TÉCNICO LISBOA

Master Thesis in Biotechnology

Poly-3-hydroxybutyrate production from carbon-rich hydrolysates of the macroalgae *Gelidium sesquipedale* using halotolerant bacteria.

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ABSTRACT

A sustainable production of the biodegradable polymer poly-3-hydroxybutyrate (P3HB) by *Halomonas boliviensis* on carbon-rich hydrolysates from the industrial waste of *Gelidium sesquipedale* was explored. A combined treatment of the algal biomass by sulfamic acid catalyzed hydrothermal hydrolysis followed by enzymatic treatment of the biomass slurry by a cellulolytic cocktail proved to be the most adequate treatment. An hydrolysate featuring a glucose concentration of approximately 25 g/L corresponding to 0.3 g glucose/g dry biomass and less than 0.02 g/L of hydroxymethyl furfural (HMF) was produced. The production of P3HB by *H. boliviensis* was conducted in shake flask and in 2L stirred-tank bioreactor. In the shake flask study, the following results were attained: a $Y_{P/S}$ of 0.153, $Prod_P$, of 0.051 $g\,L^{-1}\,h^{-1}$, a CDW of 8.3 gL^{-1} and a $P3HB$ % of 39.2, on algal hydrolysates. In the fed-batch bioreactor assay, the broth was fed with a concentrated algal hydrolysate and limited by both nitrogen and oxygen, yielding a $Y_{P/S}$ of 0.237, a $Prod_P$, of 0.109 , a CDW of 22.0 g/L and a 26.0 $P3HB$ %.

Abbreviations: P3HB, Poly-3-hydroxybutyrate; MSG, Monosodium glutamate; HMF, 5-hydroxymethylfurfural; CDW, Cell-Dry Weight; RE, Vacuum rotatory evaporator; SD, Spray-dryer

1 Introduction

Nowadays, a "plastic-less" life is hardly imaginable. Plastic polymers from fossil fuels have been used widely for decades since they offer many unique properties such as (mechanical strength, durability, lightness, flexibility, chemical inertia, etc.) in contrast to other materials (glass, wood or metals). Nevertheless, taking into consideration non-degradability and long-time persistence in the nature, the development of environmentally friendly materials is necessary [1].

Bio-based plastics (bioplastics) derived from renewable resources are promising substituent to petroleum-based plastics [2]. Some of these bioplastics may also be biodegraded by microorganisms in both aerobic and anaerobic environment [2-3].

The biodegradable polymers can be produced either by chemical synthesis or by microorganisms. Polyhydroxyalkanoates (PHA) are interesting group of the biodegradable plastics, these bio polyesters are produced by variety of bacterial species [4-5]. A wide implementation of the PHAs on the market is still limited by high production costs, which are 50 % higher in comparison to the conventional plastics [6]. A carbon source is the most expensive item in the production costs (40-50 %) of bioplastics. One way to decrease the production costs is to optimize the production with industrial waste products as feedstocks (e.g. cheese whey permeate, wood hydrolysate, sugarcane molasses, waste glycerol, corn steep liquor, etc.) [7-8].

An unexploited and yet promising feedstock is an algal biomass gathered after phycocolloid extraction (e.g. agar, carrageenan, alginate). Annually, the agar extracted from red algae overcomes 14 thousand tons, resulting in a total profit of more than one thousand billion US$ with an enormous waste being generated. Nonetheless, the algal residues still contain a non-negligible amount of carbohydrates, thus it

has a huge potential as feedstock in biotechnology industry [9].

All polymeric form of algal carbohydrates (e.g. floridean starch; cellulose and agar in some red algae species[10]) should undergo hydrolysis to mono- ; di- or oligosaccharides fraction more accessible for further fermentation processes [11].

Digestion by acids is a widely used pre-treatment technique, as it does not require an expensive equipment and the whole process can be easily scaled-up. Instead of highly-corrosive concentrated acid, it is more desirable to adapt a chemical pre-treatment with low-concentrated acids, thus avoiding a corrosion of equipment and higher rate of carbohydrates degradation generating microbial inhibitor compounds such as: furans, organic acids and phenols [12-13]. Above a certain concentration, these compounds can inhibit a bacterial growth by limitation of the protein and RNA synthesis or can also damage a microbial DNA[14].

Recently, very promising results were attained by sulfamic acid for the treatment of *Gracilaria verrucosa*. In the article written by Park et al. (2018) authors achieved almost 40% of released sugars under the optimal conditions (100 mM concertation of acid; 7.5% biomass concentration; 130°C for 90 minutes) [15].

For a further improvement of the saccharification yield, an enzymatic hydrolysis is frequently used in tandem with the chemical pre-treatment, in the so-called “combined hydrolysis” [9].

A moderately halophilic bacteria *Halomonas boliviensis* is a gram-negative, aerobic, alkalitolerant, psychrophilic bacterial specie capable of accumulating P3HB up to 80 % (w/w) of CDW [16-17]. The accumulation of P3HB by *H. boliviensis* has been reported in several studies.

