High-pressure technology for *Sargassum* spp biomass pretreatment and fractionation in the third generation of bioethanol production

Aparicio E.[1], Rosa M. Rodríguez-Jasso[1], César D. Pinales-Márquez[1], Araceli Loredo-Treviño[1], Armando Robledo-Olivo[2], Cristóbal N. Aguilar[1], Emily T. Kostas[3], Héctor A. Ruiz[1*]

[1] Biorefinery Group, Food Research Department, Faculty of Chemistry Sciences, Autonomous University of Coahuila, 25280 Saltillo, Coahuila, Mexico.

[2] Food Science & Technology Department, Universidad Autónoma Agraria Antonio Narro, Unidad Saltillo, Blvd Antonio Narro 1923, Buenavista, 25315 Saltillo, Coahuila, Mexico.

[3] Department of Biochemical Engineering, The Advanced Centre of Biochemical Engineering, Bernard Katz Building, Gower Street, London WC1H 6BT, University College London, London, United Kingdom.

*Corresponding author: Email: rrodriguezjasso@uadec.edu.mx (Rosa M. Rodríguez-Jasso) hector_ruiz_leza@uadec.edu.mx (Héctor A. Ruiz). webpage: www.biorefinerygroup.com

Abstract

Sargassum spp is an invasive macroalgae and an alternative feedstock for bioethanol production. *Sargassum* spp biomass was subjected to high-pressure technology for biomass fractionation under different operating conditions of temperature and residence time to obtain glucan enriched pretreated solids (32.22 g/100 g of raw material). Enzyme hydrolysis process at high pretreated solid loading (13 %, w/v) and enzyme loading of 10 FPU/g of glucan was performed, obtaining 43.01 g/L of glucose corresponding to a conversion yield of 92.12 %. Finally, a pre-simultaneous saccharification and fermentation strategy (PSSF) was performed to produce bioethanol. This operational strategy produced 45.66 g/L of glucose in the pre-saccharification stage, and 18.14 g/L of bioethanol was produced with a glucose to bioethanol conversion yield of 76.23 %. The development of this process highlights the feasibility of bioethanol production from macroalgal biomass in the biorefinery concept.

Keywords: Seaweed, Macroalgae, Severity Factor, Biofuels, Hydrothermal Processing

1. Introduction

The past decade has seen an overwhelming number of coastlines in the Caribbean Sea region become inundated with an influx of the brown macroalgae *Sargassum*. This unique phenomenon, which is comprised of different species of *Sargassum* including *Sargassum natans*, *Sargassum fluitans* and, *Sargassum muticum*, has been recorded in the Caribbean Sea, the Western Central Atlantic and in the Gulf of Mexico (Wang and Hu, 2016). Although coastal *Sargassum* deposits occur naturally and often regularly but with smaller quantities, records revealed that around 10,000 wet tonnes was deposited daily in the Caribbean in 2015, with the Mexican Caribbean region obtaining on average 2360 m^3 of *Sargassum* per km of coastline during the same period (Schell et al., 2015). The inundation of *Sargassum* on Caribbean and Mexican gulf beaches has been described as an 'international crisis' and the 'greatest single threat to the Caribbean', since the effects may have both negative environmental and economic consequences. The uncontrollable proliferation of *Sargassum* macroalgae is not yet fully understood, although it is believed that the global increase in CO_2 levels effecting climate change has enhanced ocean acidification and sea temperature levels (Huffard et al., 2014), thus promoting *Sargassum* blooms. As a result of this, attempts to establish efficient solutions to mitigate the build-up of *Sargassum* biomass on beach regions are being explored in order to promote coastal and economic sustainability. Studies have proposed potential valorization routes, using *Sargassum* biomass as feedstock, to produce biochemical, biofuels and pharmaceuticals in terms of circular bioeconomy and biorefinery (Kostas et al., 2017; Lara et al., 2020; Aparicio et al., 2020), yet such research is still in its infancy and requires further exploration in order to develop viable processing methodologies and technologies.

A number of studies have investigated alternative processing routes to efficiently convert different species of macroalgae into advances biofuels as bioethanol, which typically follow conventional methods of biomass hydrolysis previously developed for use with lignocellulosic feedstocks (Jung et al., 2013; Ruiz et al., 2015; Siller-Sánchez et al; 2018).

The high-pressure technology pretreatment (hydrothermal processing) for biomass fractionation is an important and promising technology that can be scale-up of large industrial processes in different mode of operation (batch and continuous) (Ruiz et al., 2013; 2017; 2020). This pretreatment applied on the macroalgal biomass increase the accessibility of hydrolyzing enzymes to exposed cell wall polysaccharides (Maneein et al., 2018). After enzymatic hydrolysis, the monosaccharides released can then converted into bioethanol using fermenting microorganisms. Nowadays, researchers are investigating novel ways to improve the conversion process and hence lower production costs (Soleymani and Rosentater, 2017; Aguilar-Reynosa et al., 2017a). This includes the modification and coupling of certain key stages of the bioethanol production process, in order to reduce water and energy cost requirements (Pinales-Márquez et al. 2020).

Strategies such as high-pressure technology pretreatment for biomass fractionation and Pre-Simultaneous Saccharification and Fermentation (PSSF) have been developed in order to merge the benefits of the traditional 'sequential hydrolysis and fermentation' processing route and 'simultaneous saccharification and fermentation' (SSF) (Aguilar et al., 2017b; Aguilar et al., 2018). PSSF incorporates a pre-saccharification time before the SSF stage. Therefore, hydrolysis is undertaken in the optimal conditions for a relatively short time before a suitable microorganism is added to initiate the fermentation process (Aguilar et al.,

2018). PSSF strategy has been applied to reduce the viscosity of slurry at high solid loadings in first- and second-generation bioethanol process, it is expected to have higher productivity and yields (Pino et al., 2018). Therefore, the high-pressure technology pretreatment and PSSF shows great potential for bioethanol production from macroalgae and may become available and suitable for commercial utilization (Konda et al., 2015; Tan et al., 2020).

In this work, the invasive *Sargassum* spp biomass was studied as a renewable material for bioethanol production, using an eco-friendly and sustainable high-pressure technology pretreatment (hydrothermal processing). Figure 1 shows the development of this process. The optimal pretreated glucan-rich solid was subjected to different conditions of enzymatic hydrolysis at high solids loading and low enzyme dosages, followed by the PSSF strategy in order to achieve the maximum glucose to ethanol conversion.

Figure 1

2. Materials and methods

2.1 *Sargassum* biomass collection and preparation

The *Sargassum* spp (Sg) used in this study was collected in Puerto Morelos, Quintana Roo, Mexico (GPS Coordinates: 20.83149359, -86.87929434), in February 2019. The Sg was washed with tap water, sun-dried for 48 hours and milled to a particle size between 0.5-2 mm using a blade mill (Blender 564A, Dual Range Pulse, Osterizer) before being preserved in dark polyethylene bags. In order to determine the moisture content, 1 g of Sg was dried at 80°C for 24 h and the differences in wet and dry weight were used to calculate the moisture content.

2.2 Physicochemical characterization of *Sargassum* biomass

The biochemical characterization of Sg biomass was determined in accordance with crude fat, crude fiber, protein, ashes and sulphate content (Dodgson, 1961; Helrich and Neal, 1990; Sluiter et al., 2008a). The polysaccharide content (glucan, galactan and fucoidan) was determined by quantitative acid hydrolysis at 72% (w/v), following the standard analytical procedures of the National Renewable Energy Laboratory (Sluiter et al., 2008b), where 0.5 g of dry biomass were places in a test tube with 5 mL of sulfuric acid at 72% (v/v), and were subsequently subjected to a 35°C water bath with manual agitation for 1 h. The samples were then diluted to 4% sulfuric acid (adding 148.67 g of distilled water), and the dilution heated in an autoclave for 1 h. Samples were then cooled before an aliquot of 1 mL was filtered with a 0.45 µm nylon filter into a vial for monomeric sugars release determination using HPLC (section 2.6) in order to estimate the content of polysaccharides in the samples: glucose as laminarin (as glucan), galactose as galactan, and fucose as fucoidan. Finally, the solid fraction was oven-dried at 50°C during 24 hours, and was weighed to determine the acid insoluble residue (del Río et al., 2019).

2.3 High-pressure technology pretreatment (hydrothermal processing) on *Sargassum* biomass

High-pressure pretreatment (hydrothermal processing) was performed in a bespoke stainless-steel pressurized batch reactor, designed (conceptual, basic and detailed engineering design) and developed by the biorefinery group (www.biorefinerygroup.com) and was equipped with stirring and proportional-integral-derivative (PID) temperature controller. The reactor is instrumented with a temperature sensor (thermocouple with a

thermowell) and a pressure sensor (dry pressure gauge) with a total working volume of 0.66 L. The reactor was heated with an electrical resistance and a water jacket cooling system was used to cool the reactor. Sg and distilled water were mixed at a ratio of 1:20 (w/v) and loaded in the reactor.

A response surface methodology (central composite design) was used in order to identify the optimal conditions to release the highest content of glucan in the pretreated solid of Sg biomass (g of glucan /100 g of Sg). Hydrothermal high-pressure pretreatment experiments were carried out at operational conditions of temperature (150 – 190 ºC), pressure (3.75 - 11.54 bar) and residence times (10-50 min) in accordance with the experimental design in Table 1, the central point was evaluated with 3 repetitions and the statistical results were analyzed using STATISTICA 8.0 software.

