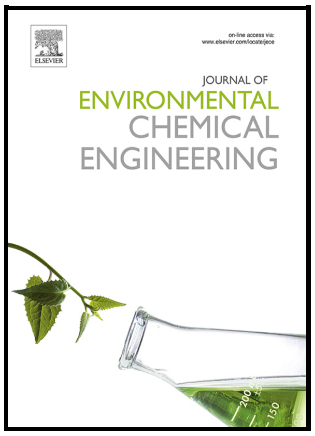

Yield evaluation of enzyme hydrolysis and dark fermentation of the brown seaweed *Rugulopteryx okamurae* hydrothermally pretreated by microwave irradiation

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Yield evaluation of enzyme hydrolysis and dark fermentation of the brown seaweed *Rugulopteryx okamurae* hydrothermally pretreated by microwave irradiation

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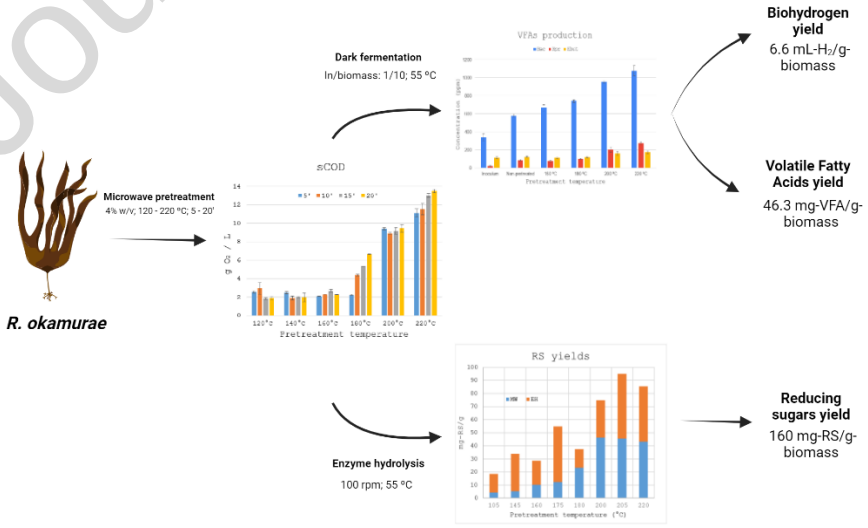
Abstract

Microvawe irradiation has been applied to solubilise the organic matter from the invasive seaweed *Rugulopterix okamurae* as pretreatment for two conventional biological processes: enzyme hydrolysis (EH) for obtaining reducing sugars (RS) and dark fermentation (DF) for obtaining volatile fatty acids (VFAs). As operational conditions for the microwave pretreatment, temperature and time has been tested between 120 - 220 °C and 5 - 20 min respectively. The maximum solubilisation yield in terms of DOC (26%) through the microwave pretreatment was registered at 220 °C at 20 min. However, the maximum concentration for RS (1.8 g/L) was obtained at 200 °C at 20 min due to the decomposition of monomeric sugars at high temperature and the total polyphenols (TP) were above 0.25 g/L (where inhibition in dark fermentation begins to be noticiable) at 180 °C. When the pretreated biomass was used for the EH the maximum accumulated RS production was 160 mg-RS/g-biomass, at 220 °C which was a 35 % better than the RS production for non-pretreated biomass. In the case of the DF tests, the maximum yield of VFAs obtained with the pretreated biomass was 46.3 mg/g-biomass at 220 °C while the maximum hydrogen yield was obtained at 200 °C (6.6 mL-H_2/g-biomass) due to the high level of TP (1.1 g/L) of pretreated biomass at 220 °C. Despite of this toxic effect, the VFAs production at 220 °C was 93% higher than in the non-pretreated biomass.

Graphical abstract



Keywords

Microwave pretreatment, hydrothermal pretreatment, dark fermentation, enzyme hydrolysis, brown seaweed.

1. Introduction

Invasive species cause major environmental, economic and social impacts (Mumford & Burgman, 2021; Yokomizo et al., 2009). In marine ecosystems, invasive species can change both the behavior of native species and their distribution, affecting the ecosystem biodiversity (Kernan, 2015; Susano et al., 2022; Tomas et al., 2011). One of the latest such episodes involves the sudden proliferation and detection in 2015 of the brown algae *Rugulopteryx okamurae,* which is native to the sea of Japan, in the Strait of Gibraltar in the south of Europe (Sempere-Valverde et al., 2021). Thus, along the southern Spanish coasts, more than 5,000 tons of *Rugulopteryx okamurae* were collected in just a few months (García-Gómez et al., 2020).