The production of P3HB polymer is triggered by limitation of some essential nutrient such as: nitrogen, phosphorus or oxygen [18]. The article written by Rivera-Terceros et al. (2015) aimed for the accumulation of P3HB by *H. boliviensis* using a variety of different c-sources, namely glucose, xylose and maltose under nitrogen limitation in shake flask and in an air-lift reactor. While in the shake flask authors obtained 60% (w/w) of P3HB content using a mixture of glucose and xylose, in the air-lift reactors that content reached only up to 40% (w/w)[19] Recently, a study published by García-Torreiro et al. (2016) describes changes of the metabolic behavior of *H. boliviensis* under different nutrient limitations. The microbial growth and P3HB production by *H. boliviensis* was examined in this study under single nitrogen and dual nitrogen and oxygen limitation. The highest P3HB accumulation (73%), P3HB concentration (35 gL^{-1}), CDW (48 gL^{-1}) and productivity 1.32 $g\,L^{-1}\,H^{-1}$ were accomplished within 40 h. under the nitrogen limitation with low oxygen supply.

This study aims for the P3HB production by *H. boliviensis* on carbon-rich hydrolysates from the *G. sesquipedale* residues in a shake flask and in bioreactor scale. In order to prepare concentrated algal hydrolysate as a feeding for a fed-batch assay, an evaporation step was implemented within this process. Two evaporation techniques (vacuum rotatory evaporator and spray-dryer) were examined and compared for such purpose in terms of the product yield, state of matter and possible impact on further degradation of carbohydrates.

2 Materials and methods

2.1 Raw materials and enzymes

2.1.1 Biomass

Gelidium sesquipedale residues after an agar extraction were gained from Iberagar SA, Portugal. The biomass was kept in sealed bags at -18 °C until use.

To prepare a powder from lyophilized *G. sesquipedale* residues, the biomass was first cleaned with a tap water to remove impurities such as rocks, sand, shelves, etc. A polished biomass was then dried on air at room temperature to remove an excess water. After 2-3 hours of drying, the biomass was put in a freezer (-80°C) overnight. A deeply frozen biomass was lyophilized for 48 hours and grinded with a coffee blender to the lyophilized powder. This powder was stored in a desiccator until use. Every assay was done using the lyophilized algal powder.

2.1.2 Enzymes

The enzymes used within this work were cellulase complex (NS 22086) and β-glucosidase (NS 22118) with enzyme activity 14.8 FPU/g and 32.0 NPGU/g, respectively both enzymes were purchased from Novozymes A/S (Denmark). The protein concentration,

determined by Bradford method using BSA as protein standard, was 120 mg/mL for (NS 22086) and 40 mg/mL for (NS 22118) [20].

2.2 Assays and methods

2.2.1 Sugar quantification

The quantification of monosaccharides, phosphate and dehydration products was performed by the High-Performance Liquid Chromatography (HPLC) equipment (Hitachi LaChrom Elite), with a column (Rezex ROA. Organic acid H+ 8% (30 mm x 7.8 mm, an autosampler (Hitachi LaChrom Elite L-2200), an HPLC pump (Hitachi LaChrom Elite L-2130) and with a Hitachi L-2490 refraction index detector and a Hitachi L-2420 UV/VIS detector. A column heater was connected externally. The temperature of column was set to 65 °C. The 5 mM H_2SO_4 eluent was used with 0.5 $mL\ min^{-1}$ flow rate in HPLC measurements.

Samples for a sugar quantification were firstly centrifuged (1-15P microcentrifuge, Sigma) for 5 minutes with 10 000 rpm. After the first centrifugation, an aliquot of supernatant (200 μL) was diluted with 200 μL of 50 mM H_2SO_4 , vortexed and centrifuged again with previous conditions. HPLC vials were then prepared with 100 μL of the supernatant and 900 μL of 50 mM H_2SO_4. After vortexing, samples were ready for HPLC analysis.

Linear regression equations were obtained from calibration curves for glucose, galactose, phosphate and HMF. Calibration data are attached in **Appendix**. The detection of sugars, phosphate was measured by RI and HMF with UV/VIS detector, respectively.

2.2.2 P3HB quantification

To quantify the concentration and P3HB content the P3HB polymer was converted into stable and volatile hydroxycarboxylic acid methyl esters, through an acid methanolysis reaction and measured by gas-chromatography (GC).

The reaction was initiated by adding 1 mL of chloroform to each Eppendorf tubes containing pellet. After, the addition of chloroform, the content was resuspended and then transferred to a glass tube. The acid methanolysis of P3HB polymer started by adding 1 mL of the “solution A” (97 mL methanol, 3 mL of 96% H_2SO_4 and 330 μL hexanoic acid) to each glass tube. These tubes were then vortexed and placed in an oven for 5 hours at 100 °C. The reaction was stopped by adding 1 mL of 60 g/L Na_2CO_3 solution to each tube. Each glass tube was vortexed and subsequently centrifuged for 5 mins at 5 000 rpm. After centrifugation 200 μL from the bottom phase (organic phase) was transferred from glass tube to a vial and these samples were stored in the freezer (-18°C) until GC measurement.