Table 1

After the reactor cooled, the solid and liquid phases were separated through filtration. The solid phase was washed with distilled water in order to remove any degradation compounds that may have been present and then oven-dried at 50 °C. The solid phase which remained post hydrothermal pretreatment was then used to calculate the solid yield (g solid recovered/100 g initial Sg) and was subsequently analyzed for polysaccharide content following the quantitative acid hydrolysis method outlined in section 2.2. An aliquot of the liquid phase after hydrothermal pretreatment was analyzed by HPLC for glucose, galactose, fucose, acetic acid, formic acid, furfural and hydroxymethylfurfural (HMF) determination (section 2.6). The oligomers fraction in the liquid phase were quantified by quantitative acid post-hydrolysis, were a sample of 2 g of the liquid phase was placed in a 100 mL vessel along with 1 g of sulfuric acid (95-97%) and 22 g of

distilled water, and the monomers released were quantified in HPLC (section 2.6). All the analyses were performed in triplicate.

The intensity of the high-pressure technology (hydrothermal pretreatment) was expressed by severity factor, taking into account operating conditions such as temperature and time, this factor can be expressed by the following equations (Chornet and Overend, 2017; Aguilar et al., 2018):

$$\log R_0 = [R_0\ Heating] + [R_0\ Isothermal\ process] + [R_0\ Cooling]$$

$$\log R_0 = \left[\int_0^{t_{máx}} \frac{T(t) - 100}{\omega}\right] + \left[\int_{ctrl}^{ctrf} exp\left[\frac{T(t) - 100}{\omega}\right] dt\right] + \left[\int_0^{t_{máx}} \frac{T'(t) - 100}{\omega}\right]$$

The severity factor was determined taking into account the heating-up and cooling profiles and the isothermal section of the process. Where [$\log R_0$] is the severity factor, $t_{máx}$ (min) is the time demanded to reach the peak of temperature, $T(t)$ and $T'(t)$ are the profile of temperature in heating and cooling steps, $ctrl$ and $ctrf$ (min) are the period needed for the overall heating-cooling process, and ω is an empirical number with a value of 14.75. The severity factor [logR_0] was calculated using numerical integration to obtain the area under of temperature vs time curve.

2.4 Enzymatic hydrolysis

2.4.1 Enzymes

The commercial enzyme cocktails Cellic CTec2 and Cellic HTec2 from *Trichoderma reesei* were used and kindly supplied by Novozymes, USA. Cellic CTec2 is a

complex enzyme cocktail consisting of β-glucosidases and hemicellulases, whereas Cellic HTec2 contains endoxylanases for hemicellulose enzyme hydrolysis. The initial cellulase activity (118 FPU/ml) for Cellic CTec2 was determined in accordance with the method of the National Renewable Energy Laboratory (NREL/TP-510-42628) (Adney and Baker, 1996).

2.4.2 Evaluation of enzymatic hydrolysis on pretreated *Sargassum* biomass

The pretreated Sg biomass from hydrothermal pretreatment were used as substrate for enzymatic hydrolysis, and non-pretreated Sg was used as a control. The assays were performed in 125 mL Erlenmeyer flasks with a working volume of 25 mL, at an incubation temperature of 50°C and an agitation speed of 150 rpm. A 50 mM (pH = 4.8) citrate buffer (pH = 4.8) was used with a cellulase/hemicellulase ratio of 1:2 (v/v) and the cellulase loading rate was 15 FPU/g glucan (Pino et al., 2019). The experimental design followed was similar to the design outlined in section 2.3.1, where the independent variables (factors studied) were pretreated solids loading (5, 9 and 13 %, w/v), and cellulase loading (5, 10 and 15 FPU/g of glucan), and glucose concentration (g/L) as dependent variable.

To avoid microbial growth during the enzymatic hydrolysis, 100 µL of tetracycline and 75 µL of cycloheximide from a 10 g/L stock solution were used according to Selig et al (34). Aliquot samples (1 mL) were taken at 0, 6, 12, 24, 48, and 72 h from the enzymatic hydrolysis process and centrifuged. The resulting supernatant was analyzed by HPLC (section 2.6) and the saccharification yield (conversion of glucan into glucose, %) was calculated using the following equation (Dowe and McMillan, 2001):

$$Saccharification\ yield\ (\%) = \frac{[Glucose] \pm 1.053[Cellobiose]}{1.111\ (f)[Biomass]} * 100$$

Where $[Glucose]$ is the residual glucose concentration in g/L, $[Cellobiose]$ is the residual cellobiose after glucan hydrolysis concentration in g/L, (f) is the glucan fraction in dry basis (g/g), 1.053 and 1.111 are factors of conversion, and $[Biomass]$ is the dry biomass concentration at the beginning of the enzymatic hydrolysis.

2.5 Fermentation of pretreated solids of *Sargassum* biomass

2.5.1 Inoculum preparation

Saccharomyces cerevisiae PE-2 was acquired from the microbiological collection of the Centre of Biological Engineering at University of Minho, Portugal and was used in these trials. The yeast was grown in a 500 mL Erlenmeyer flask with 125 mL of media consisting of 50 g/L of glucose, 10 g/L of peptone and yeast extract and incubated at 35°C at a speed of 150 rpm for 16 h. The cell suspension was aseptically centrifuged (4°C, 5600*g*, 15 min), and the solid was re-suspended in 0.9% NaCl at a final concentration of 200 g fresh yeast per L (Pereira et al., 2014, López-Sandin et al., 2021).

2.5.2 Pre-simultaneous saccharification and fermentation (PSSF) of pretreated *Sargassum* biomass

The optimal processing conditions from the enzymatic hydrolysis trial in section 2.4.2 were employed in the pre-simultaneous saccharification stage on pretreated Sg. The experiments were carried out in triplicate in 50 mL of working volume (in 250 mL Erlenmeyer flasks) under semi-anaerobic condition. After 24 h, the temperature was

adjusted from 50 °C to 35 °C and *S. cerevisiae* PE-2 was inoculated at 8 g per L of fresh yeast in NaCl (0.9% w/v) suspension. The kinetics of the fermentation were monitored for 72 h, with 1 mL samples taken at 0 and 24 h of the pre-saccharification stage for glucose quantification, and samples taken at 12, 24, 48 and 72 h of the fermentation stage for glucose and bioethanol quantification. Samples were analyzed using the HPLC method outlined in section 2.6.

The results were expressed as ethanol conversion yield (%) using the following equation:

$$Ethanol\ yield\ (\%) = \frac{[EtOH]_f - [EtOH]_0}{0.51\ [f \cdot (Biomass) \cdot 1.111]} \times 100$$

Where: $[EtOH]_f$ is the ethanol concentration at the end of the fermentation (g/L), $[EtOH]_0$ is the ethanol concentration at the beginning of the fermentation (g/L) which should be zero, 0.51 is the conversion factor for glucose to ethanol based on stoichiometric biochemistry of yeast, f is the glucan fraction of dry biomass (g/g), $(Biomass)$ is the dry biomass concentration at the beginning of the fermentation (g/L), and 1.111 is the stoichiometric factor that converts glucan to equivalent glucose (Aguilar et al., 2018).

2.6 Analytical methodology

Glucose, galactose, fucose, formic acid, acetic acid, HMF, furfural and bioethanol were analyzed via High-Performance Liquid Chromatography (HPLC) using an Agilent 1260 Infinity II Perkin Elmer(USA) equipped with a refractive index detector and a MetaCarb 87H column (300 mm x 7.8 mm, Agilent). The mobile phase was 5 mM H_2SO_4 set at a flow rate of 0.7 mL/min at 60 °C and the injection volume was 20 µL. Authentic standards of each compound of interest over a concentration range of 0 – 10 g/L were

employed to generate calibration curves and were used for reference and quantification (Pino et al., 2018). Prior to HPLC analysis, all samples were filtered through a 0.45 μm nylon filter.

3. Results and discussion

3.1 Physicochemical characterization of *Sargassum* biomass

The chemical composition (g/100 g Sargassum (d.w)) of the dried *Sargassum* spp-macroalgal biomass was: glucan (10.40 ± 0.96), galactan (4.34 ± 0.39), fucoidan (6.77 ± 0.69), acid insoluble residue (26.46 ± 0.31), sulphates (4.93 ± 0.24), Fat (0.33 ± 0.001), crude fiber (20.60 ± 0.75), protein (8.34 ± 0.39), ash (20.27 ± 0.44). Polysaccharides in the raw material were analyzed regarding glucan (as laminarin), galactan and fucoidan, and represented 10.40, 4.34 and 6.77 g/100 g d.w. respectively. The results in terms of carbohydrates are similar to Sargassum muticum (10.18 g/100 g d.w. of glucan, 2.69 of galactan, and 6.00 of fucoidan), in addition to *Himanthalia elongata* (15.69 g/100 g d.w. of total carbohydrates) (Cernadas et al., 2019; del Río et al., 2019). The polysaccharide values of *Sargassum* biomass from this study, however, are lower than those of *Sargassum horridum* (50.64 g/100 g d.w. of total carbohydrates) and *Sargassum* spp. (41.81 g/100 g d.w.) (Borines et al., 2013; Filipo-Herrera et al., 2018). This is most likely due to the fact that not all brown macroalgal polysaccharides and sugar alcohols, such as mannitol, laminarin, mannan and alginate, were compatible for quantification using the analytical technology used in this study. The ash content is comparable with the reported by del Río et al. (2019) and Sukwong et al. (2019) with values representing 11.87 and 12.43 g/100 g d.w. for *Sargassum* spp and *Gracilaria verrucosa*, respectively. The protein content is sightly lower than reported by Sukwong et al. (2019) (9.43 g/100 g d.w. for *Gracilaria*

verrucosa) and Cernadas et al. (2019) (9.98 g/100 g d.w. for *Himanthalia elongata*. Sulphate content is also similar to the reported by Cernada et al. (2019) with 3.60 g/100 g d.w. and the lipid content is in accordance with Filippo-Herrera et al. (2018) with 0.28 g/100 g d.w. for *Sargassum* horridum, and Borines at al. (2013) with 0.75 g/100 g d.w. for *Sargassum* spp. Such similarities and differences in the biochemical composition has been related to the seasonal variation, geographic location and climatic conditions (Vassilev and Vassileva et al., 2016). Those variations can be caused by seasonality in their reproductive activity. Additionally, the seasonality of the epifauna can further be associated with the increase and reduction of the macrophytes and to the variation in epiphytic coverage in macroalgae (Leite and Turra, 2003). Macrophytes could affect nutrient cycling through the transference of chemical elements from sediment to water, retention of solids and nutrients by their submerged roots and leaves, and decreasing nutrients released from sediment by protection against the wind (and wave) action.