The massive growth of *R. okamurae* is due to its biological mechanisms to ensure its survival which are superior to those present in the endemic species of the area. Specifically, this brown seaweed is capable of producing and accumulating some bioactive compounds with defensive functions against its predators, which gives it a great spatial competitiveness and allows it to proliferate faster than competitors (Casal-Porras et al., 2021).

Many studies have been carried out concerning the idea of making useful seaweed biomass, such as using macroalgae to obtain pharmaceuticals (Rushdi et al., 2020), natural products (Rushdi et al., 2022), or even biofuels (Song et al., 2015). Unlike lignocellulosic terrestrial biomass, macroalgae have a high water content (70 - 90% w/w) and high mineral content (10 - 50% of dry weight) with hardly any lignin content (Ross et al., 2008). In addition, the high carbohydrate composition of some species makes them an interesting raw material for certain fermentation processes. However, their polysaccharide content varies according to the species which is critical in the biomass usefulness (Maneein et al., 2018). *R. okamurae* is classified as brown algae, this kind of seaweed contains 30 - 50% of carbohydrates on dry weight, of which 19% is alginate, while fucoidan and laminarin are both present at 11%. Celullose is also found at 8% (Jensen, 1993; Lorbeer et al., 2015; Ross et al., 2008; Turner, 1994)

In recent years, circular economy has been gaining importance through worldwide (European Commission, 2014; Zilia et al., 2021). In this strategy, in contrast to the conventional linear economy, the aim is to convert waste products into new raw materials for the productive sector. In this way, it is intended to avoid the depletion of natural resources (Wikurendra et al., 2022). For all of these reasons, a possible interesting way of using *R. okamurae* biomass is directing it to the bioplastics production. This process could involve in a preliminary step, obtaining sugars from the biomass by enzyme hydrolysis (EH), and then, using the appropriate fermentation process to obtain bioplastics precursors such as polyhydroxyalkanoates (Dietrich et al., 2019; Heng et al., 2016; Liu et al., 2021; Obruca et al., 2015). In another additional pathway, short volatile fatty acids can be obtained by dark fermentation (DF) and could also be used as raw substrate for producing polyhydroxyalkanoates (Colombo et al., 2019; Kumar et al., 2019; Patel et al., 2021; Sekoai et al., 2021).

In any case, it is always necessary to apply conditioning and pretreatment processes to the biomass for its subsequent EH or DF processes (Xia, Cheng, Lin, et al., 2013). The pretreatment causes an efficient rupture on the cellular wall of macroalgae, not only leaving more exposed carbohydrates for possible extraction or EH, but also, causing the breakdown of protein structures and degrading them to amino acids. Consequently, the combined presence of carbohydrates and amino acids in the pretreated biomass has huge interest in the subsequent fermentation processes (Sambusiti et al., 2015; Xia, Cheng, Lin, et al., 2013).

A detailed revision of the background literature shows that microwave irradiation has been used more as an assisted extraction technique than for a hydrothermal pretreatment in the case of seaweeds. Specifically, there are a low numbers of studies applied to brown algae (Lin et al., 2019; Yin et al., 2019), and none addressing the microwave assisted hydrothermal degradation of *Rugulopteryx okamurae*. Dark fermentation and enzyme hydrolysis processes has been applied to some brown seaweed as (de la Lama-Calvente et al., 2021).

In this context, the present work studies the application of different conditions in a microwave pretreatment of the *R. okamurae* biomass, and the evaluation of the subsequent EH or DF, depending on whether sugars or volatile fatty acids are wanted respectively for later purposes. Both kind of compounds are elemental bricks in the biorefinery concept (Kamm, 2004; Thomas, 2009) and can be the starting point of the profitable use of *R. okamurae.*

2. Material and methods

2.1 Algal biomass

Seaweed was collected in a beach in the south of Spain (Tarifa, 36°00'44.1"N 5°35'53.9"W), in June 2021. The samples were washed for 24 h to remove any remaining sand or salt. The washing process was carried out by continuous rinsing with tap water. After 24 h, the conductivity of the outlet water from macroalgae washing tanks was similar when compared to the inlet one, then the release of salts had been completed. After washing, the seaweed was dried by natural convection for 48 h in a greenhouse. Once at dried, the biomass was ground to a particle size of less than 1 mm. Then, the main characteristics of the algal biomass were studied. All these features are presented in Table 1.