The samples were analyzed by gas chromatograph (Agilent Technologies 5890 series II) equipped with an FID detector and a 7683B injector. The oven, injector and detector temperatures were set to 60, 120 and 150 °C. The GC column used within this study was a HP-5 from Agilent J&W Scientific with a 30 meters in length and 0.32 mm of internal diameter. The data acquisition and integration were performed by the Shimadzu CBM-102 communication Bus Module and Shimadzu GC solution software (Version 2.3).

An identification of polymer was achieved by using an internal standard of 3-methyl hydroxybutyrate from Sigma-Aldrich. For a quantification, the calibration curve of 3-methyl hydroxybutyrate was measured in a concentration range from 0 to 8 g/L. The calibration data for P3HB quantification is enclosed in **Appendix**.

2.3 Combined hydrolysis of algal biomass

Combination of chemical pre-treatment under the most promising conditions followed by enzymatic hydrolysis was applied for a sufficient hydrolysis of algal polysaccharides.

2.3.1 Chemical pre-treatment

The chemical digestion of algal biomass was optimized by altering the reaction parameters such as type of catalyst, concentration of catalyst, concentration of biomass and time. An overview of the chemical agents with set of parameters that were explored, is displayed in **Table 1**. All chemical pre-treatment assays were performed the autoclave at 121°C. The biomass was weighted to three 50 mL glass flasks and 10 mL of acid was added in each flask to attain biomass concentrations of 21.5, 43 and 86 g/L. These flasks were then sealed with a rubber cover and aluminum cap and placed in the autoclave.

Table 1. Chemical pre-treatment conditions.

Catalyst	Catalyst concentration %(w/v)	Time (minutes)	T (°C)
Sulfuric acid	0.25;0.5;0.1	15;30;60	121
Phosphoric acid	0.5;1.0;1.5	15;30;60	121
Sulfamic acid	0.5;1.0; 1.5	30;60;90	121

After chemical hydrolysis, aliquots from the three samples were centrifuged (10 000 rpm, 5 minutes) and prepared for the HPLC analysis for sugar quantification.

2.3.2 Enzymatic hydrolysis

Enzymes were firstly pre-diluted 10 times with deionized water to ease further working with those originally viscous solutions. The enzymatic cocktail used for the hydrolysis contained: 1.2 mg of cellulase complex (NS 22086) and 0.3 mg of β-glucosidase (NS 22118), corresponding to an enzyme load 2.8 mg cellulase complex/ g biomass (0.04 FPU/g) and 0.7 mg β-glucosidase/ g biomass (0.022 NPGU/g). The enzymatic hydrolysis was executed in an incubator at 50 °C for 30 hours at 660 rpm [21]. Samples were taken before the addition of enzymes and in the end of enzymatic hydrolysis, centrifuged at 10 000 rpm for 5 minutes and prepared for the sugar quantification.

2.3.3 Concentration of algal hydrolysates

To produce concentrated *G. sesquipedale* hydrolysate to use as a feed in fed-batch cultivations, a concentration step was implemented.

Table 2 – Process conditions applied for the concentration of the hydrolysates from *G. sesquipedale residues.*

Vacuum Evaporator		Spray-Dryer	
Process parameter	**Value (Unit)**	**Process parameter**	**Value (Unit)**
Pressure	50 ± 5 (mbar)	T_{inlet}	150 (°C)
$T_{Water\ Bath}$	50 ± 5 (°C)	T_{out}	93 ± 2 (°C)

Two possible concentration techniques were studied, namely: vacuum rotatory evaporator (BUCHI Rotavapor R-3) and Spray dryer (BUCHI Mini Spray Dryer B-290), using the conditions given in **Table 2**.

2.4 Cultivation of *Halomonas boliviensis*

The halotolerant bacteria *Halomonas boliviensis* DSM 15516, a strain able to accumulate P3HB, was used within the framework of this work.

The inoculation medium for *H. boliviensis* contained: 45 g/L of *NaCl*; 25 mL/L of 100 g/L $MgSO_4 \times 7\ H_2O$; 0.55 g/L K_2HPO_4; 2.3 g/L NH_4Cl; 15 g/L *Tris*; 3 g/L *MSG*, 0.005 g/L $FeSO_4 \times 7\ H_2O$. Solutions of *NaCl* (300 g/L) and $MgSO_4 \times 7\ H_2O$ (100 g/L). The pH was adjusted to 7.5 with 35% (w/w) HCl.

The inoculum of *H. boliviensis* was prepared in sterilized 500 mL shake flask to a total volume of 100 mL. Growth of the inoculum occurred at 30 °C and 170 rpm until the exponential phase was reached, which was after 24 (O.D.$_{600\ nm}$ = 5).