3.2 High-pressure pretreatment of *Sargassum* spp

Throughout this study, water was used as the reaction medium as the operational conditions such as temperature and residence time are critical factors that affect the efficiency of the pretreatment. Hydrothermal pretreatment possesses great attributes, including the sole use of hot water or steam, without the requirements of any additional solvents. Technoeconomic models, which have been generated to compare different pretreatments, have revealed that hydrothermal pretreatments require the lowest capital and the fact that fewer inhibitory compounds are produced from the process gives way to fewer environmental penalties, including lower greenhouse gas emissions and upgrade water-quality effects from biorefineries (Ruiz et al., 2013; 2017, 2020).

In this study, the severity factor was calculated to be between 3.71 – 4.23, in both temperature and time parameters of the pretreatment and offers a practical manner to compare the results with other reports using different parameters of temperature, time and acids. Severity factors calculated in this trial were found to be within the severity ranges previously described by Rodríguez-Jasso et al. (2013) who conducted hydrothermal pretreatment on *Fucus vesiculosus* biomass, as well as Cernadas et al. (2019) on *Himanthalia elongata* biomass. The Figure 2 shows temperature profiles for high-pressure pretreatment and operational conditions: severity factor.

*****Figure 2*****

Table 1 shows the composition of the liquid and solid phases of all pretreatment experiments conducted in this study. It is evident that the pH of the generated liquid phases decreased as the pretreatment severity increased. This is most likely due to the auto-ionization of water, resulting in the generation of hydronium ions liberated from the water used, and the SO_3H groups from fucoidan present in *Sargassum* biomass, which would also induce this phenomenon (Ruiz et al., 2013). Pino et al. (2019) reported that sugar degradation products, which acts as inhibitor agents during enzyme hydrolysis and fermentation, may be prevented by controlling the pH in the pretreatment processing. The pH variation in this study was between 6.03 and 6.80.

For the operational conditions of central point (170ºC, 30 min, 6.91 bar), the composition of the liquid phase was: galactose (0.16 ± 0.01 g/L), mannitol (0.22 ± 0.01 g/L), fucose (0.32 ± 0.009 g/L), and glucose was not detected. The degradation compounds that were detected in this study were formic (6.22 ± 0.22 g/L) and acetic acid (0.25 ± 0.01 g/L), with formic acid being present at higher levels. Acetic acid was detected at significantly lower levels, whilst no traces of furfural and HMF were identified.

Oligosaccharides were detected at slightly higher concentrations than the aforementioned monosaccharides, with gluco-oligosaccharides (GlO), galacto-oligosaccharides (GaO) and fuco-oligosaccahrides (FuO) being present between 0.00 – 2.45 g/L, 0.00 – 1.05 g/L and 0.00 – 1.64 g/L, respectively. The hydrothermal high-pressure pretreatment that had a severity factor of 3.84 generated a liquid phase containing the highest concentration of GlO, 2.45 g/L, across all pretreatments. A study by Del Rio et al. (2019) quantified similar levels of GlO (2.58 g/L) in the liquid phase that was generated after a pretreatment of 140 °C – in isothermal condition, however FuO detection was greater, ca. 10 g/L.

The solid yields (%) of the remaining residues generated after hydrothermal high-pressure pretreatment decreased as the severity of the pretreatment increased, ultimately enriching the glucan content which can then be hydrolyzed by enzymes into glucose. A severity factor of 4.23, which corresponded to the most severe pretreatment, generated a residue containing the greatest enrichment of glucan, 32.33 g/100 g d.w. The insoluble solid residue was the major component in the solid phase, which should mainly be comprised of ash and other non-quantifiable compounds.

Analysis of variance (ANOVA) was analyzed for the mathematical model fitting (shown below) where “T” is temperature and “t” is time.

$$\%\ Glucan = 1.01T + 6.61t - 2.15T^2 + 0.87t^2 + 4.56Tt + 20.20$$

The ANOVA indicated that the model obtained explains 95% of the results (R^2), which is in accordance with the adjustment determination coefficient R^2adj = 0.90. The glucan obtained is lower than the content achieved by Schultz-Jensen et al. (2013) who

employed hydrothermal treatments at 180, 190, and 200 °C and obtained glucan yields of 55, 60, and 64 g/100 d.w. from the macroalgae *Chaetomorpha linum*, respectively. However, the study that also analyzed plasma assisted treatment, obtained yields of 38, 36, and 38 g/100 d.w. of glucan for 20, 40, and 60 minutes of treatment. del Río et al. (2019) reached the maximum of 14.91 g/100 g d.w. of glucan through hydrothermal pretreatment on *Sargassum* spp. at 170 °C, which is much lower than the yield obtained in this research.

3.3 Enzyme hydrolysis of pretreated *Sargassum* biomass

The operational condition at 190 °C and 50 min pretreatment (severity factor: 4.23) was selected as the optimal pretreated biomass for enzymatic hydrolysis. Preliminary tests were then performed on pre-treated and non-pretreated Sg biomass using a 1 % (w/v) glucan loading rate (ca. 15 FPU/g glucan) of Cellic CTec 2, and Cellic CTec 2 in combination with Cellic HTec 2 at a ratio of 1:2. A maximum concentration of only 0.86 g/L of glucose was liberated from the non-pretreated Sg biomass when enzymatically hydrolyzed with either Cellic CTec 2 or Cellic CTec 2 and Cellic HTec 2 (Figure 3a). On the contrary, it appeared that glucose concentrations significantly increased with the use of Cellic CTec 2, and Cellic CTec 2 and Cellic HTec 2, on Sg biomass that had been pretreated at 190 °C for 50 min, with concentrations of 4.17 g/L and 9.69 g/L being obtained, respectively. This ultimately indicates that pretreatment makes the glucan fraction more accessible to enzymes for hydrolysis to glucose. Additionally, the combination of the two enzyme cocktails resulted in a 57 % higher yield of liberated glucose from the pretreated Sg biomass, compared to the use of Cellic CTec 2 alone (Figure 3b).

Figure 3a and b

Pengilly et al. (2015) reported that is difficult to estimate the real synergistic effects between the two enzymatic complexes, however accessory enzymes, such as the glycoside hydrolases family and oxidative enzymes which are present in Cellic CTec2, help to improve glucan conversion into glucose, as well as the cellulases present in the Cellic HTec2 cocktail. Moreover, the combination of enzymes increases fiber porosity, resulting in an increment of shorter fibers and fiber swelling, and this increases the available surface area for cellulases to act upon. It is worth to mention that no studies with specialized enzymes for macroalgae have been reported, therefore, comparisons have mainly been made between lignocellulosic biomass for ethanol production, where the main polysaccharide used in enzymatic hydrolysis process is glucan as cellulose. Romaní et al. (2014) mentioned that the supplement of hemicellulases can improve the enzymatic hydrolysis yields in second generation biomasses, even though, the low quantity of xylan in the pretreated solid, xylanases increases the accessibility of cellulose eliminating the hemicellulose redeposited on the solid. Blends of enzymatic complexes can also improve the glucose concentration and yields, such as in the works described in Yang et al. (2017) who utilized pretreated empty fruit bunches as substrate and Cellic CTe2, Cellic HTec2, and polyethylene glycol (PEG) as enzyme blend. Glucose yields increased from 48.3 % (Cellic CTec2 only) to 72.5 % (Cellic CTec2, Cellic HTec2, and PEG) at 21.7 % of pretreated solids w/v, and at 16.3 % of pretreated solids, they enhanced from 52.8 (Cellic CTec2 only) to 75.4 % (Cellic CTe2, Cellic HTec2, and PEG). They suggested that PEG stabilizes and activates Cellic CTec2 and HTec2, and also affects structure and viscosity, thus increasing the interaction between enzymes and biomass. The study concludes that the addition of accessory agents like Cellic HTec2 and PEG is effective to improve the efficacy in enzymatic hydrolysis using commercial cellulase. Cho et al. (2013) testing *Entermorpha*

intestinalis, using Celluclast 1.5 L and Viscozyme L, when the enzymatic complexes was mixed, they found higher efficiency than the use of one cocktail with 73% of the theoretical yield being reached.

