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

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Abstract

Polyhydroxyalkanoates (PHAs) are bio-based and biodegradable plastics, whose production is expanding in the plastic market. Within this family of polymers, poly-3-hydroxybutyrate (P3HB) is the most studied molecule, being mostly used in the packaging industry. *Halomonas elongata* is a halophilic bacterial species capable of synthesizing P3HB in an environment with excess of carbon under nutrient limitation. Residues of *Ulva lactuca* after protein extraction containing a total carbohydrate content of 44.0 % dw, were used as a carbon-rich source in the culture media for P3HB accumulation by *H. elongata,* after saccharification to monomeric sugars. A combined hydrolysis using an acidic thermal treatment followed by enzymatic hydrolysis with cellulolytic enzymes, resulted in a glucose-rich hydrolysate with a sugar recovery yield of 81.2 % (g sugar hydrolysate/g sugar biomass) in total sugars (83.2 % in glucose) for the optimum condition, during a 6.3-fold scale-up. Microbial growth inhibitor 5-hydroxymethylfurfural (HMF) was detected in negligible amounts in the hydrolysate. However, after concentration of the hydrolysate, a new compound with similar chemical composition of HMF's was formed and HMF was no longer detectable. The concentrated algae residue hydrolysate was assessed for P3HB production by *H. elongata* under unsterile conditions using different concentrations of the nitrogen sources. Results showed maximum productivity of 0.027 g/(L.h), a polymer concentration of 4.5 g/L and a yield of polymer over the consumed substrate ($Y_{P/S}$) of 0.21 g P/g $S_{cons.}$ with 1.0 g/L MSG.H_2O and 1.0 g/L NH_4Cl in the culture medium. Cell growth was only observed after 72h of incubation due to growth inhibition that might have been caused by the presence of the new compound in the concentrated hydrolysate. In this work, residues of *U. lactuca* were shown to be a low-cost, sustainable alternative carbon source for P3HB production by *H. elongata*.

Keywords: *Ulva lactuca, Halomonas elongata,* Polyhydroxyalkanoates, poly-3-hydroxybutyrate, acidic pre-treatment, enzymatic hydrolysis

Introduction

Plastic pollution has become a matter of great concern worldwide. With the world population growing, plastic demand is increasing and so is its generated waste. Addressing this issue at its source by rethinking the processes of plastic production is a necessary step towards a greener future [1]. Nowadays, bioplastics (bio-based and/or biodegradable) are becoming more popular, even though they still account for a very small percentage of the global plastic production (1% in 2019). They are currently applied as packaging materials (contributing to 53% of bioplastic production in 2019) and in other sectors from agriculture to electronics [2]. Among these, PHAs, which are bio-based and biodegradable plastics produced by microbial fermentation, are strong candidates to potentially replace conventional plastics, due to having similar chemical properties to oil-based plastics [3]. PHAs are constituted of R(-)-3-hydroxyalkanoic acid monomers that can range from C3 to C14 carbon atoms, varying in saturation, branching and side groups, which result in different polymer properties and thus, different applications [4]. They accumulate in bacterial cells in the form of granules as food and energy reserves when there is excess of carbon and a deficit of nutrients in the environment, such as nitrogen, oxygen, phosphorus, or magnesium [5]. Within this family, the most well characterized polymer is P3HB, whose properties resemble the ones of polypropylene plastic. Regardless of all the environmental advantages, PHAs commercial use is still lacking. While plastic is cheap, used worldwide and its production process is well developed, biodegradable plastics have a higher production cost, do not have all the properties that make conventional plastic such a good working material and there are still questions regarding its full degradation [6]. Regarding production costs, product recovery processes and the substrate used for the fermentation require high investments, whereas substrate can account for up to 50% of the overall production cost. Therefore, research focused on using low-value substrates from industrial and agricultural wastes has been carried out in recent years to achieve a competitive market price [7].

A very promising alternative carbon source that has a quick growth, requires few resources, and does not compete with food industry is algae. In particular, marine macroalgae, also known as seaweeds, appear as a good substitute for agricultural feedstocks in biorefinery. They have high carbohydrate contents, do not require arable land nor freshwater for their cultivation and have rapid growth rates derived from their superior photosynthetic efficiency [8]. Furthermore, contrary to terrestrial biomass, seaweeds lack lignin in their composition, which makes them easier to depolymerize without the need of resorting to harsher hydrolysis conditions, potentially reducing processing costs [9]. Macroalgae can be divided into three groups: green, red and brown, accordingly to their thallus colour. Regarding carbohydrates, each group possesses characteristic types of polysaccharides: ulvan is seen in green algae, carrageenan and agar in red algae whereas alginate, fucan and laminarinan are present in brown algae [10].