Table 1: Main chemical features of the brown seaweed R. okamurae for a significance p<0.05. ADF: Acid Detergent Fibre; ADL: Acid Detergent Lignin; ILF: Insoluble Lignin Fraction; NDF: Neutral Detergent Fibre; AEF: Acetone Extractable Fraction. The percentage was expressed at dry basis.

Parameter	Units	Value
TS	g/kg	862 ± 1.06
VS	g/kg	746 ± 1.90
ADF	%	13.7 ± 1.34
ADL	%	13.6 ± 0.37
ILF	%	4.49 ± 0.60
NDF	%	64.2 ± 0.99
AEF	%	4.02 ± 0.29

ADF fraction corresponds to hemicellulose, fucoidan, laminarin and alginate, while ADL refers to cellulose and soluble lignin, ILF represents the insoluble lignin, NDF to proteins, mucilages, pectins and soluble salts and finally AEF refers to the lipid fraction. The values obtained in the fibre analysis correspond well to those described in literature; the lipid (AEF) fraction constitutes 4.02 % of the total dry weight (0.3 - 4.5 % in brown algae) and proteins and soluble salts (NDF) represents 64.20 % (proteins constitute 4.3 – 24 % and mineral salts 14 – 44 % in brown algae) (Li et al., 2021).

Moreover, the values corresponding to the total carbohydrate composition (27.29 %, ADF + ADL) were also included into the usual carbohydrate range described in brown seaweed (12.5 - 56.5 %) (Li et al., 2021). Despite this, some seaweeds as *Saccharina japonica* reaches higher

carbohydrate composition values (75 %) (Vishchuk et al., 2012). The release of this carbohydrate content is crucial for downstream hydrolytic or fermentative processes.

2.2 Microwave pretreatment

To perform the microwave pretreatments, a Milestone® Flexiwave 1900 was used. Thus, samples of 2 g of biomass suspended in 50 mL of deionized water were pretreated in all the tests. The suspensions were subjected to plateau temperatures ranging from 120 °C to 220 °C (120, 140, 160, 180, 200, and 220 °C). Those maximum temperatures were maintained during times in the range of 5 to 20 minutes (5, 10, 15, and 20 min). The maximum temperatures were reached by heating the samples during 10 minutes from the room temperature and later they were chilled from the plateau temperature to the room temperature by forced ventilation in the corresponding time.

After obtaining the pretreated biomass at the different conditions, several chemical parameters were analysed in the supernatant liquid in order to establish the level of solubilisation: Dissolved Organic Carbon (DOC), soluble Chemical Oxygen Demand (sCOD), Reducing Sugars (RS) and Total Polyphenols (TP).

2.3 Enzyme hydrolysis

After some of the above mentioned conditions of the microwave pretreatment, two trials of EH were carried out in triplicate: a) hydrolysis of the solid and liquid obtained together (total suspensions enzyme hydrolysis); b) hydrolysis of the remaining solid only, once filtered and dried in an incubator at 40 °C during 12 h (filtered solids enzyme hydrolysis).

In the first case, the 50 mL of each pretreated suspension were introduced into a 100 mL Erlenmeyer flask. Also the weight of potassium phosphate necessary to create directly in the medium a phosphate buffer (pH 5.0) was added (not affecting the total enzyme hydrolysis volume), with the purpose of adjusting the medium to the optimum conditions of the enzymes working conditions. Then, all the flasks were sterilized (120 °C, 20 min) using an autoclave to avoid subsequent contamination. After that, 688 µL of CellicCTec2 enzyme cocktail was added to each flask. The trading enzyme cocktail CellicCTec2 developed by Novozymes®, which contains several hydrolytic enzyme activities (exo-cellulases, endo-cellulases, β-glucanohydrolases, etc.) was used in this study. This has been reported as a very good broad-spectrum hydrolytic enzyme cocktail (Cannella et al., 2012; Rodrigues et al., 2015). Finally, the enzyme hydrolysis was performed at 50 °C and 100 rpm using an orbital shaker incubator (Thermo Fisher Scientific®, MaxQ^{TM} 6000). All EH conditions were decided in according to the supplier recommendations.