The highest saccharification yields resulted from a low pretreated solid loading (4 % (w/v)) with a high enzyme loading rate (16 FPU/g glucan, Cellic CTec 2 and Cellic HTec 2 at a ratio of 1:2). Such conditions, an excess amount of enzyme in the presence of a low pretreated Sg biomass loading rate, enabled the efficient depolymerization of all the glucan that is present in the Sg biomass. The ANOVA was analyzed for the mathematical model fitting (shown below) where "P" is the pretreated solid loading and "E" is enzyme loading.

$$\%\, Saccharification\ yield = 7.56E - 3.29P - 2.22E^2 + 0.81p^2 - 2.12EP + 91.22$$

The ANOVA indicated that the model obtained explains 90% of the results (R^2), which is in accordance with the adjustment determination coefficient R^2adj = 0.81.

Figure 4 shows the concentration of glucose that was liberated using Cellic CTec2 and Cellic HTec2 (at a ratio of 1:2) with varying FPU levels and solids loading after 72 hours on pretreated Sg biomass. The highest levels of glucose, 43.07 g/L, 42.65 g/L and 36.57 g/L, were liberated from solids loading content conditions of 13 % (w/v), but with differing FPU levels of 15, 10 and 5, respectively. Such values correspond to conversion yields of 92.12, 91.33, 78.32 %.

Pino et al. (2019) studied the enzymatic hydrolysis of pretreated solids from agave bagasse, using Cellic CTec2, and reported glucose yields of 40 and 70 g/L (corresponding to 80 % conversion yield) when using conditions of 10 and 15% (w/v) solids loading at 15 FPU, respectively.

The differences are attributed to the type of biomass used, where the glucan in pretreated lignocellulosic biomass was 46.46 %, compared to 32.33 % in the *Sargassum* used in this study. With regard to the conversion yield, it is clear that hydrothermal pretreatment aids the enzymatic susceptibility of *Sargassum* and the conversion yield, which may be attributed to the macroalgae cell wall that does not contain any lignin. Aguilar et al. (2018) studied the pretreatment of lignocellulosic biomass and found that 78.0 g/L of glucose was liberated, with a conversion yield of 98%, when a 10% (w/v) of pretreated solids loading content was used with 20 FPU/g of pretreated solid of cellulase loading. This conversion yield could be attributed to the high amount of enzyme used in the study, mainly because there was an excessive quantity of enzyme used for the amount of substrate in the medium, and the high concentration of glucose is caused by the 65.87% of glucan present in the residue after the pretreatment. del Río et al. (2019) pretreated *Sargassum muticum* where 6.01 g/L of glucose was liberated with a conversion yield of 94%, when using 14.3 kg/100 total weight of pretreated solid loading and 20 FPU of enzyme loading. The concentration and yield of glucose are similar to the obtained in the preliminary test undertaken in this study, where a low solid loading content but high enzyme FPU was used.

Figure 4

3.4 Fermentation of pretreated Sargassum biomass

3.4.1 Pre-simultaneous saccharification and fermentation strategy

Fermentation was performed under the pre-simultaneous saccharification and fermentation (PSSF) strategy aiming to maximize bioethanol production using optimal conditions obtained from the previous stages of pretreatment and enzymatic hydrolysis;

190°C – 50 minutes, and 10 FPU/g of glucan and 13% (w/v) of pretreated solids, respectively. According to Gonçalves et al. (2016), the PSSF compared to other strategies for bioethanol production, such as separate hydrolysis and fermentation, and simultaneous saccharification and fermentation, has higher productivity and conversion rates, as long as the pre-saccharification time improve the conversion yields. Romaní et al. (2016) mentioned that the PSSF strategy it is suitable for pretreated solids loading contents, as the viscosity of the slurry during the pre-saccharification stage becomes significantly reduced, therefore improving the mass transfer in the conversion to bioethanol.

As seen in Figure 5, the concentration of glucose after 24 hours is 45.66 ± 0.75 g/L with a yield of 97.78 ± 1.62 %. After 12 hours of fermentation, the glucose had been entirely consumed and 18.14 ± 1.11 g/L of bioethanol was produced, with a conversion yield of 76.23 ± 4.68%. The concentration of glucose that was achieved during the PSSF is clearly higher than the glucose yields obtained from the separate enzymatic hydrolysis stage (39.52 g/L at 24 h). This could be attributed to a number of reasons, which include an improvement in the heterogeneity of the enzymatic reactants such as the enzymes, water, buffer, in addition to the pretreated biomass at the beginning of the enzymatic hydrolysis and during the first hours of the pre-saccharification stage. The insufficient contact between reactants due to the random distribution of the material in the flask lead to a disadvantageous mass transfer. A premix could offer more effective contact amongst the enzymes and substrate at a high solid loading content and robust reactors that are suitable for efficient mixing of biomass slurries are needed to mitigate such mass transfer drawbacks (Pino et al., 2018, 2019). For example, Pino et al. (2019) used a horizontal reactor that was designed for enzymatic hydrolysis at high solids loading on second generation biomass which enhanced mixing effectiveness. Although it appeared that all the

glucose has been consumed by 12 hours of fermentation, the bioethanol yield was only 76 %; making it unclear whether the maximum yield of bioethanol had peaked earlier.

On the contrary, in the works of Tan and Lee (2016) who used a PSSF strategy obtained a bioethanol yield of 92.7% in less than 3.5 hours as well as del Río et al. (2019) who likewise acquired an 81% bioethanol yield in 8.5 hours following SSF strategy.

Figure 5

After 36 hours of fermentation, it appeared that the concentration of bioethanol decreased, and a final concentration of 10.39 ± 1.17 g/L was obtained. This may due to ethanol evaporation or the formation of alternative by-products, by the yeast, which are not quantifiable by the analytical systems used in this study. Furthermore, it may be possible that the yeast started to consume the bioethanol it had produced, as studies have suggested that bioethanol producing yeast strains have the ability to assimilate bioethanol when favorable carbon sources such as glucose become depleted (Kostas et al., 2020).

The concentration and conversion yields obtained in this study are similar to yields reported in other studies. Titres obtained in this study are higher than those achieved by del Río et al. (2019), who achieved bioethanol yields of 12.23 and 14.10 g/L when *Sargassum muticum* and *Saccharomyces cerevisiae* PE-2 were utilized in a SSF strategy, corresponding to a conversion yield of 81%. Our results are similar to Hou et al. (2015), who worked under two fermentation strategies: SSF and SHF on *Laminaria digitata* as substrate, using a commercial yeast *S. cerevisiae*, they produced 14.7 and 20.7 g/L ethanol (corresponding to 50.5 and 70.6 % of conversion yield) using SSF and SHF, respectively. They attributed the lower ethanol yield to the efficiency during the enzymatic hydrolysis stage. Lee et al. (2013) produced 6.65 g/L of ethanol using a thermotolerant *S. cerevisiae* DK 410362 under SSF at 3-6% (w/v) of solid loading. In another study, Kim et al. (2015)

applied SSF and SHF strategy with the strain *S. cerevisiae* KCTC 7906 to ferment autoclave-treated solids of *Gelidium amansii* reporting 3.33 and 3.78 g/L of ethanol concentration, when 2% (w/v) of pretreated solids were used under SHF and SSF strategy respectively, these results correspond to 74.3 and 84.9% of conversion ethanol yield. The authors also investigated SSF using 15% (w/v) of pretreated solids and they produced 25.70 g/L of ethanol concentration corresponding to 76.9% of conversion ethanol yield.

4. Conclusions

This study described the development of a process using *Sargassum* spp as substrate after high-pressure pretreatment, and its promising ability to serve as a feedstock for bioethanol production. Under PSSF strategy, final bioethanol concentration of 18.14 g/L was achieved (76% of theoretical yield). The results from this study suggest that *Sargassum* spp biomass, which can be easily obtained from coastal beaches, is a competitive future feedstock to produce biofuels as bioethanol under a biorefinery concept and bioeconomy, also it is necessary to look for strategies of process scaling-up in the near future.

E-supplementary data for this work can be found in e-version of this paper online

Acknowledgments

This work was financially supported by the Innovation Incentive Program (PEI)—Mexican Science and Technology Council (SEP-CONACYT) with the Project (Ref. PEI-251186), titled: Study of the variation temporary space of Fucoidan from *Sargassum* SP. Elizabeth Aparicio Velazco is grateful to the National Council for Science and Technology (CONACYT, Mexico) for supporting her Master Fellowship (grant number: 924192).