2.4.1 P3HB production in shake flasks

The medium for the production of P3HB by *H.boliviensis* in shake flasks assays contained: 45 g/L *NaCl*; 50 mL/L of 100 g/L $MgSO_4 \times 7\ H_2O$; 2.2 g/L K_2HPO_4; 0.005 g/L $FeSO_4 \times 7\ H_2O$ and 15 g/L *Tris*. Various concentrations of NH_4Cl (0 or 1 g/L) and *MSG* (0; 5; 10 g/L) were tested. The pH was adjusted to 7.5 with a 35% (w/w) HCl.

For the growth on *G. sesquipedale* hydrolysates a 20-fold concentrated mineral medium was prepared. The pH was not adjusted in this concentrated medium but only after dilution in the shake flask.

The assays for P3HB production were performed in the 500 mL shake flasks containing the mineral medium supplemented with glucose or *G. sesquipedale* hydrolysate up to volume of 100 mL. A volume of a previously prepared inoculum was added to attain 5 % (v/v) concentration. Samples from the shake flasks were periodically taken for sugar, polymer, optical density; pH and CDW quantification. Each assay was done in duplicate.

2.4.2 P3HB production in bioreactor

The inoculum for the fed-batch assay was prepared as described in section (**2.4**) to a total volume of 65 mL (5% (v/v) of the bioreactor initial volume).

Fed-batch cultivations were carried out in 2L stirred-tank bioreactors (New Brunswick Bioflo 115) using the BioCommand Batch Control software, which enabled control, monitoring and data acquisition. The pH was controlled at 7.5 with 10% NH_4OH or 2.5 M NaOH.The gas flow used was 2.0 L/min, unless stated otherwise. The dissolved oxygen (DO (%)) set point was set at 20% saturation, unless stated otherwise. The agitation speed (max. 1200 rpm) was set in cascade with the DO (%). The initial volume of cultivation was 1.3L including all medium components and inoculum.

The bioreactor medium for P3HB production triggered by nitrogen and oxygen limitation. contained: 45 g/L *NaCl*; 5 mL of 100 g/L $MgSO_4 \times 7 H_2O$; 15 g/L K_2HPO_4; 0.005 g/L $FeSO_4 \times 7 H_2O$, 10 g/L *MSG* and 25 g/L glucose to attain 1.3 L total working volume.

2.4.3 Cultivation parameters

The yield of product over substrate $Y_{P/S}$ in shake flasks and in bioreactors was calculated according to **Eqs. 2** and **3**, respectively. Variations in sample volumes were neglected in the shake flask assays.

$$Y_{P/S}\ (\mathrm{g\,P}/\,\mathrm{g}\,S_{cons.}) = \frac{[P_{max}]}{[S_{cons.}]} \qquad (2)$$

$$Y_{P/S}\ (\mathrm{g\,P}/\,\mathrm{g}\,S_{cons.}) = \frac{m_{P_{sum.}}}{m_{S_{cons.}}} \qquad (3)$$

Where $[P_{max}]$ represents the maximal concentration of product (P3HB polymer), $[S_{cons.}]$ or $m_{S_{cons.}}$ the concentration or mass of consumed substrate and $m_{P_{sum.}}$ sum of product mass in each sample until the maximal mass of the polymer was attained. Maximum productivity for both shakes flasks and bioreactors was calculated regarding **Eq. 4.** The unit $t_{s\,max}$ is a time of cultivation, when $[P_{max}]$ was acquired.

$$Prod_{max\,P}\ (g\,L^{-1}H^{-1}) = \frac{[P_{max}]}{t_{s\,max}} \qquad (4)$$

P3HB content (%) and residual CDW (g/L) were acquired by **Eq. 5** and **6**.

$$P3HB\ cont.(\%) = \frac{P3HB\ (g\,L^{-1})}{CDW\ (g\,L^{-1})} \qquad (5)$$

$$CDW_{residual}\ (g\,L^{-1}) = CDW - P3HB \qquad (6)$$

3 Results and discussion

3.1 Algal hydrolysate preparation

3.1.1 Chemical pre-treatment of algal biomass

Acid-catalyzed hydrothermal hydrolysis of *Gelidium* residues was carried out using the conditions stated in **Table 1** [21], three most promising conditions are summarized in the **Figure 1**.

During the chemical pre-treatment, merely galactose was released, showing that the mild acid conditions used are not strong enough to hydrolyze cellulose into glucose. Nonetheless, even in mild conditions HMF is released, and this is caused by degradation of the anhydro-L-galactose (AHG) even in the mild acidic environment [22]. Besides HMF, no other degradation products were detected. The hydrolysis with sulfuric and phosphoric acid were able to release 64.0% and 44.2% of the total galactose. However, the dehydration of the agar monomers to HMF was significantly higher using these acid catalysts comparing to sulfamic acid.

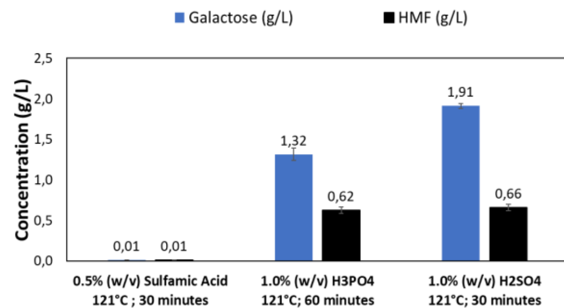

Figure 1. The chemical pre-treatment of algal biomass by mild-concentrated acids. The concentration of biomass used for this assay was 43.2 g/L.