In the second case, the dry remaining solid of each pretreated condition was introduced into a 100 mL Erlenmeyer flask and 45 mL of phosphate buffer (pH 5.0) was added. Later on, the flasks were sterilized as was abovementioned. Finally, 688 µL of CellicCTec2 enzyme cocktail was put on to each flask and the EH was performed in the same conditions as before.

Apart of these two types of trials with the microwave-pretreated biomass, an assay of EH of the biomass without any microwave pretreatment was also carried out. In all the cases, during the first 3 hours, 500 µL samples of each flask were taken every 30 minutes. After that, samples were taken at 6, 8, 10, 24, 30 and 50 h. RS was determined on each sample from enzyme hydrolysis tests.

2.4 Dark fermentation

In parallel, a series of DF tests was carried out in triplicate with some of the pretreated biomass. In these tests, the effluent of an acidogenic reactor was used as inoculum. The acidogenic reactor had been operating at pH 5.5, a temperature of 55 °C and hydraulic retention time (HRT) of 6 days (Ren et al., 2007) in order to produce the suitable inoculum. This reactor was daily fed in a semi-continuous mode with exhausted sugar beet pulp (ESBP), supplied by a nearby sugar factory of AB Sugar® located in Jerez de la Frontera (Spain) (Bravo et al., 2021; Romero-García, 2022). The inoculum has the physicochemical characteristics described in Table 2.

Table 2: Main physicochemical features of the inoculum used in dark fermentation essays for a significance p<0.05 calculated in dry basis. All the results are expressed at dry basis.

Parameter	Units	Value
TS	g/kg	17.7 ± 0.52
VS	g/kg	8.44 ± 0.67
TA	g/kg	1253 ± 87.0
sCOD	g/kg	1023 ± 1.18
TP	g/kg	13.4 ± 1.05
TVFA	g/kg	276 ± 5.08

The assays of DF were carried out in 250 mL vials operating in batch mode. The loading volume of vials was 150 mL (135 mL of the pretreated suspension plus 15 mL of inoculum) and the rest of the vial volume was occupied by the produced biogas.

Apart of the DF tests of the microwave-pretreated biomass, two controls were performed as a test reference: a) The inoculum control (100 % of the loading volume filled by inoculum); b) The non-pretreated biomass control (135 mL of a suspension 4 % Dry weight (DW) of non-pretreated biomass in deionized water plus 15 mL of inoculum).

Initially, pH was adjusted to 7.5 to prevent from pH dropping below 5.0 due to acid production. Then, vials were incubated at 55 °C for 9 days. During the first 3 days, the overpressure of the headspace was daily measured using an digital manometer (Omega® HPP350) and the biogas produced was evacuated and the composition was determined by gas chromatography in a Shimadzhu® GC-2014 equipped with a thermal conductivity detector and a packed column with molecular sieve packing of 0.3 cm diameter and 3 m length (Carbosieve® Supelco® Merk kGaA®). Nitrogen was used as carrier gas at flow rate of 50 mL/min (Gómez-Quiroga et al., 2019). Afterwards, measurements were only performed on days 6 and 9. Biohydrogen yield was estimated as the ratio between the cumulative hydrogen production expressed in mL at normal conditions and the added seaweed biomass in each assay expressed in grams. Other chemical parameters were analysed both at the beginning and at the end of the fermentation process: sCOD, DOC, VFAs, TS, VS, TA and TP.

2.5 Analytical methods.

Most of the analytical techniques used in this work were performed according to APHA-AWWA-WPCF standard methods (APHA-AWWA-WPCF, 2017). Thus, the following determinations of this reference were applied: Total Solids (TS), Volatile Solids (VS), soluble Chemical Oxygen Demand (sCOD), Dissolved Organic Carbon (DOC), Total Alkalinity (TA) and pH. Moreover, quantification of Total Polyphenols (TP) were measured according to the Folin-Ciocalteu method described in literature (Espada-Bellido et al., 2016) and quantification of Reducing Sugars (RS) were performed using the dinitrosalicylic acid (DNS) method (Serna, 2019). Volatile Fatty Acids (VFAs) were determined by gas chromatography in a Shimadzhu® GC-2010 equipped with a flame ionisation detector and a capillary column (Nukol® Supelco® Merk kGaA®) with a diameter of 0.25 mm and 30 m length. Hydrogen was used as a carrier gas and detector fuel, at flow rate 50 mL/min. Synthetic air at flow rate of 400 mL/min and nitrogen at flow rate of 30 mL/min were used as oxidising and make-up gas respectively (Gómez-Quiroga et al., 2019).