Analogously to lignocellulosic biomass, seaweeds can be pre-treated and saccharified into fermentable sugars. Various pre-treatment methods such as dilute acid, alkali,

hydrothermal, microwave, ultrasound or even white-rot fungi treatments have been investigated [11]. Depending on the severity of the conditions used for the chosen pre-treatment, toxic compounds derived from sugar degradation may be formed, which could hinder the subsequent hydrolysis or fermentation steps [12]. The mostly synthesized toxic compounds are furans. Particularly, 5-hydroxymethylfurfural and furfural are formed through dehydration reactions of, respectively, glucose and xylose. These cause inhibition in microbial cells by reducing specific growth rates and their productivity [11,13].

In this work, residues of the green algae *Ulva lactuca* after protein extraction were used as a carbon-rich feedstock to produce P3HB by *Halomonas elongata*. *U. lactuca* is a macroalgae belonging to the *Ulva* genus. It is often associated with green tides formation due to eutrophication derived from human activity, which hinder marine ecosystems and local tourism [13,14].

Table 1 – *U. lactuca species general composition [15,16].*

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

As part of the green macroalgae, *U. lactuca* possesses ulvan as its main cell wall polysaccharide. Since ulvan has a complex structure composed of ulvanobioses and ulvanobiuronic acids, commonly cellulolytic enzymes cannot break its chemical bonds and do not release sugars like rhamnose or xylose [14]. As such, a dilute acid pre-treatment was performed to achieve higher sugar yields.

H. elongata is a halophilic microorganism that can tolerate salt concentrations between the range of 3% to 20% NaCl and grow at high pH values [17]. This allows the use of unsterile conditions for PHA production which, in larger scales can lower production costs. Besides, this bacterium can grow on different carbon sources so, by having *U. lactuca* residue hydrolysates, not only glucose but xylose and rhamnose are available for bacterial intake [18].

Materials and Methods

Raw material

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

Synthetic seawater

The synthetic seawater (SW) was used to prepare the acidic solutions of HCl and H_2SO_4 applied in *Ulva lactuca*'s chemical pre-treatment, to assess about the influence of seawater salts on the algae acidic hydrolysis. For this, solutions containing 2.7% NaCl, 0.33% $MgSO_4$, 0.25% $MgCl_2$, 0.1% $CaCl_2$ and 0.07% KCl were prepared using Milli Q® water to make up the total volume [19].

Enzymes

The commercial enzymes used for this work were cellulase complex (NS 22086), β-glucosidase (NS 22118), xylanase (NS 22083) and xylanase/ β-glucanase mixture (NS 22002), all from Novozymes® (Bagsværd, Denmark). NS 22086 has a Filter Paper Unit activity of 111.2 FPU/mL and NS 22118 had a cellobiase activity of 20.1 CBU/mL [26].

Microorganism

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

Chemicals

The chemicals used were sulphuric acid >95% (Fischer Chemical), hydrochloric acid 37% (Honeywell Fluka), sodium hydroxide (Fischer Chemical), dextrose monohydrate (COPAM, Portugal), glycerol (ACROS Organics), calcium chloride dihydrate (Merck), magnesium chloride hexahydrate (Merck), ammonium chloride (Merck), magnesium chloride 99% (Fagron), di-potassium hydrogen phosphate 99% (PanReac AppliChem), potassium chloride (Merck), magnesium sulphate hepta-hydrate (LabChem), sodium chloride 99.5% (Fischer Chemical), calcium carbonate min. 99% (Merck), Tris (Eurobio Scientific) and 5-hydroxymethylfurfural 98% (Sigma Aldrich).

Chemical pre-treatment of algal residues

Different concentrations (0.25, 0.5, 1 and 1.5% w/v) of dilute H_2SO_4 and dilute HCl solutions were tested, using either synthetic seawater or Mili Q® water to prepare the solutions. The chemical hydrolysis was performed at 121°C for 15 or 30 minutes in the autoclave. The algal biomass was weighted into 100 mL Schott flasks and 40 mL of acid were added, resulting in a biomass concentration of 50 g/L. After each hydrolysis, the flasks were cooled down at room temperature and the pH was adjusted between the range of 3-6 with NaOH 8M to avoid HPLC column damage. Samples were taken and prepared for sugar quantification in HPLC. All trials were done in duplicate.

Combined hydrolysis

Combined hydrolysis was carried out with chemical pre-treatment prior to enzymatic hydrolysis, using the optimum pre-treatment condition. After the chemical pre-treatment, the pH was adjusted to 4.8 using NaOH 8M right after flask cooling and Milli Q® water was added to make up a biomass concentration of 44.4 g/L, under sterile conditions. Before and right after adding the enzymes, a sample was taken from each flask and was prepared for HPLC quantification, allowing the subtraction of sugar contribution from the enzyme preparations. The enzymatic hydrolysis was then carried out by adding either cocktail A, B or C (see **Table 2**) in each of the testing concentrations over the chemically

pre-treated biomass to assess the most suitable option. The process was conducted at 50°C and stirring speed of 600 rpm in an incubator with magnetic stir plates. Samples were collected at different times and prepared for HPLC analysis throughout a period of 48h.