Although the condition with sulfamic acid was unable to release galactose from agar it was further chosen in the pre-treatment step, due to the low amount of HMF produced. This is a reasonable decision for processing this type of biomass because the amount of agar in the residues and thus of released galactose is quite low and thus it might be neglected for further valorization to P3HB.

3.1.2 Inhibitory effect of HMF on H. boliviensis growth

Concerning the results from the chemical pre-treatment, it was necessary to understand the magnitude of the HMF inhibitory effect on the microbial growth. Therefore, an assay of *H. boliviensis* growth (section *2.4.1.*) in mineral medium supplemented with 20 g/L glucose and different concentrations of HMF was performed in 500 mL shake flasks (**Figure 2**).

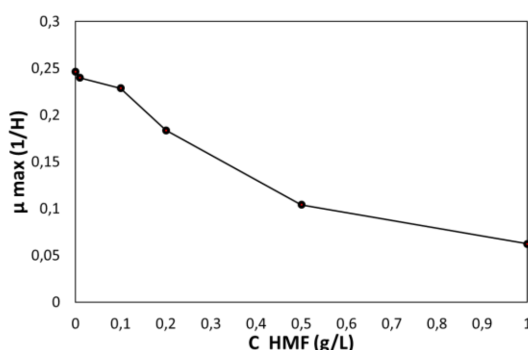

Figure 2. The inhibitory effect of HMF on *H. boliviensis* growth with P3HB production medium and glucose 20 g/L.

The study of the influence of HMF on *H. boliviensis* growth, suggests that above 0.1 g/L the inhibitory effect on *H. boliviensis* growth is significant. At 0.1 g/L HMF the relative growth rate decreased approximately to 92.8 % of the maximum attained in the control, i.e. same cultivation medium supplemented with 20 g/L of glucose and no HMF. Therefore, the chemical pre-treatment condition with 0.5% (w/v) sulfamic acid at 121 °C for 30 minutes was chosen for the further production of hydrolysates, as under this condition not more than 0.01 g/L of HMF was released (**Figure 1**

3.1.3 Combined hydrolysis of algal biomass

With the best knowledge about the optimal condition for chemical pre-treatment, a combined hydrolysis of algal biomass was observed. To find out the highest amount of glucose released during combined hydrolysis, an assay with different biomass concentrations was explored (**Figure 3**). The increase of biomass concentration to 86.4 g/L resulted in 24.1 g/L concentration of the total sugar released and HMF concentration beyond 0.01 g/L (**Figure 3**). Two monosaccharides, glucose and galactose, were released during the

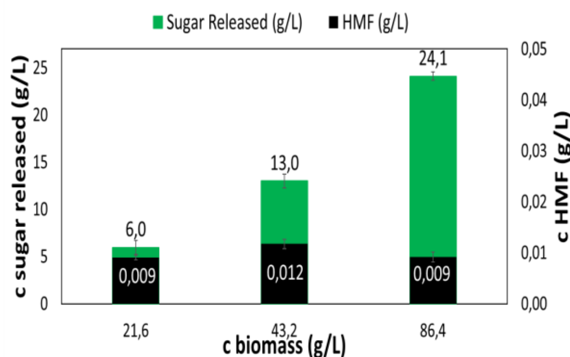

Figure 3. The combined hydrolysis of algal biomass with different biomass concentrations.

combined hydrolysis of *G. sesquipedale* residues by 0.5% (w/v) sulfamic acid. However, glucose was predominantly released in the all conditions. Only a small concentration 0.3 g/L of galactose was released with 21.6 g/L biomass concentration.

3.2 P3HB production by *H. boliviensis*

3.2.1 P3HB production in shake flasks

Shake flasks experiments were carried out to access the microbial growth and P3HB production by *H. boliviensis* on hydrolysates from *G. sesquipedale* residues. The glucose concentration of the prepared algal hydrolysate varied between 24 and 26 g/L and the concentration of HMF did not exceed 0.02 g/L.

3.2.1.1 H. boliviensis growth and P3HB production on algal hydrolysates

To determine the best medium composition to promote P3HB production by *H. boliviensis* , a 20-fold concentrated mineral media supplemented with algal hydrolysates and different concentrations of nitrogen source (0 or 1 g/L NH_4Cl and 0,5 and 10 *MSG*) was carried out.

The results reported in **Figure 4** indicate that the medium containing 10 g/L *MSG* and no NH_4Cl promoted P3HB production by *H. boliviensis* on algal hydrolysates. The production medium “D” was favored, due to a higher P3HB content (%), over the production medium “C” **(Table 3)**.

