of feedstock, biocompatible, 100% biodegradable to either carbon dioxide and water or methane under

aerobic or anaerobic conditions[3]. The most well-known and better characterized PHA structure is the homopolymer PHB [3] that can be synthesized by a wide range of Gram-positive and Gram-negative bacteria. Most species produce PHAs under excess of carbon source and limitation of one essential nutrient [4]. Under these conditions, bacteria accumulate PHB intra-cellularly as granules that can serve as carbon, energy, or reducing-power source.

However, PHAs have a high production cost, limiting factor for their commercialization at large scale. One factor that contributes significantly to this is the price of the carbon source. To reduce raw materials costs, inexpensive and sustainable carbon sources can be used. Those feedstocks materials include terrestrial crops and lignocellulosic materials and algal biomass, which have great potential as C source to be used in biological processes [5]. As carbon source algal biomass is more advantageous because it does not use arable land and potable water, since it can assimilate nutrients directly from seawater or wastewater, and it has higher biomass productivity and yield when compared with lignocellulosic biomass [5]. Besides this, algal biomass is easier to process by hydrolysis due to the lack of lignin that requires a hard pre-treatment that increases process costs due to the high degree of polymerization and complexity [5]. Algal biomass can be categorized into two main categories macroalgae and microalgae biomass, based on morphology. 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. The green alga *U. lactuca* was chosen as model seaweed because it is commonly found in green tides that annually hit the shore worldwide, being thus an inexpensive raw material [6]. *H. elongata*, a halophilic bacteria, was chosen as PHA producer because it can grow in glucose, rhamnose and xylose the main sugars released from *Ulva* polysaccharides namely cellulose, starch and ulvan [7].

2. Materials and Methods

2.1. Materials

U. 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. *H. elongata* DSMZ 2581 was selected because of its ability to produce PHB from *U. lactuca* sugars. All remaining reagents were from different suppliers with p.a. grade.

2.2. Methods

2.3. Determination of Total Carbohydrates

The carbohydrates of *U. lactuca* were determined based on the method provided by the National Renewable Energy Laboratory [8].

2.4. Ulvan Extraction

Ulvan was extracted from 6.0 g of *U. lactuca* as described by Yaich H. et al. (2013) [9].

2.5. Enzymatic Hydrolysis of U. lactuca

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 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 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.

2.6. 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 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 or 45 minutes for treatment with H_2SO_4 or TFA, respectively. 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 (2.5. Enzymatic Hydrolysis of *Ulva lactuca*). All trials were performed in duplicate.

2.7. Strain Storage

H. elongata cultures 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.

2.8. 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 [10] 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 *H. elongata* cryovials, the culture was incubated at 35°C and 200 rpm orbital shaking overnight.

2.9. 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 was obtained from 200 ml of 100 g/l of *U. lactuca* 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, 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 inoculate 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 . 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 gas chromatography (GC) and HPLC. All trials were performed in duplicate.

2.10. Analytical Methods

2.10.1. Quantification of Protein

The Bradford 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 Bradford reagent were added to 150 µl of adequately diluted enzyme preparation into a microplate. This mixture was incubated at room temperature for 10 minutes. Then the absorbance was measured at 595 nm by a microplate reader and the protein concentration was calculated based on a calibration curve of bovine serum albumin.

2.10.2. Quantifications by HPLC

Glucose, rhamnose and xylose, as well as 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 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.

2.10.3. Biomass Quantification

Cellular growth was monitored by measuring the OD_{600} in a spectrophotometer Hitachi U-2000. The 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.

2.10.4. Total Nitrogen Quantification

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

2.10.5. Quantification by GC

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 by 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 Enzyme Preparations

Firstly, the protein concentration of the preparations from Novozymes and Sigma-Aldrich was determined for all the enzymes used in this work. The obtained results are present in table 1.