DOC, sCOD and VFAs analyses were performed on samples centrifuged at 3,200 g for 10 minutes (Orto Alresa® Consul 21R) and afterwards filtered to a particle size of 0.47 µm (Hahnemühle®, GF52 glass fibre filter). In the case of VFAs measurement, an additional filtration was applied using a 0.22 µm particle size filter (Scharlab®, PTGE syringe filter). DOC was performed using an Analytik-Jena® multi N/C 3100.

Fibre analysis was performed using Van Soest method (Van Soest et al., 1991) using a fibre analyser Foss® Fibretec 8000.

3. Results and discussion

3.1 Pretreatment optimization

After microwave pretreatment of algal biomass under the conditions described above, sCOD, DOC, TP and RS were studied in order to identify the best pretreatment conditions for the subsequent processes: EH and DF.

3.1.1 Solubilised organic matter (DOC, sCOD)

In Figure 1, sCOD and DOC values has been showed for the evaluation of the organic matter solubilisation process.

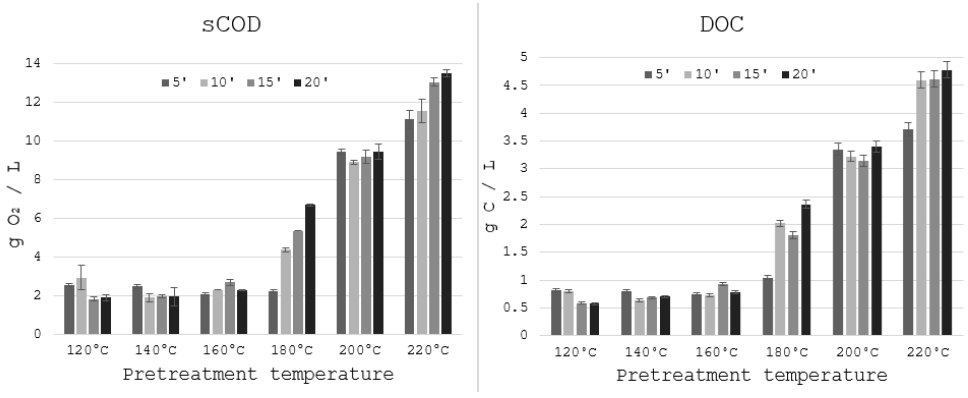


Fig 1: Solubled organic matter measured as sCOD and DOC for each microwave pretreatment condition: temperature and time.

Taking into consideration the content of volatile solids of the algal biomass and assuming that 52 % of that organic matter corresponds to carbon (Navarro et al., 1993), the solubilised organic matter can be calculated as the percentage of theoretical total dissolved organic matter by measuring the amount of Dissolved Organic Carbon (DOC) detected in the extract. Solubilisation levels higher than 5 % were not observed in the first three pretreatment temperatures. However, from 180 °C onwards, a large progressive increase was observed until the pretreatment of 20 min under 220 °C, reaching then almost 27 % of the total organic matter. This may indicate that 180 °C is the minimum temperature where the solubilisation of the organic matter is quantitative.

In most cases, the majority of organic matter was already solubilised in the first 5 minutes of pretreatment, but in general, no clear dependence was observed between pretreatment time and organic matter solubilisation. However, a high dependence was observed in the case of temperature in terms of the solubilisation level.

On the other hand, after a regression analysis, it was found that sCOD and DOC results are strongly linearly related (R^2 = 0.98). The regression slope was 2.78 g sCOD/g DOC. This parameter can provide an estimation of the type of organic matter solubilised by the microwave pretreatment. To perform this estimation, the molecular formulas of some polymers present in brown algae such as laminarin, fucoidan, cellulose and alginate were obtained (National Center for Biotechnology Information, 2022). The following values for the sCOD:DOC ratio were obtained by stoichiometric calculations: 2.66, 3.50, 2.66 and 2.88 respectively. This ratio was also calculated for the corresponding monosaccharides of their decomposition, being glucose for laminarin and cellulose, mannuronic acid and guluronic acid for alginate and mainly fucose for fucoidan (Stiger-Pouvreau et al., 2016). Values of 2.66, 2.44, 2.44 and 2.88 were obtained respectively.