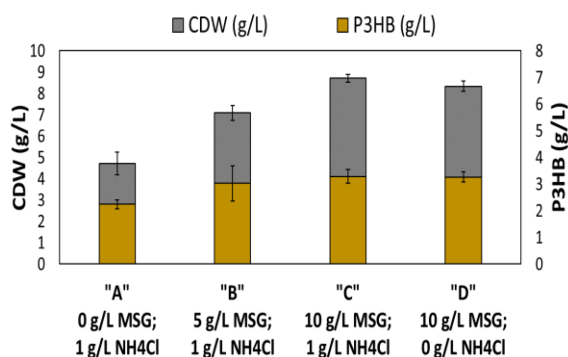

Figure 4. The *H. boliviensis* growth and P3HB production on *G. sesquipedale* hydrolysates with production media containing variable concentrations of nitrogen source (0 or 1 g/L NH_4Cl and 0; 5; 10 or 20 g/L *MSG*).

Table 3. Cultivation parameters from the assay of *H. boliviensis* growth and P3HB production on *G. sesquipedale* hydrolysate with production media (PM) containing variable concentrations of nitrogen source: PM A (1 g/L NH_4Cl and 0 g/L *MSG*); PM B (1 g/L NH_4Cl and 5 g/L *MSG*); PM C (1 g/L NH_4Cl and 10 g/L *MSG*) and PM D (0 g/L NH_4Cl and 10 g/L *MSG*).

Unit	$Y_{P/S}$	$Prod_P$	CDW	P3HB %
PM A	0.113	0.047	4.7	56.7
PM B	0.149	0.057	7.1	52.2
PM C	0.158	0.056	8.7	37.7
PM D	**0.155**	**0.056**	**8.3**	**39.2**

3.2.1.2 H. boliviensis growth and P3HB production on concentrated algal hydrolysates

The concentration of *G. sesquipedale* was performed using a vacuum rotatory evaporator (RE) or a spray-dryer (SD). The composition of concentrates so obtained and their effect on the growth and P3HB by *H. boliviensis* in shake flasks is reported in **Figure 5**. The production medium “D” was used within this study case.

Referring the results of HMF inhibitory effect on *H. boliviensis* (section *3.1.2.*) the initial concentration of HMF in shake flasks (**Table 4**) for both conditions was slightly above 0.1 g/L, therefore a significant limitation of the *H. boliviensis* growth was not expected.

Table 4. Composition of the concentrates and initial concentration of glucose and HMF in the shake flasks.

	$C_{Concentrate}\ (g\ L^{-1})$		$C_{Shake\ Flask}\ (g\ L^{-1})$	
	Glu.	**HMF**	**Glu.**	**HMF**
RE	181.8	1.347	17.86	0.135
SD	153.4	0.903	17.31	0.105

Observing the results from *H. boliviensis* growth on concentrates (**Figure 5**), a difference in the *H. boliviensis* growth in both conditions is significant, as the highest CDW acquired were 8.7 and 6.4 g/L in the case of *RE* and *SD* concentrate, respectively. This disproportion influenced the final P3HB content (%) as in the condition with RE concentrate the highest P3HB content reached 26.5 %, while with SD concentrate that parameter rose up to 40.4 %. Also, higher P3HB concentration was attained with SD concentrate (2.58 g/L) than in the case of RE concentrate, 2.19 g/L. Despite a limited microbial growth in the assay with SD concentrate, the attained production yield 0.153 $Y_{P/S}$ and productivity 0.051 $(g\ L^{-1}\ h^{-1})$ $Prod_P$ were higher in this case. Concerning, the impact of both evaporation techniques on algal hydrolysates, the results obtained with SD hydrolysates showed better results. Other comparisons are also in favor of the SD, such as energy input, which is significantly higher for the RE evaporation than in the case of SD. Also, the final dry powder obtained by SD has much more desirable properties such as: longer shelf life, lower costs for storage and transportation, than the viscous liquid concentrate, obtained from RE evaporation. Nonetheless, due to the high relative stickiness of glucose, the solids attached to parts of the equipment caused a considerable loss of product, the yield of product attained being $Y_P = 62.56\ \%$ [23].