Table 1. Protein concentrations of the enzymes preparations 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

3.2. Total Carbohydrates in U. lactuca

The total carbohydrates content of *Ulva lactuca* was determined. As expected, glucose is the most abundant, representing 11.1%±0.1 of dry weight (dw), then rhamnose 10.6%±0.3 and xylose 3.6%±0.1. These values are in agreement with previous results (11.3%, 9.0% and 2.9% of glucose, rhamnose and xylose, respectively) [11], having in mind that seaweeds composition has large variations according to growth location and harvest time (growth stage and season).

3.3. Enzymatic Hydrolysis of U. lactuca

The feasibility of performing the enzymatic hydrolysis in water rather than buffer was established (data not shown). The maximum reducing sugars decreased about 1.1 fold but ensure the compatibility of the hydrolysate with the fermentation microorganism. The use of sterilized *U. lactuca*, to avoid microbial contamination of the hydrolysates observed previously, allowed an increase of carbohydrates concentration and the reaction plateau was reached in less time, maybe due to greater accessibility of enzymes to the polysaccharides. The sterilization also allowed residual rhamnose release, not quantifiable with the calibration curve used. In the enzymatic hydrolysis, using an enzymatic cocktail composed of cellulase, β-glucosidase, α-amylase and glucoamylase (figure 1 (▲)), it was possible to release 106.4% ± 6.2 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 same procedure was also performed with some differences in the enzyme cocktail, first, it was used only cellulase and β-glucosidase (●) and then α-amylase was substituted by xylanase (■). 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, the simultaneous use of the four enzymes prevented the inhibitory effect of cellobiose on cellulase activity, the inhibitory effects of oligosaccharides and maltose on α-amylase activity [12].

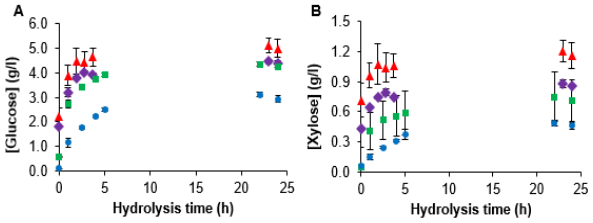

Figure 1. The concentration of released glucose (**A**) and xylose (**B**) (g/l), analysed by HPLC, over the enzymatic hydrolysis of *U. lactuca* at 43.2 g/l, after sterilization at 121°C for 20 minutes. The hydrolysis was performed in 10 ml of distilled water with pH adjusted to 4.8 and at 50°C under 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 (♦). Glucuronidase has been described as a catalyst to ulvanobiouronic acid hydrolysis from ulvan, releasing rhamnose and glucuronic acid [13]. However, rhamnose release during enzymatic hydrolysis was not observed rhamnose concentration did not increase during enzymatic hydrolysis (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 [13]. 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.

3.4. Combined Hydrolysis of U. lactuca using H_2SO_4 Pre-treatment

An acid pre-treatment was carried out aiming at the release of rhamnose from *U. lactuca* [5]. 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 before enzymatic hydrolysis (data not shown). Besides this, only after this pre-treatment rhamnose was present, in an amount that, remained unaltered during the enzymatic hydrolysis, further highlighting the inability of the enzymatic cocktail to release rhamnose. The presence of HMF in this hydrolysate was also detected in HPLC runs (data not shown), at concentration 0.04 g/l. The presence of HMF in the hydrolysates is a factor to take into account and evaluate its possible inhibitory effect on microbial cultivations.