The obtained ratio seems to indicate that fucoidan was not solubilised in extension during pretreatments, on the contrary the ratio would have resulted higher. Also, alginate does not seem to be much solubilised either. Despite having a ratio quite close to the one obtained, in the case of solubilisation of alginate, it was expected to observe a lower resulting ratio due to the presence of mannuronic acid and glucuronic acid. All this indicates that the microwave pretreatment has mainly solubilised cellulose and laminarin and, thus, glucose resulting from their degradation.

In the current study, a maximum COD value of 13.5 g/L was obtained. This is slightly lower than the one in others studies for brown algae in the same conditions, in which 15 g/L of COD were obtained from *Laminaria japonica* (Yin et al., 2019).

3.1.2 Solubilised total polyphenols (TP)

Results obtained in the Total Polyphenols (TP) analysis are shown in Figure 2. As it can be observed, the polyphenols solubilisation shows a very similar behavior to the organic matter previously analysed. Thus, at the first three pretreatment temperatures, not many polyphenols were solubilised. However, from 180 °C onwards, greater quantities of polyphenols were progressively solubilised. The higher polyphenols concentration obtained in this study was around 1 g/L, at 220 °C.

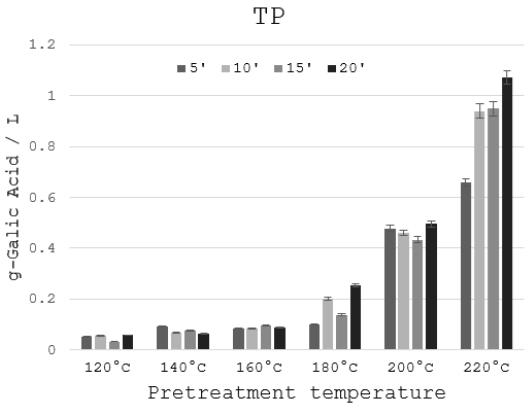


Fig 2: Solubilised total polyphenols for each microwave pretreatment condition.

3.1.3 Total reducing sugars (RS)

Hidrothermal pretreatment of algal biomass can promote the extraction of reducing sugars in the form of polysaccharides and monosaccharides. The most common in these seaweeds are galactose, xylose and glucose (Rodriguez-Jasso et al., 2011; Y. Yuan & Macquarrie, 2015). Extracting reducing sugars from biomass increases the bioavailability of substrates that are more easily assimilated by microorganisms during later processes, such as alcohol fermentation or DF (Han et al., 2015, 2017). Results obtained in this study from the analysis of RS in the extracts are shown in Figure 3.

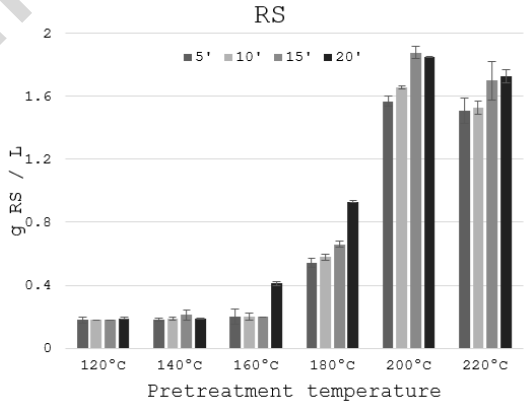


Fig 3: Soluble reducing sugars for each microwave pretreatment condition.

Figure 3 shows that higher values of soluble reducing sugars were obtained at higher temperatures, reaching the maximum concentration at 200 °C. This may be due to the glucose degradation at high temperatures, which can produce sugar caramelisation or even

compounds such as 5-hydroxymehyl furfural (5-HMF). This and other furfurals are a well-known inhibitors of hydrogen production in dark fermentation (Sun et al., 2020).