Table 2 *- Tested concentrations in %(v/v) used in U. lactuca's combined hydrolysis for each enzymatic cocktail and their respective contained enzymes*

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

Scale-up of combined hydrolysis

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

Concentration and storage of the hydrolysate

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

Strain storage

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

Inoculum medium preparation

The inoculation medium for *H. elongata* consisted in 45 g/L NaCl, 2.5 g/L $MgSO_4.7H_2O$, 20 g/L glucose, 3 g/L K_2HPO_4, 4 g/L NH_4Cl, 8.9 g/L $MSG.H_2O$, 1 mL/L trace elements and 15 g/L Tris. Firstly, Tris was dissolved in Milli Q® water and 37% (w/w) HCl was added to adjust the pH to 7.5. Then, the medium was prepared by sequentially adding the components in the following order: NaCl, K_2HPO_4, NH_4Cl, $MSG.H_2O$ and trace elements. This trace elements solution was composed of 10 mL/L 25% (w/w) HCl, 0.19 g/L $CoCl_2.6H_2O$, 0.1 g/L $MnCl_2.4H_2O$, 1.5 g/L $FeCl_2.4H_2O$, 0.07 g/L $ZnCl_2$, 0.062 g/L H_3BO_3, 0.036 g/L $NaMoO_4.2H_2O$, 0.024 g/L $NiCl_2.6H_2O$ and 0.017 g/L $CuCl_2H_2O$. Stock solutions of $MgSO_4.7H_2O$ (100 g/L) and of glucose (500 g/L) were prepared separately to avoid, respectively, precipitation and enhanced thermal degradation during sterilization in the autoclave. The inoculum medium was prepared in 500 mL Erlenmeyer flasks for a total volume of 100 mL corresponding to: 93.5 mL of culture medium, 2.5 mL of 100 g/L $MgSO_4.7H_2O$ and 4 mL of 500 g/L glucose. A 2 mL cryovial of *H. elongata* was added. Inoculum incubation was carried out in an AGITORB 200 orbital shaker, supplied by Aralab®, at 30°C and 200 rpm for 17-18 hours where, after it, the $O.D._{600}$ was measured to calculate the volume of inoculum required for the respective assay in study. The incubation conditions remained the same throughout every assay, unless stated otherwise.

Effect of HMF concentration on cell growth

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

Effect of C/N ratio on the production of P3HB

In order to assess the effect of the C/N ratio on the production of P3HB, an assay was conducted testing various

C/N ratios, where the concentration of the accessible nitrogen components (NH_4Cl and $MSG.H_2O$) in the mineral medium was altered while the sugar concentration remained unchanged. The composition of this culture medium consisted in: 45 g/L NaCl, 2.5 g/L $MgSO_4.7H_2O$, 3 g/L K_2HPO_4, (0 or 1) g/L NH_4Cl, (0, 1.0, or 8.9) g/L $MSG.H_2O$, 1.0 mL/L trace elements and 15 g/L Tris. Stock solutions of NH_4Cl (250 g/L) and $MSG.H_2O$ (500 g/L) were prepared separately to adjust the adding volume according to the tested assays. The stock solutions and the culture medium were sterilized in the autoclave at 121°C for 20 minutes. The carbon source consisted of the optimized concentrated hydrolysate produced from the combined hydrolysis of *U. lactuca* residues treated with 1.5% (w/v) HCl solution prepared with Milli Q® for 30 minutes at 121°C in the autoclave, followed by an enzymatic hydrolysis with a 0.25% (v/v) cellulase complex and β-glucosidase mixture (Cocktail A) at 50°C and pH 4.8 for 24 hours.

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

Quantification of total carbohydrates in biomass

The determination of total carbohydrates of Ulva lactuca residues was done according to an adaptation of the National Renewable Energy Laboratory (NREL) protocol "Determination of Total Carbohydrates in Algal Biomass" [21]. This method consists in a two-step acidic hydrolysis where, in the first step, triplets of 0.5 g of biomass residues are weighted out in Erlenmeyer flasks and 5 mL of 72% (w/w) sulphuric acid are added in each. Then, the flasks were incubated at 30°C and 100 rpm for one hour in an orbital shaker. The second step consists in autoclaving the samples at 121°C for one hour after diluting the hydrolysate to a final 4% (w/w) sulphuric acid concentration with 139 mL of Milli Q® water. After cooled down to room temperature, 10 mL of each triplet are taken into Falcon tubes to then get neutralized to pH 6-8 with calcium carbonate. To separate the suspended solids, centrifugation at 1932 g for 10 minutes is done and the supernatant is recovered and prepared for HPLC carbohydrate quantification.