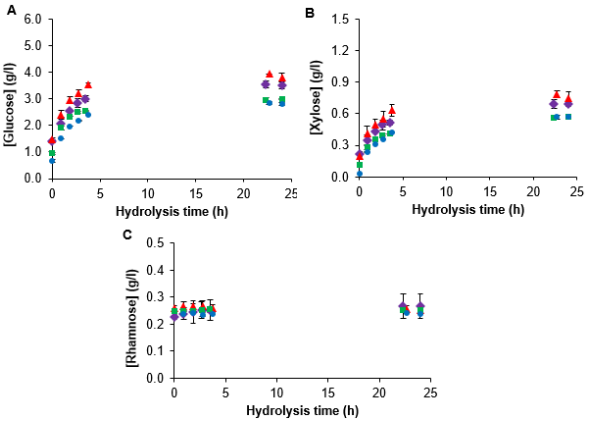

Figure 2. The concentration of released glucose (**A**), xylose (**B**) and rhamnose (**C**) (g/l), analysed by HPLC, over the enzymatic hydrolysis of *U. lactuca* at 43.2 g/l, after a chemical pre-treatment with 1.0% (w/V) of H_2SO_4 at 121°C for 30 minutes. The hydrolysis was performed in a total volume 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 order to make a comparison between enzymatic hydrolysis and combined hydrolysis, the same enzyme cocktails used for the experimental run described in figure 1 were used on the hydrolysate from acid pre-treatment with 1.00% (w/v) of H_2SO_4 (figure 2). 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.

3.5. Glucuronidase Activity Assay

In an effort to evaluate the glucuronidase activity an assay was performed using ulvan as substrate. Firstly, ulvan was extracted from *U. lactuca*. Then the evaluation of glucuronidase activity was performed combining acid-hydrolysis using TFA 2 M and enzymatic hydrolysis using β-glucuronidase as described in literature by Quemener B. et al. (1997) [13]. As happened before the rhamnose concentration did 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.

3.6. Influence of Chemical Pre-treatment with TFA 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. The maximum glucose and xylose was also obtained when using the four-enzyme cocktail with α-amylase (figure 3). Despite the obtained glucose and xylose concentrations were lower than when used H_2SO_4 1.0% (w/v) as well as without any chemical pre-treatment the increase in rhamnose, the second monosaccharide more abundant in *U. lactuca*, allowed a higher final concentration of carbohydrates in the hydrolysate, 9.3 g/l.

However, higher HMF concentrations were present compared to the treatment with 1.0% (w/v) H_2SO_4, namely 0.06 g/l. Additionally, furfural, another possible inhibitor, was also detected by HPLC analysis. 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 for *H. boliviensis* (0.1 g/l) [14].

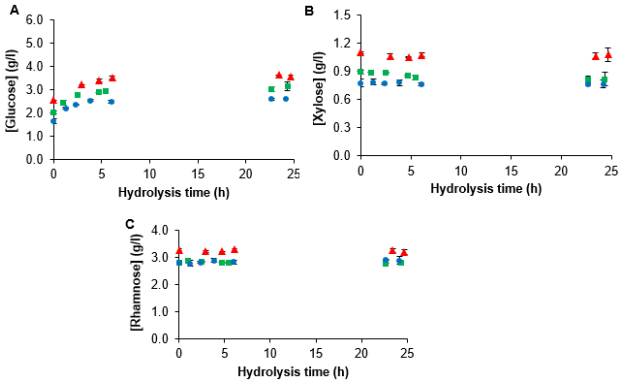

Figure 3. The concentration of released glucose (**A**), xylose (**B**) and rhamnose (**C**) (g/l), analysed by HPLC, over the enzymatic hydrolysis of *U. lactuca* at 43.2 g/l, after a chemical pre-treatment with TFA 2 M at 121°C for 45 minutes. The hydrolysis was performed in a total volume of 40 ml 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 (▲).

3.7. Halomonas elongata Cultivation

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 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 during acid treatment 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.

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 (figure 4).

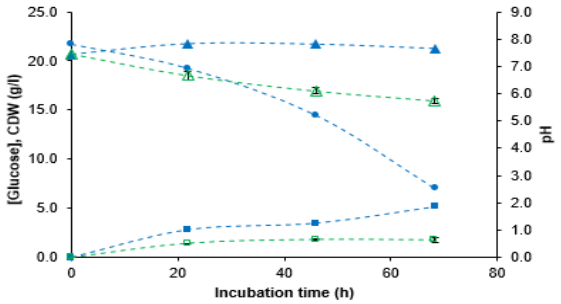

Figure 4. Glucose concentration (g/l), obtained by HPLC analysis, CDW (g/l) and pH during incubation time of H. elongata cultures at 35°C and 200 rpm orbital shaking, represented by circles (● and ○) squares (■ and □) and triangles (▲ and Δ), respectively. The cultures were inoculated in the pre-inoculum 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 by the filled ones (●, ■ and ▲).