3.1.4 Pretreatment simulation and selection

The values of the parameters studied in previous sections shall be critical when selecting the pretreatment conditions, particularly to improve the subsequent EH or DF processes. Thus, a significant increase in the obtained sCOD and DOC implies a higher amount of soluble organic matter in the liquid fraction. Consequently, the bioavailability of substrates for subsequent processes also increases (Oliveira et al., 2014). On the other hand, an increase in the TP obtained can produce a large inhibition of the hydrogen production during the DF process (Ghimire et al., 2015, 2016).

DF studies show inhibition of hydrogen production from polyphenols concentrations of 0.2 - 0.25 g/L (Ntaikou et al., 2009; Sharma & Melkania, 2017), although, the inhibition seems to be more pronounced (almost 50 % of inhibition) at TP concentrations of 1 g/L (Sharma & Melkania, 2017). Thus, higher concentrations or TP are not desirable in DF trials.

In order to estimate a suitable range of pretreatment conditions to be used for the DF or the EH processes, a simulation of the resultant parameters (DOC, TP and RS) was carried out. To perform this simulation, it can be assumed that the global process through which substances are solubilised during the hydrothermal pretreatment corresponds to a first order kinetic model, with kinetic constant k. Moreover, it can also be assumed that the temperature influence follows the equation of Arrhenius with the activation energy E_a and the reference constant k_O (Equations 1 and 2). Y corresponds to the solubilised organic matter at t time and Y_m means the maximum organic matter to be solubilised, which is content in the biomass pretreated.

$$Y = Y_m\left(1 - e^{-kt}\right) \quad (Eq.1) \qquad\qquad k = k_o\, e^{-\frac{E_a}{RT}} \quad (Eq.2)$$

Then, the model parameters can be obtained (Ea and kO) by fitting them to the experimental data for each variable (DOC, TP and RS) to the following linear equation (Equation 3):

$$ln\left(-\frac{ln\left(1-\frac{Y}{Y_m}\right)}{t}\right) = ln(k_o) - \frac{E_a}{R}\left(\frac{1}{T}\right) \quad (Eq.3)$$

In order to develop this linear regression, data at 220 °C were not included for both the RS and the DOC calculations, due to the evidence that there was some degradation of solubilised sugars at this temperature and degradation processes can overlap the temperature influence on solubilisation processes. Thus, the R^2 values obtained in the linear regressions were 0.99 for DOC, 0.96 for TP and 0.94 for RS. The theoretical curves and the experimental data for the different parameters are shown in Figure 4. The quantities of each compound obtained in water without any pretreatment are represented at 22 °C.

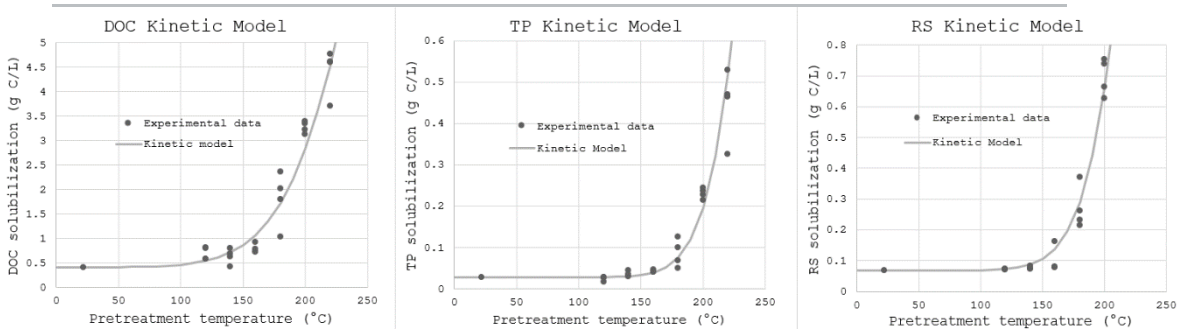


Fig 4: Theoretical curve fitting to experimental data for DOC, TP and RS.