References

1. Adney, B., Baker, J., 1996. Measurement of Cellulase Activities. NERL/TP-510-42628. National Renewable Energy Laboratory, Golden, CO.
2. Aguilar-Reynosa, A., Romaní, A., Rodríguez-Jasso, R.M., Aguilar, C.N., Garrote, G., Ruiz, H.A., 2017a. Microwave heating processing as alternative of pretreatment in second-generation biorefinery: An overview. Energy Convers. Manag., 136:50-65.
3. Aguilar-Reynosa, A., Romaní, A., Rodríguez-Jasso, R.M., Aguilar, C.N., Garrote, G., Ruiz, H.A., 2017b. Comparison of microwave and conduction-convection heating autohydrolysis pretreatment for bioethanol production. Bioresour. Technol. 243:273–83.
4. Aguilar, D.L., Rodríguez-Jasso, R.M., Zanuso, E., Rodríguez, D., Amaya-delgado, L., Sanchez, A., Ruiz, H.A. 2018. Scale-up and evaluation of hydrothermal pretreatment in isothermal and non-isothermal regimen for bioethanol production using Agave bagasse. Bioresour. Technol. 112-119.
5. Aparicio, E., Rodríguez-Jasso, R., Lara, A.B., Loredo-Treviño, A., Aguilar, C.N., Kostas, E.T., Ruiz, H.A., 2020. Biofuels production of third generation biorefinery from macroalgal biomass in the Mexican context: An overview, in: Dolores, M.T., Kraan, S., Dominguez, H. (Eds.), Sustainable seaweed technologies cultivation, biorefinery, and applications. Amsterdam, Netherlands, Elsevier, pp. 393-446.
6. Borines, M.G., de Leon, R.L., Cuello, J.L., 2013. Bioethanol production from the macroalgae *Sargassum* spp. Bioresour. Technol. 138, 22-29.
7. Cernadas, H., Flóres-Fernández, N., González-Muñoz, M.J., Domínguez,H., Torres, M.D., 2019. Retrieving of high-value biomolecules from edible *Himanthalia elongata* brown seaweed using hydrothermal processing. Food Bioprod. Process. 117, 275-286.
8. Cho, Y., Kim, M.J., Kim,S.K., 2013. Ethanol production from seaweed, *Enteromorpha intestinalis*, by separate hydrolysis and fermentation (SHF) and simultaneous saccharification and fermentation (SSF) with *Saccharomyces cerevisiae*. Ksbb Journal. 28, 366-371.
9. Chornet, E., Overend, R.P., 2017. How the severity factor in biomass hydrolysis came about. In: Ruiz, H.A., Thomsen, M.H., Trajano, H.L. (Eds.). Hydrothermal processing in biorefineries. Springer Cham, p 1–3.
10. Del Río, P.G., Domínguez, E., Domínguez, V.D., Romaní, A., Domingues, L., Garrote, G., 2019. Third generation bioethanol from invasive macroalgae *Sargassum muticum* using autohydrolysis pretreatment as first step of a biorefinery. Renew. Energy. 2019, 728-735.
11. Dodgson, K.S., 1961. Determination of inorganic sulphate in studies on the enzymic and non-enzymic hydrolysis of carbohydrate and other sulphate esters. Biochem J.78:312–9.
12. Dowe, N., McMillan, J., 2001. SSF Experimental Protocols-Lignocellulosic Biomass Hydrolysis and Fermentation. National Renewable Energy Laboratory Golden, CO. NERL/TP-510-42630.
13. Filipo-Herrera, D.A., Hernández-Carmona, G., Muñoz-Ochoa, M., 2018. Monthly variation in the chemical composition and biological activity of *Sargassum horridum.* Bot. Mar. 61, 91-102.
14. Helrich, K., Neal.,M.J.E., eds. 1990. Association of Official Analytical Chemists. In Official Methods of Analysis. Washington, DC.

15. Hou, X., Hansen, J.H., Bjerre, A.B., 2015. Integrated bioethanol and protein production from brown seaweed *Laminaria digitata.* Bioresour. Technol. 197, 310-317.
16. Huffard, C.L., Thun, S.V., Sherman, A.D., Saeley, K., Smith, K.L., 2014. Pelagic *Sargassum community* change over a 40-year period: temporal and spatial variability. Mar. Biol. 161, 2735-2751.
17. Jung, K.A., Lim, S-R., Kim, Y., Park, J.M., 2013. Potential of macroalgae as feedtocks for biorefinery. Bioresour. Technol. 135, 182-190.
18. Kim, H.M., Wi, S.G., Jung, S., Song, Y., Bae, H.J., 2015. Efficient approach for bioethanol production from red seaweed *Gelidium amansii*. Bioresour. Technol. 175,128-134.
19. Konda, N.V.S.M., Singh, S., Simmons, B.A., Klein-Marcuschamer, D., 2015. An Investigation on the economic feasibility of macroalgae as a potential feedstock for Biorefineries. Bioenerg. Res. 8, 1046-1056.
20. Kostas, E.T., White, D.A., Cook, D.J., 2017. Development of a bio-refinery process for the production of speciality chemical, biofuel and bioactive compounds from *Laminaria digitata.* Algal Res. 28, 211-219.
21. Kostas, E.T., White, D.A., Cook, D.J., 2020. Bioethanol production from UK seaweeds: investigating variable pre-treatment and enzyme hydrolysis parameters. Bioenerg. Res. 13, 271–285.
22. Lara, A., Rodríguez-Jasso, R.M., Loredo-Treviño, A., Aguilar, C.N., Meyer, A.S., Ruiz, H.A., 2020. Enzymes in the third generation biorefinery for macroalgae biomass, in: Singh. S.P., Singhania, R.R., Li, Z., Pandey, A., Larroche, C. (Eds.), Biomass, Biofuels, Biochemicals. Advances in Enzyme Catalysis and Technologies. Elsevier, pp. 363–396.
23. Leite, F. P. P., Turra, A., 2003. Temporal variation in *Sargassum* biomass, *Hypnea* epiphytism and associated fauna. Braz. Arch. Biol. Technol. 46, 665-671.
24. Lee, J.Y., Li, P., Lee, J., Ryu, H.J., Oh, K,K., 2013. Ethanol production from *Saccharina japonica* using an optimized extremely low acid pretreatment followed by simultaneous saccharification and fermentation. Bioresour. Technol. 127, 119-125.
25. López-Sandin, I., Zavala-García, F., Levin, L., Ruiz, H.A., Hernández-Luna., C.E., Gutiérrez-soto, G., (2021). Evaluation of bioethanol production from sweet sorghum variety Roger under different tillage and fertilizer treatments. Bioenerg. Res. In press. https://doi.org/10.1007/s12155-020-10215-7
26. Maneein, S., Milledge, J.J., Nielsen, B.V., Harvey, P., 2018. A review of seaweed pre-treatment methods for enhanced biofuel production by anaerobic digestion of fermentation. Fermentation. 4,100.
27. Pengilly, C., García-Aparicio, M.P., Diedericks, D., Brienzo, M., Gorgens, J.F., 2015. Enzymatic hydrolysis of steam-pretreated sweet sorghum bagasse by combinations of cellulase and endo-xylanase. Fuel. 154,352-360.
28. Pereira, F.B., Romaní, A., Ruiz, H.A., Teixeira, J.A., Domingues, L., 2014. Industrial robust yeast isolates with great potential for fermentation of lignocellulosic biomass. Bioresour. Technol. 161,192-199.
29. Pinales-Márquez, C., Rodríguez-Jasso R.M., Araújo, R.G., Loredo-Treviño, A., Nabarlatz, D., Gullón, B., Ruiz, H.A, (2020). Circular bioeconomy and integrated biorefinery in the production of xylooligosaccharides from lignocellulosic biomass: A review. Ind. Crops. Prod. 162, 113274.

30. Pino, M.S., Rodríguez-Jasso R.M., Michelin, M., Flores-Gallegos, A.C., Morales-Rodríguez, R., Teixeira, J.A., Ruiz, H.A., (2018). Bioreactor design for enzymatic hydrolysis of biomass under the biorefinery concept. Chem. Eng. J. 347,119-136.
31. Pino, M.S., Rodríguez-Jasso, R.M., Michelin, M., Ruiz, H.A., 2019. Enhancement and modeling of enzymatic hydrolysis on cellulose from agave bagasse hydrothermally pretreated in a horizontal bioreactor. Carbohydr. Polym. 211, 349-359.
32. Rodríguez-Jasso, R.M., Mussato, S.I., Pastrana, L., Aguilar, C.N., Teixeira, J.A., 2013. Extraction of sulfated polysaccharides by autohydrolysis of brown seaweed *Fucus vesiculosus*. J. Appl. Phycol. 25, 31-39.
33. Romaní, A., Ruiz, H.A., Pereira, F.B., Domingues, L., Teixeira, J.A, 2014. Effect of hemicellulose liquid phase on the enzymatic hydrolysis of autohydrolyzed *Eucalyptus globulus* Wood. Biomass Conv. Bioref. 4, 77-86.
34. Romaní, A., Ruiz, H.A., Teixeira, J.A., Domingues, L., 2016. Valorization of Eucalyptus wood by glycerol-organosolv pretreatment within the biorefinery concept: An integrated and intensified approach. Renew. Energy. 95, 1-9.
35. Ruiz, H.A., Conrad, M., Sun, S.N., Sanchez, A., Rocha, G.J.M., Romaní, A., Castro, E., Torres, A., Rodríguez-Jasso, R.M., Andrade, L., Smirnova, I., Sun, R.C., Meyer, A.S., 2020. Engineering aspects of hydrothermal pretreatment: From batch to continuous operation, scale-up and pilot reactor under biorefinery concept. Bioresour. Technol. 299, 122685.
36. Ruiz, H.A., Rodríguez-Jasso, R.M., Aguedo, M., Kádar, Z., 2015. Hydrothermal pretreatments of macroalgal biomass for Biorefineries. In: Prokop, A., Bajpai, R.K., Zappi, M.E., (Eds.) Algal biorefineries: Products and refinery design. Springer Cham, pp. 467-491.
37. Ruiz, H.A., Rodríguez-Jasso, R.M., Fernandes, B.D., Vicente, A.A., Teixeira, J.A., 2013. Hydrothermal processing, as an alternative for upgrading agricultural residues and marine biomass according to the biorefinery concept: A review. Renew. Sust. Energ. Rev. 21, 35-51.
38. Ruiz, H.A., Thomsen, M.H., Trajano, H.L., 2017. Hydrothermal processing in Biorefineries. Springer International Publishing., Cham, Switzerland.
39. Schell, J.M., D.S. Goodwin, and A.N.S. Siuda., 2015. Recent *Sargassum* inundation
40. Schultz-Jensen, N., Thygesen, A., Leipold, F., Thomsen, S.T., Roslander, C., Liholt, H., Bjerre, A.B., 2013. Pretreatment of the macroalgae *Chaetomorpha linum* for the production of bioethanol – Comparison of five pretreatment technologies. Bioresour. Technol. 140, 36-42.
41. Siller-Sánchez, A., Ruiz, H.A., Aguilar, C.N., Rodríguez-Jasso, R.M., 2018. Biorefinery Approach for Red Seaweeds Biomass as Source for Enzymes Production: Food and Biofuels Industry. In: Parameswaran, B., Varjani, S., Raveendran, S., (Eds.) Green bio-processes. Springer, Singapore, pp. 431-446.
42. Sluiter, A., Hames, R., Ruiz, R., Scarlata, C., Sluiter, J., Templeton, D., 2008a. Determination of Ash in Biomass – NREL/TP-510-42622. NREL analytical procedure. National Renewable Energy Laboratory.
43. Sluiter, A., Hames, R., Ruiz, R., Templeton, D., Crocker, D., 2008b. Determination of structural Carbohydrates and lignin in biomass - NREL/TP-510-42618. NREL analytical procedure. National Renewable Energy Laboratory.
44. Soleymani, M., Rosentrater, K.A., 2017. Techno-economic analysis of biofuel production from macroalgae (seaweed). Bioengineering. 4:92.