Ash, moisture and total solids

To determine the ash, moisture and total solid contents, the protocol "Determination of Total Solids and Ash in Algal Biomass" from NREL was followed [22]. Briefly, it consists in preconditioning crucibles by drying them overnight at 575°C in a muffle furnace and weighing them after cooling down in room temperature in a desiccator. Afterwards, 100 mg of sample were weighed in each crucible (performed in triplicate) and left drying in a convection drying oven at 60°C for at least 18 hours. Total solids and moisture are then determined by weighing the crucibles with the dried samples. For ash content, the same crucibles with the dried samples were burnt in a muffle furnace following a ramping program that consisted in: ramping from room temperature to 105°C and hold for 12 minutes, then ramping to 250°C at 10°C/minute and hold for 30 minutes and finally ramping to 600°C at 14°C/minute and hold for 16 hours, allowing the temperature to drop back to 105°C in the end, before removing the samples. The crucibles with the ashes were weighed and the ash content was determined.

Quantifications by High Performance Liquid Chromatography

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

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

Nitrogen quantification in the algae hydrolysate

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

P3HB quantification

To quantify the accumulated P3HB produced by *H. elongata*, an acidic methanolysis was done in order to convert the polymer into hydrocarboxylic acid methyl esters, which are then analysed through Gas Chromatography (GC). 1.2 mL samples were taken to Eppendorf tubes and, after centrifugation at 9167 g for 5 minutes (SIGMA 1-15P, Sartorius), the supernatant was discarded and the pellet washed with 1 mL Milli Q® water and centrifuged again in

the same conditions. The supernatant was again removed and the pellet was stored at -20°C until further use. The acidic methanolysis reaction consists in the addition of 1 mL of chloroform to the stored pellets, resuspension of the cells until complete detachment from the bottom of the Eppendorf tubes, sample transfer to Pirex glass tubes and addition to each tube of 1 mL of "solution A", which contains 97 mL methanol, 3 mL 96% H_2SO_4 and 330 µL hexanoic acid. The tubes were then vortexed and incubated at 100°C for 5 hours in an oven and throughout this time the samples were resuspended once. Afterwards, the samples were neutralized by adding 1 mL of 60 g/L Na_2CO_3 and centrifuged at 432 g for 5 min in a Heraeus Labofuge 200 from Thermo Scientific. More Na_2CO_3 solution was added if necessary to balance the tubes in the centrifuge. Finally, 200 µL of the organic phase (bottom phase) were taken and the P3HB concentration was determined using GC.

Quantifications by Gas Chromatography

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

Results

Characterization of *U. lactuca* residues

The analysis of the composition of *U. lactuca* residues after protein extraction was essential to follow up the hydrolysis steps. For the acidic pre-treatment, a mixture of residues from batches U1.00618M and U1.00319M was used while for the combined hydrolysis and the remaining work a mixture of the residues from all three batches was used. **Table 3** displays these results.

Jard et al. (2013) reports similar results for total solid and total sugar contents for *Ulva lactuca* of 83.3% and of 31.4% dw, respectively [23]. Amamou et al. (2018) reports a slightly higher total solid content of 90% while a lower total sugar content of 25.8% dw. It is important to note that the reported results were obtained for the residues of *U. lactuca* after protein extraction and not for the entire algae, meaning there should be a higher value of total carbohydrates in the latter, which account for the soluble carbohydrates that were removed in the process. For ash content, Amamou et al. reports 32% dw, similar to the results obtained. Glucose, xylose and rhamnose were reported to constitute 15.2 ± 1.0% dw, 3.1 ± 0.2% dw and 7.5 ± 0.1% dw of *U. lactuca*'s content, respectively [24]. It is important to note that the chemical composition of macroalgae can vary due to many uncontrolled factors such as geographical origin, harvest season, environment growth or even the method utilized to quantify their composition.

Table 3 *- Chemical composition of the residues of U. lactuca after protein extraction from both batches U1.00618M and U1.00319M and from all of the three batches U1.00618M, U1.00319M and U1.00321MB2401.*

Batches U1.00618M and U1.00319M		**Batches U1.00618M, U1.00319M and U1.00321MB2401**	
Parameter (Unit)	**Value**	**Parameter (Unit)**	**Value**
Moisture (%)	6.94±0.75	Moisture (%)	11.14±1.81
Total Solids (%)	93.04± 0.75	Total Solids (%)	88.86±1.81
Ash (% dw)	25.32±0.70	Ash (% dw)	21.88±0.54
Total Carbohydrates (% dw)	32.67±0.40	Total Carbohydrates (% dw)	44.02±1.01
Glucose (% dw)	13.30±0.27	Glucose (% dw)	28.66±0.98
Xylose (% dw)	4.32±0.11	Xylose (% dw)	3.96±0.15
Rhamnose (% dw)	15.05±0.27	Rhamnose (% dw)	11.39±0.17
Glucuronic Acid (% dw)	21.76±0.06	Glucuronic Acid (% dw)	21.15±0.05

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

Chemical pre-treatment of the algae residues

To optimize the conditions before proceeding to the combined hydrolysis, different concentrations of HCl and H_2SO_4 solutions prepared either with Milli Q® water (RO) or synthetic seawater (SW) and durations of thermal treatment were tested.