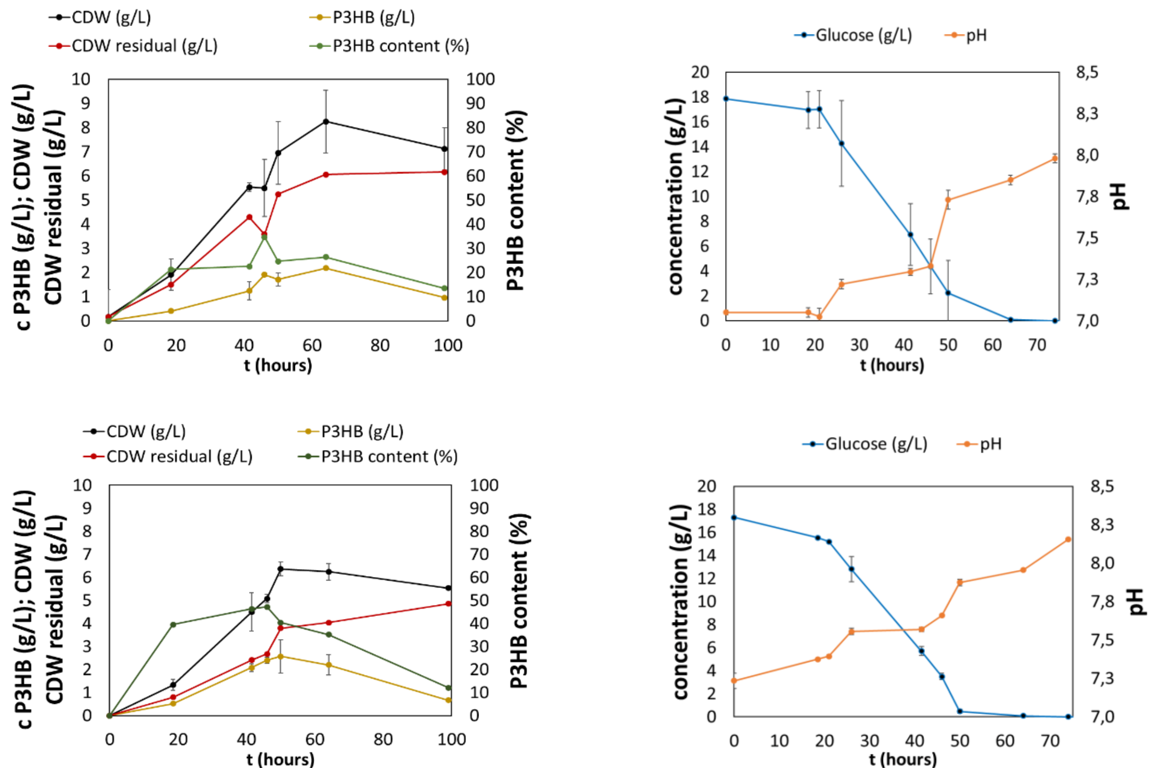

Figure 5. The *H. boliviensis* growth and P3HB production on concentrates from *G. sesquipedale* hydrolysates, acquired by two evaporation techniques Vacuum rotatory evaporator (**top two images**) and Spray-dryer (**bottom two images**). Microbial growth and P3HB production are accessed on production medium "D".

3.2.2 P3HB production in bioreactor

Fed-batch assays were carried out in a STR bioreactor to attain high yields and productivities. The production medium imposing the P3HB production by nitrogen limitation was mentioned in the section (*2.4.2.*). The dual limitation by nitrogen and oxygen was chosen as a suitable limitation strategy to trigger the P3HB production. In the end of the batch phase, the base $10\,\%\,NH_4OH$ was substituted with $2.5M\,NaOH$ to initiate the nitrogen limitation. Simultaneously, to access oxygen limitation the DO was reduced from 20.0 to 2.0 % and the gas flow from 2.0 to 0.8 $L\,min^{-1}$. Cultivation with the algal concentrate was compared to a similar cultivation with commercial glucose. In this case the glucose feeding had the same glucose concentration as the algal hydrolysate (**Table 7**).

Regarding the results of the fed-batch study (**Figure 6**), similar $Y_{P/S}$ were attained in both cases with the algal hydrolysate as feed (0.237) than with glucose feeding 0.275.

Table 7. Composition of the final concentrate (***FC***) from *G. sesquipedale* hydrolysates and glucose simulate (***S***), both as the feeding solutions fed-batch assay.

	V (mL)	$c_{glucose}$ $(g\,L^{-1})$	c_{HMF} $(g\,L^{-1})$
FC	420	165.1	0.086
S	420	160.0	-

Surprisingly, the highest concentration of P3HB achieved in the case of the algal concentrate feeding was 5.72 g/L almost 1.5 times higher than in the study with glucose feeding (3.56 g/L). Although, a low volume and glucose concentration did not allow to continue with the process longer than for 42h, the production of P3HB (g/L) was steadily continuing in the case of glucose feeding, whilst in the case of the algal hydrolysate the P3HB production halted after 50 h. However, due to a very slow production in the assay with glucose