45. Sukwong, P., Sunwoo, I.Y., Lee, M.J., Ra, C.H., Jeong, G.T., Kim, S.K., 2019. Application of the severity factor and HMF removal of red macroalgae *Gracilaria verrucosa* to production of bioethanol by *Pichia stipitis* and *Kluyveromyces marxianus* with adaptive evolution. Appl. Biochem. Biotechnol. 187, 1312–1327.
46. Tan, I.S., Lam, M.K., Foo, H,C., Lim, S., Lee, K.T., 2020. Advances of macroalgae biomass for the third generation of bioethanol production. Chinese. J. Chem. Eng. 28, 502-517.
47. Tan, I.S., Lee, K.T., 2016. Comparison of different process strategies for bioethanol production from *Eucheuma cottonii*: An economic study. Bioresour. Technol. 199, 336-346.
48. Vassilev, S.V., Vassileva, C.G., 2016. Composition, properties and challenges of algae biomass for biofuel application: An overview. Fuel. 181,1-33.
49. Wang, M., Hu, C., 2016. Mapping and quantifying *Sargassum* distribution and coverage in the Central West Atlantic using MODIS observations. Remote Sens. Environ. 183, 350-677.
50. Yang, J., Kim, J.E., Kim, H.E., Yu, J.H., Cha, Y.L., Kim, K.H., 2017. Enhanced enzymatic hydrolysis of hydrothermally pretreated empty fruit bunches at high solids loadings by the synergism of hemicellulase and polyethylene glicol. Process Biochem. 58, 211-216.

Figure captions

Figure 1. General flow diagram for ethanol production using *Sargassum* spp biomass fractiontion and high-pressure technology.

Figure 2. Heating-up and cooling profiles of each high-pressure pretreatment.

Figure 3. Enzymatic hydrolysis suceptibility kinetic for pretreated and unpretreated biomass using Cellic Ctec2 and Cellic HTe2 at 1% of glucan and 15 FPU´s of cellulase. a) Glucose concentration (g/L). b) Glucose yield (%).

Figure 4. Enzymatic hydrolysis at high solid loading of pretreated macroalgal biomass and low enzyme loading using Cellic CTec2 and Cellic HTec 2 (1:2 ratio).

Figure 5. Kinetic profiles for bioethanol production using pre-simultaneous saccharification and fermentation strategy and pretreated macroalgal biomass as susbtrate.

Figure 1

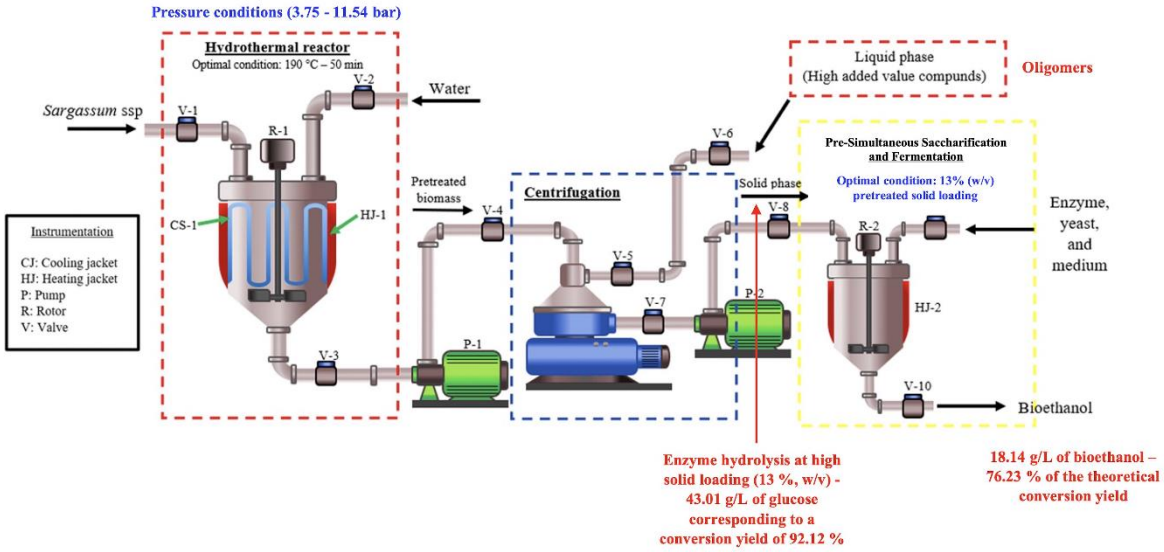

Pressure conditions (3.75 - 11.54 bar)
Hydrothermal reactor
Optimal condition: 190 °C – 50 min
Sargassum ssp
V-1
R-1
V-2
Water
CS-1
HJ-1
V-3
Instrumentation
CJ: Cooling jacket
HJ: Heating jacket
P: Pump
R: Rotor
V: Valve
Pretreated biomass
V-4
P-1
Centrifugation
V-5
V-6
Liquid phase
(High added value compunds)
Oligomers
V-7
P-2
Solid phase
V-8
Pre-Simultaneous Saccharification and Fermentation
Optimal condition: 13% (w/v) pretreated solid loading
R-2
HJ-2
Enzyme, yeast, and medium
V-10
Bioethanol
Enzyme hydrolysis at high solid loading (13 %, w/v) - 43.01 g/L of glucose corresponding to a conversion yield of 92.12 %
18.14 g/L of bioethanol – 76.23 % of the theoretical conversion yield

Figure 2

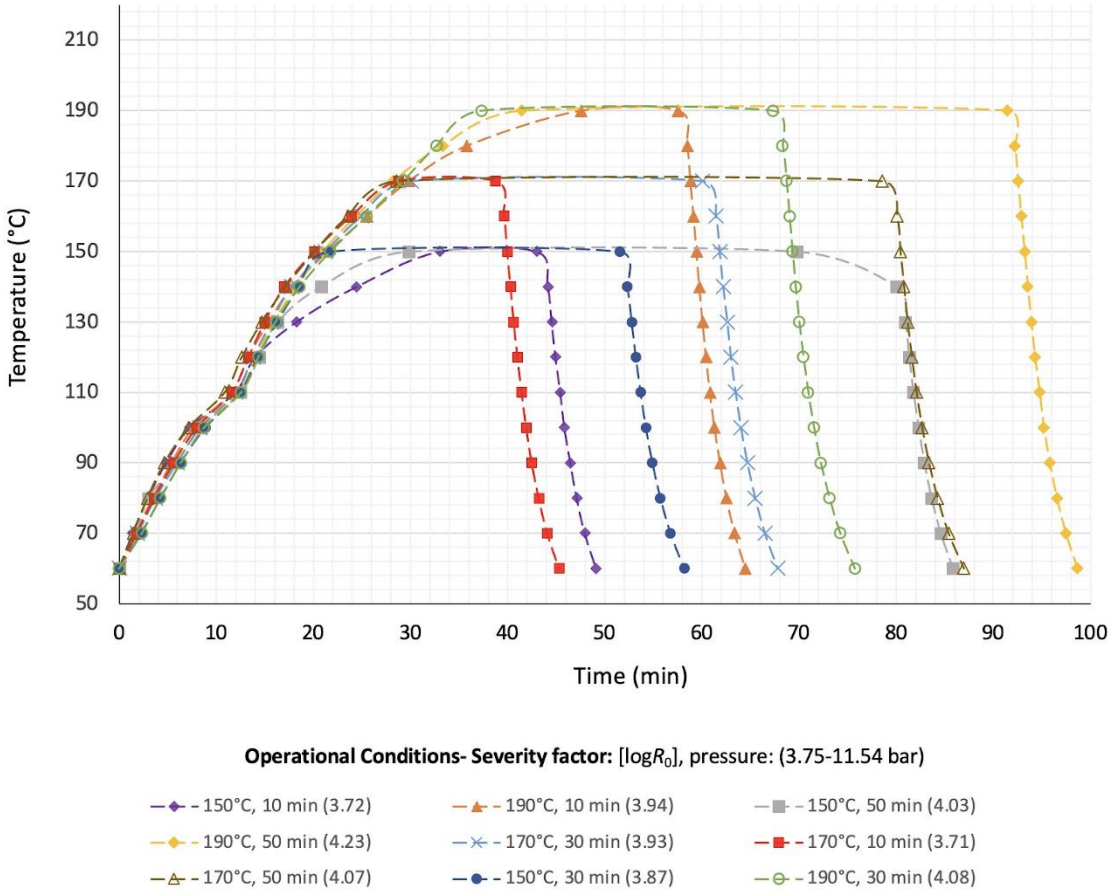

Temperature (°C)
210
190
170
150
130
110
90
70
50
0
10
20
30
40
50
60
70
80
90
100
Time (min)
Operational Conditions- Severity factor: [logR_0], pressure: (3.75-11.54 bar)
150°C, 10 min (3.72)
190°C, 10 min (3.94)
150°C, 50 min (4.03)
190°C, 50 min (4.23)
170°C, 30 min (3.93)
170°C, 10 min (3.71)
170°C, 50 min (4.07)
150°C, 30 min (3.87)
190°C, 30 min (4.08)