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

HMF's concentration is lower for the RO water (0.038 g/L) than for the SW (0.043 g/L) and thus, the 30 minute 1.5% (w/v) HCl prepared with Milli Q® water thermal treatment was considered the best option.

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

Regarding sugar release, as expected, 1.5% H_2SO_4 conditions have the highest concentration values where, in

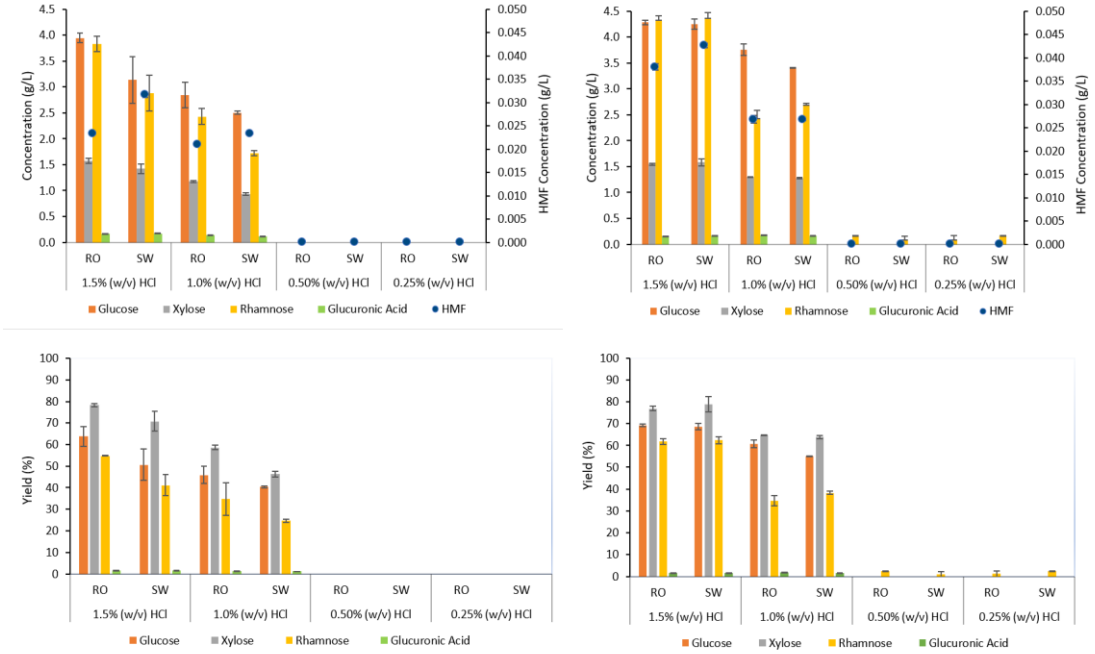

Figure 1 *- Chemical pre-treatment of U.lactuca's residues performed with HCl solutions of 0.25, 0.50, 1.0 and 1.5% (w/v) concentrations prepared either with Milli Q® (RO) water or with synthetic seawater (SW) for biomass concentration of 50 g/L tested for 15 or 30 minutes in the autoclave at 121ºC. At the top are depicted the concentrations of released algae components of interest for 15 minutes of thermal treatment (top left) and for 30 minutes (top right) and in the bottom is each process components respective yields (bottom left corresponding to 15-minute process and bottom right to the 30-minute one).*