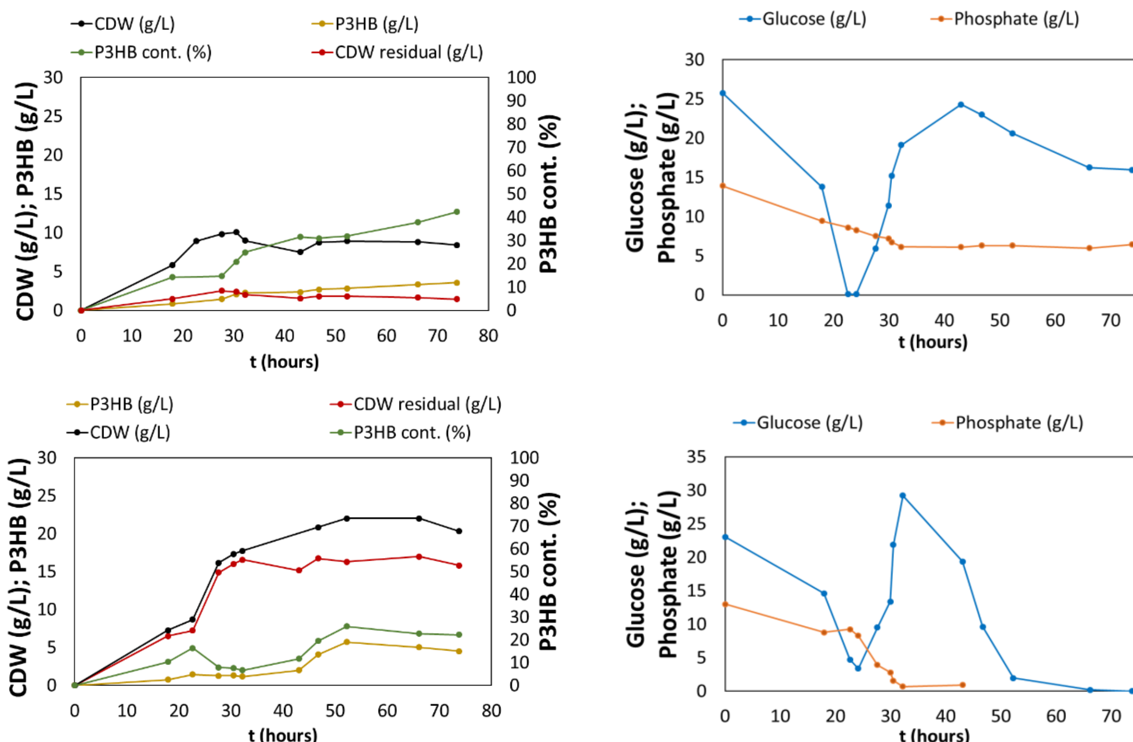

Figure 6. The fed-batch cultivation of *H. boliviensis* under the dual limitation of nitrogen and oxygen to impose the P3HB production. The limitation began at 25 h. of cultivation. The **top two** images represent the simulate assay with glucose feeding, whereas the **bottom ones** are from the assay with feeding with algal hydrolysate.

simulation, the $Prod_{max\ P}$ was higher in the case of feeding by carbon-rich concentrate.

A huge disproportion can be seen in the $CDW\ (gL^{-1})$ in both feeding conditions. These results were strongly influenced by the state of concentrate itself. During the concentration some insoluble agglomerates, probably originated from oligosaccharides and proteins, were formed. Thus, throughout the feeding, a change of $CDW\ (g\ L^{-1})$ was inevitable and it was difficult or almost impossible to predict the $CDW\ (g\ L^{-1})$ corresponding to the microbial growth only. To avoid this problem, a centrifugation step should be implemented to minimize the presence of the precipitates formed during the concentration. Due to the CDW differences, the $P3HB\ cont$ (%)differs significantly. However, the polymer content in with algal hydrolysates should be higher than 26.0 % as the highest P3HB concentration was 5.72 g/L surpassing the one with glucose feeding, 3.56 g/L.

4 Conclusion and future prospects

The waste biomass of *G. sesquipedale*, proved to be an underexplored and yet promising material for biorefinery purposes [21]. Acid catalyzed hydrothermal hydrolysis using a diluted sulfamic acid solution followed by enzymatic hydrolysis with cellulolytic enzymes produced a glucose-rich hydrolysate with a non-inhibitory HMF concentration. The produced algal hydrolysates were shown to be a good carbon source for the production of P3HB by *H. boliviensis.* The best production and growth parameters in shake flask assays and the algal hydrolysates were obtained with a mineral medium supplemented with 10 g/L MSG. In these conditions the highest $Y_{P/S}$, $Prod_P$, CDW, $P3HB$ %, $c\ P3HB$ were 0.155, 0.056 $(g\ L^{-1}\ h^{-1})$, 8.3 $(g\ L^{-1})$, 39.2 % and 3.27 $(g\ L^{-1})$, respectively. For the bioreactor assays a concentrated algal hydrolysate was produced to be used as feeding. This was accomplished using vacuum evaporation or a spray-dryer. Regarding the lower energy input and the state of the concentrate (dry powder), concentration using a spray-dryer was proved to be a more suitable option. Moreover, better growth and P3HB yields were attained with this concentrate in shake flask assays. During the fed-batch cultivation, 420 mL concentrated hydrolysate

(165.1 g/L glucose and 0.086 g/L HMF) was used as feeding. P3HB production was promoted under a dual limitation by nitrogen and oxygen. Under these conditions, a CDW of 22.0 g/L containing 26.0 % (g/g) polymer as well as a yield $Y_{P/S}$, of 0.237 and a productivity $Prod_P$, of 0.109 g L^{-1} h^{-1} were obtained.

In future studies the pre-treatment step should take place with agitation instead of the static treatment in the autoclave. Also, the concentration step should be analyzed much more in depth, in particular the possible formation of other degradation products besides HMF and a way to overcome the issue of glucose stickiness. Finally, optimization of fed-batch cultivations on glucose and concentrated hydrolysates to attain higher P3HB productivities should be followed

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