Figure 3 a

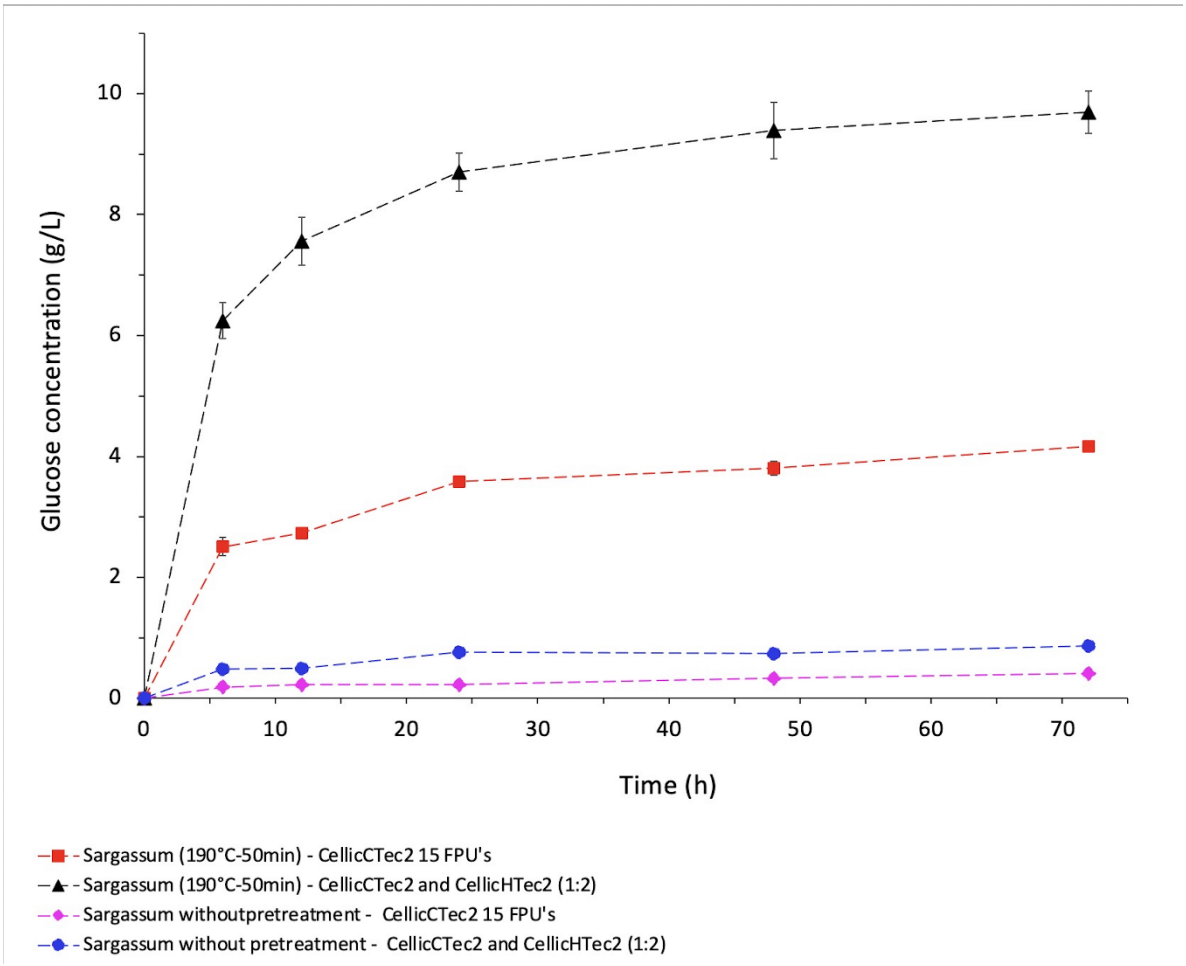

Glucose concentration (g/L)
10
8
6
4
2
0
0
10
20
30
40
50
60
70
Time (h)
Sargassum (190°C-50min) - CellicCTec2 15 FPU's
Sargassum (190°C-50min) - CellicCTec2 and CellicHTec2 (1:2)
Sargassum withoutpretreatment - CellicCTec2 15 FPU's
Sargassum without pretreatment - CellicCTec2 and CellicHTec2 (1:2)

Figure 3 b

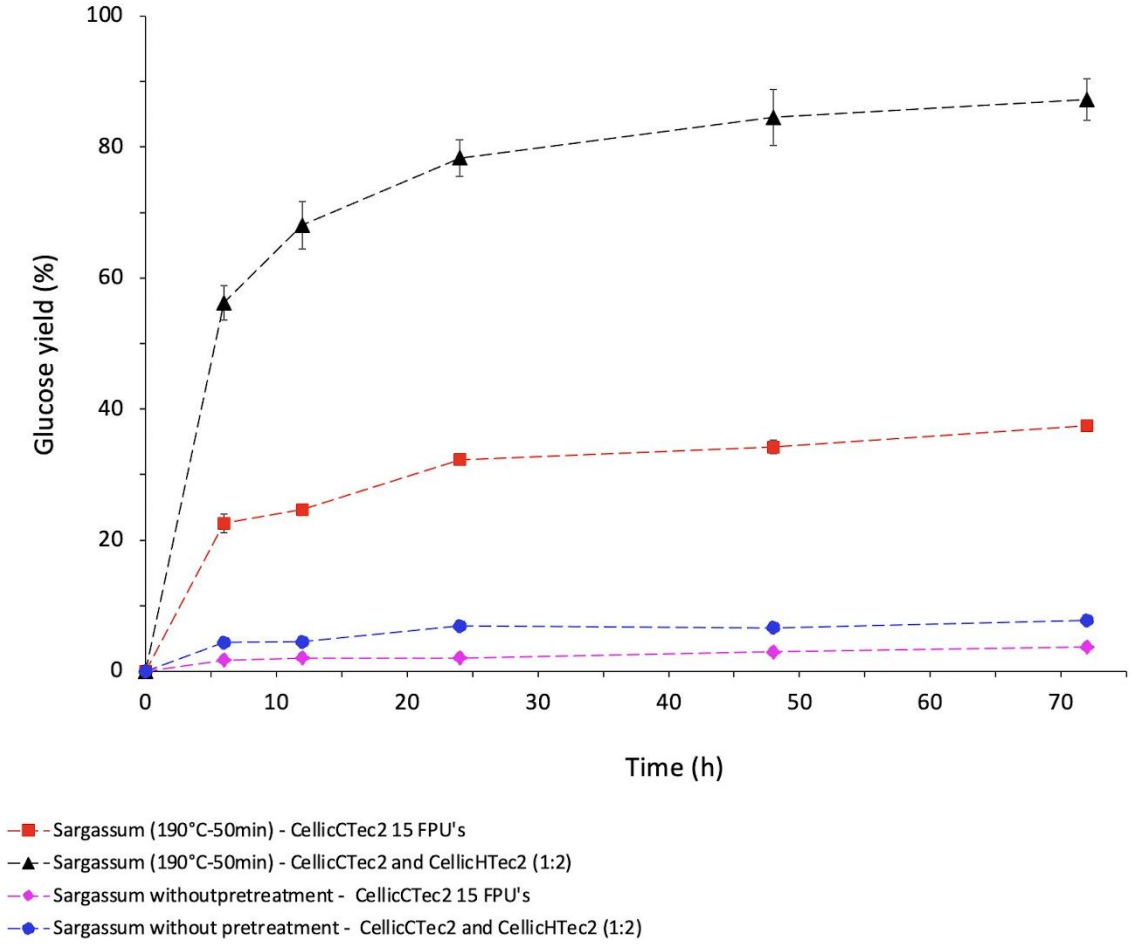

100
80
60
40
20
0
Glucose yield (%)
0
10
20
30
40
50
60
70
Time (h)
Sargassum (190°C-50min) - CellicCTec2 15 FPU's
Sargassum (190°C-50min) - CellicCTec2 and CellicHTec2 (1:2)
Sargassum withoutpretreatment - CellicCTec2 15 FPU's
Sargassum without pretreatment - CellicCTec2 and CellicHTec2 (1:2)