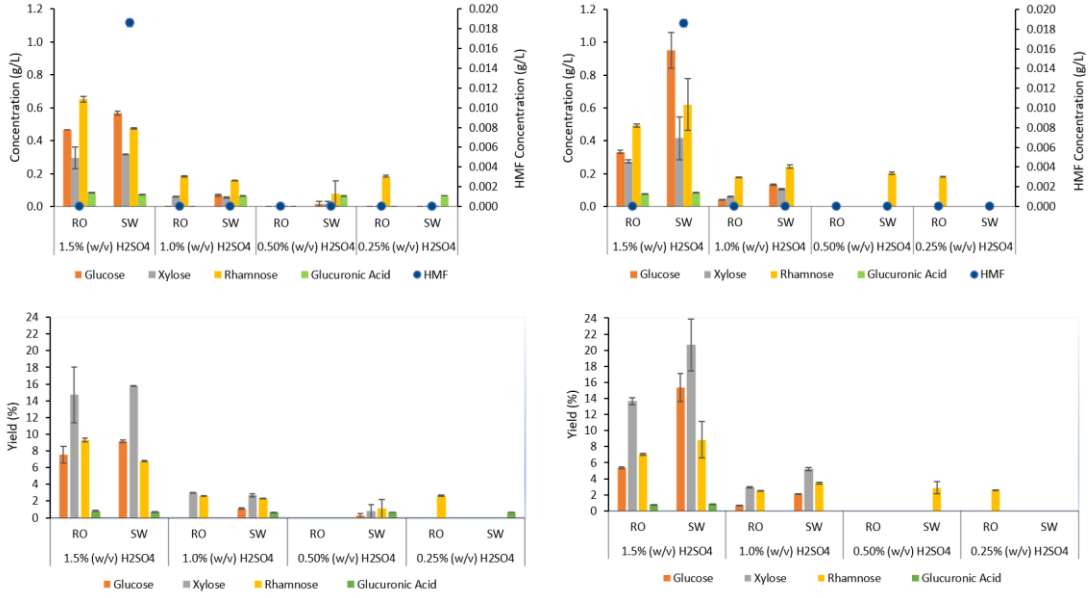

Figure 2 *- Chemical pre-treatment of U.lactuca's residues performed with H_2SO_4 solutions of 0.25, 0.50, 1.0 and 1.5% (w/v) concentrations prepared either with Milli Q® (RO) water or with synthetic seawater (SW) for biomass concentration of 50 g/L tested for 15 or 30 minutes in the autoclave at 121ºC. At the top are depicted the concentrations of released algae components of interest for 15 minutes of thermal treatment (top left) and for 30 minutes (top right) and in the bottom is each process components respective yields (bottom left corresponding to 15-minute process and bottom right to the 30-minute one).*

the 15-minute treatment, the SW one reaches a slightly higher value for glucose concentration, 0.57 g/L (9.2%), than the RO water one, which reaches 0.47 g/L (7.5%), although for rhamnose, the opposite happens, reaching a

concentration of 0.47 g/L (6.8%) in SW and of 0.65 g/L (9.3%) in RO. For the 30-minute process, SW reached the highest values of released sugar, where glucose reached 0.95 g/L, xylose 0.42 g/L and rhamnose 0.62 g/L, corresponding to yields of 15.4%, 20.7% and 8.9%, respectively. Hence, between the 15 minute and the 30-minute processes, the latter seems to be better when using the 1.5% H_2SO_4 solution prepared with seawater. Greetham et al. (2020) tested diluted acid pre-treatments in 10% (w/v) *Ulva linza sp* with 1%, 2%, 3% and 5% H_2SO_4 prepared with either RO water or SW and, autoclaved for 15 minutes at 121°C [19]. For every acid concentration, SW was reported to have a higher sugar release than the one seen in RO water results, which, in this study, is observed for the 30-minute treatment with H_2SO_4, however, not in the 15 minute one.

The 30-minute thermal treatment done with 1.5% HCl prepared with Milli Q® water was chosen for the next stage of combined hydrolysis.

Inhibitory effect of HMF on *H. elongata's* growth

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

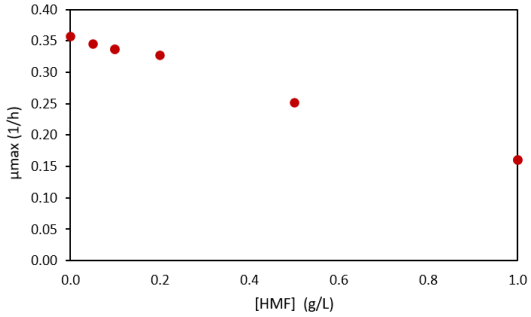

Figure 3 *- Inhibitory effect of HMF on H. elongata's growth where the maximum specific growth rate (μ_{max}) is shown for the different tested HMF concentrations of 0.0 g/L, 0.05 g/L, 0.1 g/L, 0.2 g/L, 0.5 g/L and 1.0 g/L, all done in the same medium as the inoculum for 20 g/L of glucose. To determine each μ_{max}, $O.D._{600}$ was measured over a period of 10h covering the exponential growth phase and a plot with the logarithmic values of the $O.D._{600}$ for the points in exponential phase was drawn. The slope from each linear regression obtained gave the μ_{max} values.*

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

Combined hydrolysis of *U.lactuca* residues

To further increase the amount of released sugars during the hydrolysis, combined hydrolysis of the algae residues was performed. The tested conditions were carried out with the mixture of algae residues from three different batches.

Regarding the enzymatic cocktails, it is expected that cocktail A increases the amount of released glucose from the algae residues, while cocktail B, in addition, is expected to increase the amount of xylose. Cocktail C is used to test if further cellulose degradation may occur by β-glucanase hydrolytic action, apart from glucose and xylose release due to cellulase, β-glucosidase and xylanase activity. Rhamnose release concentration is not expected to increase since no enzyme that breaks the bonds between glucuronic acid and rhamnose and iduronic acid and rhamnose, such as β-glucuronidase, nor xylose and rhamnose bonds, such as ulvan lyase, are used.

Graphical representations of the evolution of sugar concentrations throughout the experimental time are not depicted in this extended abstract. However, a summary of these results is shown in **Tables 4**, **5** and **6**.

Table 4 *– Maximum reached concentrations of released sugars in 0.25% (v/v) enzymatic hydrolysis step of combined hydrolysis and their respective incubation times and achieved recovery yields. Sugar concentrations and recovery yields account solely for the outcome of enzymatic hydrolysis.*

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

For concentration 0.25% (v/v), there is an increase of released glucose for every cocktail while rhamnose concentration remains almost unchanged, as expected. Xylose concentration reaches the highest concentrations in cocktail B. However, cocktail A has the highest recovery yields after the 48h of hydrolysis.