Up to 46 hours of incubation *H. elongata* had similar consumption of glucose in both assays, 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 stopped growing keeping the same glucose and cell concentration. 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, since *H. elongata* has an optimal pH of 8 [15]. As expected, the obtained biomass, analysed by GC analysis, did not accumulate PHB, since the concentration of nitrogen 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 lack of standard) the hydrolysate was treated with 10 g/L activated carbon prior to sterilization. However, *H. elongata* also was not able to grow in the culture containing the treated hydrolysate, maybe due to high concentrations of HMF and specially furfural that increased after sterilization even after activated carbon treatment that removed all the HMF present and reduce 3.6 fold the furfural concentration, i.e the area corresponding to furfural. The sterilization, due to carbohydrates degradation, increased HMF concentration to 0.05 g/l and furfural concentration increased 2.8 times, almost the initial concentration before activated carbon treatment. Moreover, 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 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, the hydrolysate was also prepared using 100 g/l of *U. lactuca*. As before the releasing yields of each monosaccharide decreased. The hydrolysate obtained contained 10.9 g/l of carbohydrates (9.3 g/l, 1.7 g/l and 0.2 g/l of glucose, xylose and rhamnose, respectively), lower than in the hydrolysates produced with TFA treatment, despite a higher glucose titre. HMF concentration

was 0.05 g/l and furfural was not detected by HPLC. *H. elongata* was able to grow in the culture containing this hydrolysate (figure 5).

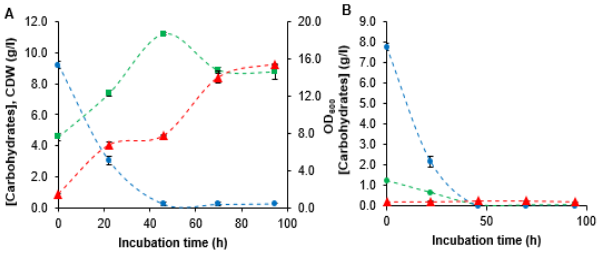

Figure 5. A) Total carbohydrates concentration (●), obtained by HPLC analysis, CDW (■) (g/l) and OD_{600} (▲) during incubation time of *H. elongata* cultures at 35°C and 200 rpm orbital shaking. **B)** Glucose (●), xylose (■) and rhamnose (▲) concentration during incubation time of *H. elongata* cultures at 35°C and 200 rpm. 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.

After 48 hours of incubation, glucose was fully depleted and 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 during the cultivation. 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 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. The obtained biomass was analysed to quantify the PHB production. Unfortunately, no accumulation of PHB was observed, probably due to low C/N ratio. The nitrogen content on the hydrolysate was analysed and a value of 1125.3 mg N/l obtained. 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 culture medium should be approximately 50.0 g/l.

4. Conclusion

Different hydrolysis conditions to obtain monomeric sugars hydrolysate for further bioplastic production were evaluated. 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 also 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 was achieved with a chemical pre-treatment with TFA 2 M followed by enzymatic treatment. However, acid pre-treatment triggers HMF and furfural production. In the hydrolysate treated with 1.0% (w/v) of H_2SO_4, furfural was not detected and HMF had lower titres. *H. elongata* was able to grow only in cultivations using the hydrolysate prepared with 1.0% H_2SO_4. 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. Also, the results of hydrolysis and subsequent *H. elongata* fermentation can be compared with *Saccharophagus degradans* fermentation. *S. degradans*, a gram-negative bacterium has been shown to use directly several algal polysaccharides as C source and accumulate PHAs.

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6. References

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