Figure 4

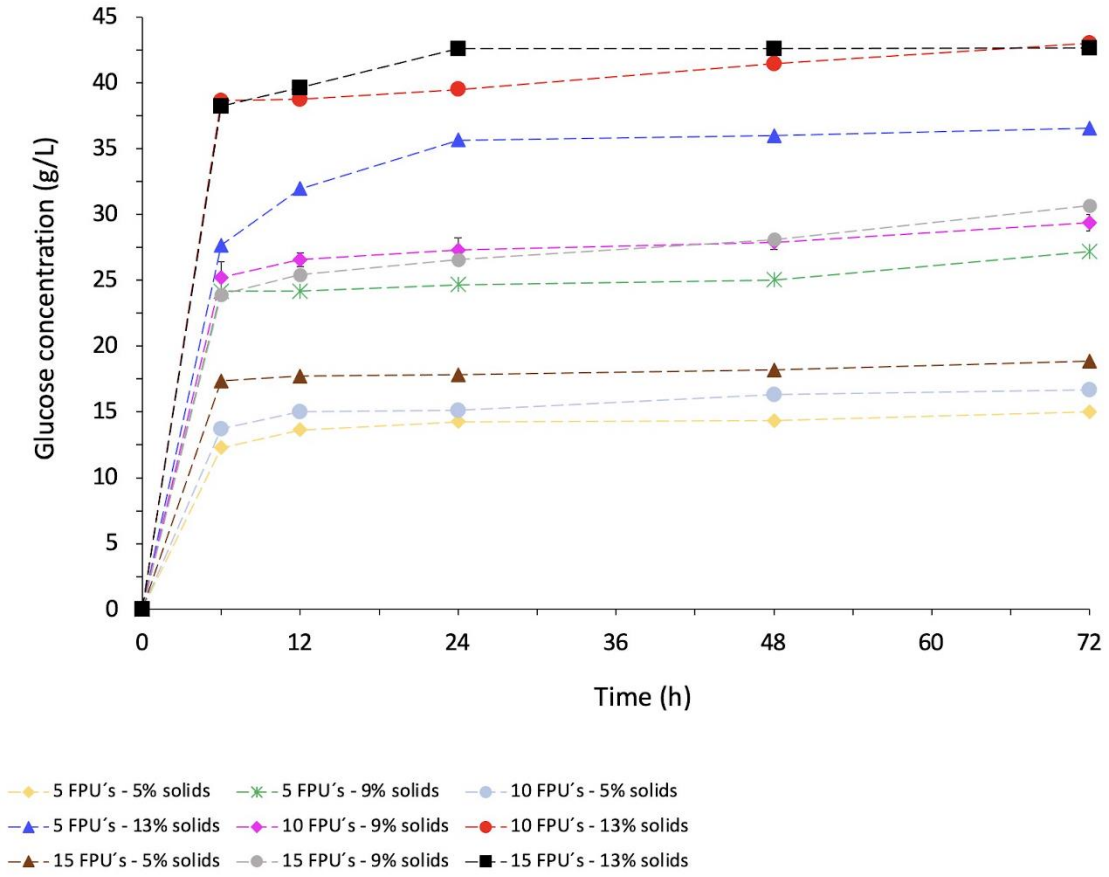
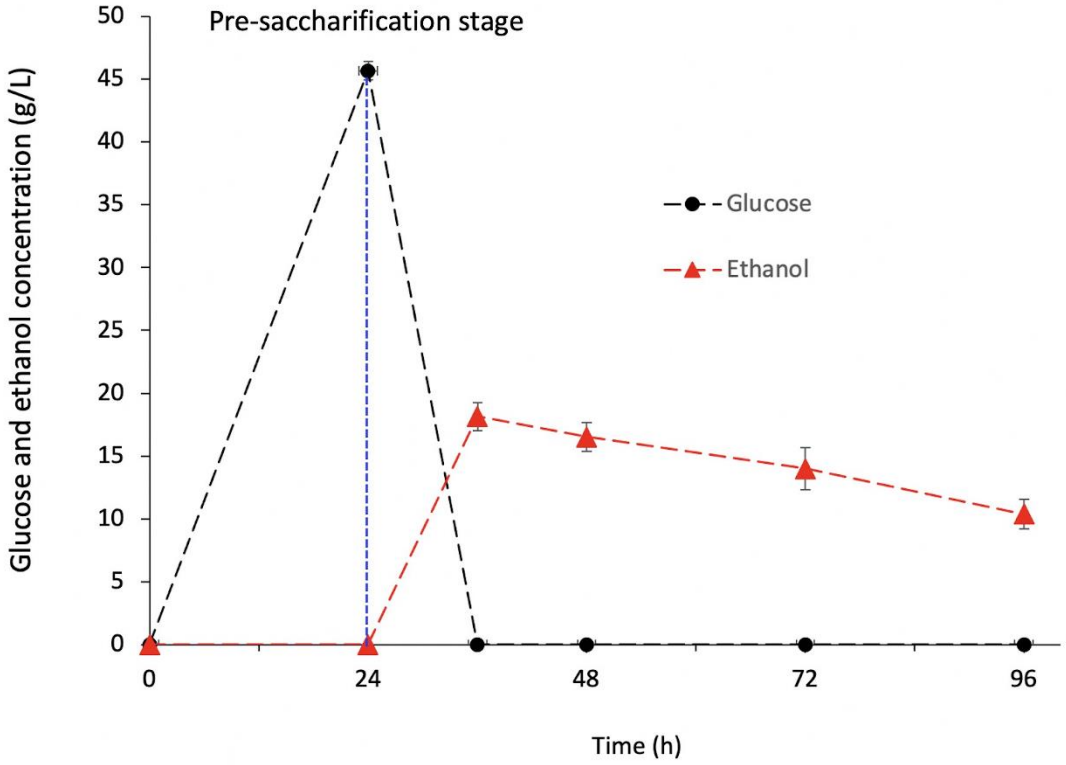

Glucose concentration (g/L)
45
40
35
30
25
20
15
10
5
0
0
12
24
36
48
60
72
Time (h)
5 FPU´s - 5% solids
5 FPU´s - 9% solids
10 FPU´s - 5% solids
5 FPU´s - 13% solids
10 FPU´s - 9% solids
10 FPU´s - 13% solids
15 FPU´s - 5% solids
15 FPU´s - 9% solids
15 FPU´s - 13% solids

Figure 5


Pre-saccharification stage
Glucose and ethanol concentration (g/L)
Time (h)
Glucose
Ethanol
0
5
10
15
20
25
30
35
40
45
50
0
24
48
72
96

Highlights

- The fractionation with high pressure of an invasive macroalgae is proposed.
- Hydrothermal pretreatment was effective pretreatment of *Sargassum* biomass.
- Cellulase and hemicellulase was employed for enzymatic hydrolysis on *Sargassum.*
- Theoretical bioethanol yield of 76.23% reached by PSSF strategy

E-supplementary data

High-pressure technology for *Sargassum* spp biomass pretreatment and fractionation in the third generation of bioethanol production

Aparicio E.[1], Rosa M. Rodríguez-Jasso[1], César D. Pinales-Márquez[1], Araceli Loredo-Treviño[1], Armando Robledo-Olivo[2], Cristóbal N. Aguilar[1], Emily T. Kostas[3], Héctor A. Ruiz[1*]

[1] Biorefinery Group, Food Research Department, Faculty of Chemistry Sciences, Autonomous University of Coahuila, 25280 Saltillo, Coahuila, Mexico.

[2] Food Science & Technology Department, Universidad Autónoma Agraria Antonio Narro, Unidad Saltillo, Blvd Antonio Narro 1923, Buenavista, 25315 Saltillo, Coahuila, Mexico.

[3] Department of Biochemical Engineering, The Advanced Centre of Biochemical Engineering, Bernard Katz Building, Gower Street, London WC1H 6BT, University College London, London, United Kingdom.

Table S1. Experimental design enzymatic hydrolysis of pretreated *Sargassum* spp: Experimental parameters.

Assay	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
Pretreated solid loading (%, w/v)	5	9	13	5	9	13	5	9	13
Enzyme loading (FPU/glucan)	5	5	5	10	10	10	15	15	15

Factor	Sum of squares	d.f	Mean square	F-value	*p*-value
T	6.13	1	6.13	1.63	0.25
t	262.21	1	262.21	69.70	0.0004*
T^2	11.66	1	11.66	3.10	0.13
t^2	1.93	1	1.93	0.51	0.51
Tt	83.01	1	83.01	22.07	0.005*
$Error$	18.81	5	18.81		
$Total$	382.07	10	382.07		
R^2	0.95				
R^2adj	0.90				

d.f., Degree of freedom
* Significant

Table s2. Analysis of variance for glucan concentration model as function of temperature (T) and time (t).

Table s3. Analysis of variance for saccharification yield (%) model as function of pretreated solids loading (P) and enzyme loading (E).

Factor	Sum of squares	d.f	Mean square	F-value	p-value
E	342.80	1	342.80	36.27	0.002*
p	65.08	1	65.08	6.88	0.05*
E^2	12.49	1	12.49	12.49	0.30
p^2	1.65	1	1.65	1.65	0.69
Ep	18.05	1	18.05	1.91	0.23
$Error$	47.25	5	47.25		
$Total$	485.80	10	485.80		
R^2	0.90				
R^2adj	0.81				

d.f., Degree of freedom

* Significant