For concentration 0.50% (v/v), cocktail C seems to release more glucose than the other cocktails. As previously mentioned, further xylose release was expected when cocktails B and C were used, although this was not the case with cocktail B. Rhamnose concentration has low variation and comparing with the data in Table 5, the recovery yields are either 0% or near it, as expected by the absence of an enzyme that cleaves the bonds associated with this sugar.

For 1% (v/v) enzymatic treatment, cocktail C reached higher maximum concentrations for every sugar although, in rhamnose, this difference was observed for incubation time of t=1h, which does not imply there was an increase of released sugar as it can be seen by a recovery yield of 0%.

Table 5 *- Maximum reached concentrations of released sugars in 0.50% (v/v) enzymatic hydrolysis step of combined hydrolysis and their respective incubation times and achieved recovery yields. Sugar concentrations and recovery yields account solely for the outcome of enzymatic hydrolysis.*

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

Glucose release profile seems similar for every cocktail and, after 48h of incubation, each glucose concentration is almost the same. Cocktail A reaches a higher recovery yield of xylose, although it does not reach the highest concentration obtained with the enzymatic hydrolysis step.

***Table 6** - Maximum reached concentrations of released sugars in 1.0% (v/v) enzymatic hydrolysis step of combined hydrolysis and their respective incubation times and achieved recovery yields. Sugar concentrations and recovery yields account solely for the outcome of enzymatic hydrolysis.*

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

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

Scaled-up combined hydrolysis

After optimizing the combined hydrolysis conditions, the production of the hydrolysate to be used in P3HB production fermentation assays was performed in a 6.3-fold scaled up combined hydrolysis. Results of released sugar yields of the whole process are shown in **Table 7**.

***Table 7** - Released sugar and inhibitor concentrations in g/L and sugar yield in % and of total released sugars in g/g dw and total sugar yield in % (g released sugars/g total sugars in biomass) after the 6.3-fold scaled up combined hydrolysis of U. lactuca residues (44.4 g/L biomass).*

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

Concentration of hydrolysates produced from algae residues

Before initiating the concentration step, the hydrolysate was centrifuged and vacuum filtered to remove most of suspended particles in the mixture and the filtrate was distributed among 6 Schott flasks of 100 mL. To concentrate, the hydrolysate was then placed in an oven at 60ºC for 82 hours. The processes results are shown in **Table 8**.

Two batches of hydrolysate were produced beginning with a total volume of 1126 mL (563 mL per batch) resulting in a total final volume of 82 mL of concentrated hydrolysate, which, altogether, resulted in a glucose concentration of 119.02 ± 0.19 g/L.

***Table 8** – Summarized results of centrifugation and filtration and of concentration processes related to 2 batches of hydrolysate from U. lactuca*

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

After sample analysis by HPLC, it was noticed that the peak corresponding to HMF, that had previously been analysed at the end of the combined hydrolysis, was no longer in the chromatogram but instead, a peak near HMF's retention time had appeared. This could indicate that a component with similar molecular structure of HMF could have possibly been produced with HMF's degradation by heat exposure.

P3HB production assays

The production of P3HB by *H. elongata* with the prepared hydrolysate was tested in 250 mL shake flasks, where the nitrogen content varied in each experimented condition to check upon its influence in the polymer production. According to the results obtained with LCK338 Laton Total Nitrogen test kit, the hydrolysate had 4.8 g/L of total nitrogen, however, part of that N might not be available for bacterial intake. Since a reduced nitrogen content increases P3HB production, the lesser available nitrogen in the hydrolysate the better. Four fermentation conditions were tested for P3HB production, and each medium component is described in **Table 9**.

***Table 9** – P3HB production media used for each tested condition in H. elongata fermentation.*

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

To follow cell growth, neither optical density at 600 nm nor cell dry weight quantification were adequate methods due to the interference of suspended particles present in the hydrolysate and thus, are not represented in Figure 4.

A significant glucose consumption is observed only after 72h of fermentation for every condition and the pH does not change significantly until after 72h of incubation time as well. *H. elongata* growth seems to be inhibited for at least 3 days, although, after 72h, condition A already showed signs of biomass growth through pellet observation. This could be the reason why a higher P3HB production in condition A is observed, while the other conditions showed a negligible P3HB production after 96h of cultivation. Growth inhibition is probably due to the unknown component similar to HMF

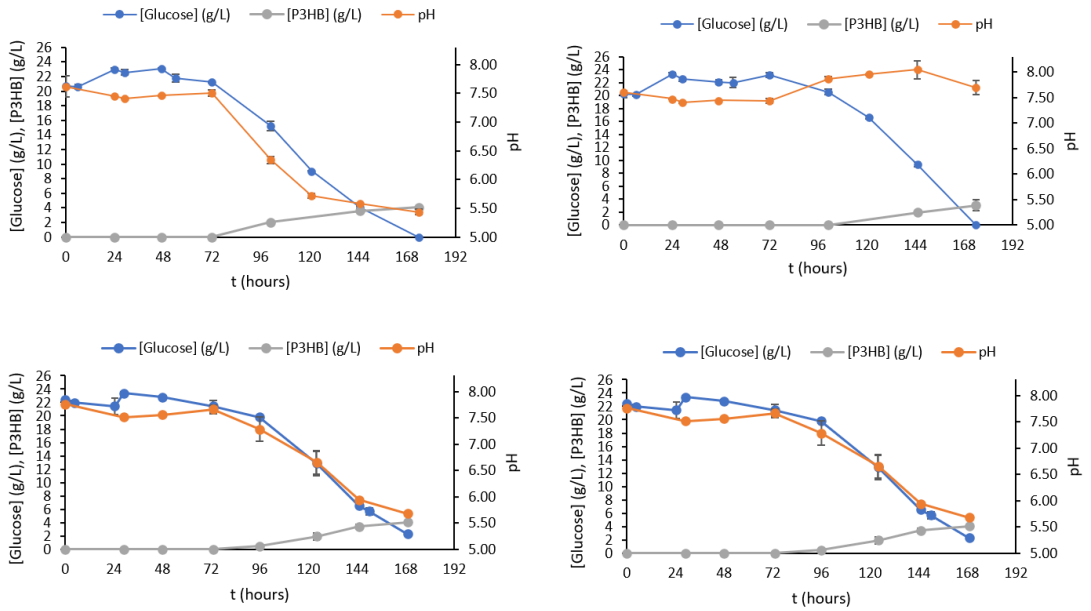

Figure 4 *- P3HB production by H. elongata using U. lactuca residue hydrolysate as a carbon rich source, replacing the use of pure glucose. In the left axis the glucose and polymer concentrations are represented, in g/L, while in the right axis the pH values are represented throughout the fermentation time. The top part figure represents Conditions A (top left) and B (top right) which were done simultaneously in parallel, while the bottom part represents tested Conditions C (bottom left) and D (bottom right), also performed at the same time in parallel. Condition A consists in a fermentation using hydrolysate and mineral media (as described in Effect of C/N ratio on the production of P3HB) without MSG and NH_4Cl; Condition B, C and D consist in the same as Condition A, but the MSG and NH_4Cl concentrations are, respectively, 8.9 g/L and 0.0 g/L, 0.0 g/L and 1.0 g/L, and 1 g/L for both. The assays were done at 30ºC with an agitation speed of 200 rpm.*

which is present in the medium through the concentrated hydrolysate. It is hypothesized that this component might be levulinic acid because it results from HMF degradation after heat exposure and also inhibits microbial growth [25].

Table 10 *– Comparison between each medium used to produce P3HB by H. elongata using algae residues hydrolysate in terms of yield of product over substrate, in g P/g $S_{cons.}$, maximum productivity, in g/(L.h), and maximum P3HB concentration, in g/L, respectively.*

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

Conditions A and C show the same P3HB maximum concentration, almost the same maximum productivity and similar yields. The yields diverge more than the productivity due to conditions A and B starting with higher glucose concentration (approx. 22 g/L) than conditions C and D (approx. 20 g/L). Condition D has the highest values in each parameter while condition B has the lowest. It is clear that MSG influences polymer production the most within the tested conditions. The lower polymer production attained in condition B seems to be caused by the high MSG concentrations and thus higher N concentrations in the cultivation medium. Similar results were attained by Tůma et al. (2020), using *Gelidium sesquipedale* residues and *Halomonas boliviensis* [26]. Condition D, however, with 1 g/L of MSG and 1 g/L NH_4Cl, increases the nitrogen content over the already existent one from the hydrolysate and still has higher P3HB production than condition A and C. Therefore, MSG has a greater impact than NH_4Cl in the polymer production and, to optimize it, there should be a balance between the C/N ratio and the added MSG content, since condition A has a higher C/N ratio (11.5 considering hydrolysate's total nitrogen availability) than condition D (8.41 considering hydrolysate's total nitrogen availability). Tůma et al. (2020), reported 2.7 g/L of P3HB produced for 1 g/L NH_4Cl and 0 g/L MSG for *H. boliviensis* using a similar production medium with *G. sesquipedale* residue hydrolysate as a carbon rich source. Within their tested conditions, the ones with lower nitrogen content that still contained MSG reached higher P3HB concentrations, corroborating the obtained results [26].

Conclusions

The present work demonstrates that high yields of released sugars from *U. lactuca* residues after protein extraction are attained with combined hydrolysis using mild HCl conditions in the chemical pre-treatment, which result in low amounts of microbial growth inhibitor HMF. It is feasible to use the resulting hydrolysate as low-value C-source to produce P3HB by *H. elongata* under unsterile shake flask fermentations. However, further research in the concentration step should be carried out, to avoid the formation of other microbial growth inhibitors before scaling-up the fermentation step.

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