After fitting, the values of the kinetic parameters obtained in each case were the following: for DOC, E_a = 21.7 kcal/mol and $k_O = 2.9 \cdot 10^6$ s^{-1}; for TP, E_a = 27.5 kcal/mol and $k_O = 8.7 \cdot 10^8$ s^{-1}; and for RS, E_a = 25.6 kcal/mol and $k_O = 2.7 \cdot 10^8$ s^{-1}. It was observed that the chemical processes involved in solubilisation of polyphenols or sugars were kinetically quite similar. Thus, they have an activation energy of 25 - 28 kcal/mol and a reference constant of 2 - 9 $\cdot 10^8$ s^{-1}. Moreover, the global process of organic matter solubilisation was also kinetically similar, with a global activation energy of 21 - 22 kcal/mol and a global reference constant of 2 - 3 $\cdot 10^6$ s^{-1}. It can be inferred that the other organic matter present in this biomass (apart from RS and TP) was more easily solubilised thermally due to its slightly lower activation energy.

It is important to point out that temperature is not here a fixed variable, since it varies during treatment. First, raising in ramp; secondly, staying in a plateau; and, finally, decreasing in exponential cooling. Thus, the total organic matter that is solubilised during a specific treatment comes as a consequence of the sum of the successive steps applying a fixed temperature during a differential time. In this way, it was calculated the numerical value of solubilised organic matter (Y) obtained by the simultaneous combination of equations 1 and 2, at infinitesimal intervals (t) of fixed temperatures (T), following the proper temperature curve in each case. The results tightly agree with the experimental data and provide us of a robust kinetic model of the microwave pretreatment.

In pretreatments, it is intended to solubilise the highest amount of organic matter, while keeping polyphenol content in the extract as low as possible. For this purpose, the theoretical kinetic curves obtained previously can be combined to produce the corresponding curve to the TP/DOC ratio plotted in Figure 5. When observing this theoretical curve, a minimum is declared at 155 °C and a maximum at 240 °C. The minimum ratio indicates the temperature at which the highest solubilisation is obtained with the minimum polyphenol content. On the other hand, the maximum ratio indicates the temperature at which the highest amount of polyphenols would be obtained per unit of solubilised organic matter.

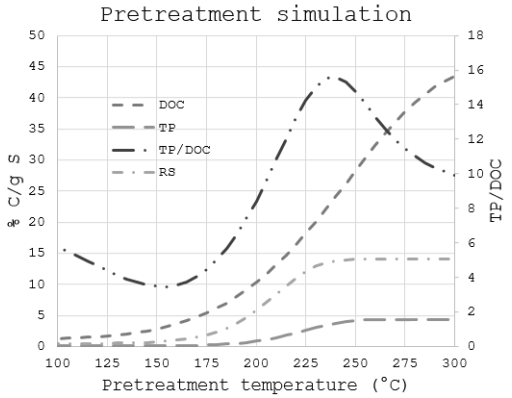


Fig 5: Pretreatment simulation based on the kinetic model. TP/DOC is represented in the secondary axis.

Taking into consideration this temperature range, and the fact that TP concentrations higher than 1 g/L were reached at 207 °C, the following pretreatment conditions were selected for the subsequent assays of EH and DF: 160, 180, 200 and 220 °C, with temperature plateau times of 20 min.

3.2 Enzyme hydrolysis

Sugar yields obtained after 24 hours in different experiments of EH were compared. As it was mentioned in Section 2.3, thirteen samples were analysed at different EH times from each assay. It was elucidated that the maximum reducing sugars concentration were reached at 24 hours.

Two different types of EH tests were carried out: first, EH was performed on the pretreated biomass as a whole (solid and liquid together in suspension); whereas secondly, EH was performed on only the solid phase (once filtered and dried).

Studying whether there are significant differences between both experiments has some interest due to it has been described an inhibition by product in EH (Andri et al., 2010; Yu et al., 2012). This inhibition may occur if reducing sugars solubilised during pretreatment add up to the reducing sugars solubilised during hydrolysis. The results are shown in Table 3.

Table 3: Enzyme hydrolysis yields (over non-pretreated biomass control) of total suspensions and filtered solids for different pretreatment conditions for a significance p<0.05.

Total suspensions		Filtered solids	
Temperature (°C)	Yield (mg-RS/g)	Temperature (°C)	Yield (mg-RS/g)
160	18.5 ± 3.5	105	14.3 ± 1.7
180	14.1 ± 12.0	145	28.6 ± 5.7
200	28.5 ± 11.7	175	42.7 ± 9.4
220	42.2 ± 6.25	205	49.5 ± 5.6

3.2.1 Enzyme hydrolysis yields

In Table 3, it can be observed that pretreatment has similar effect on the EH of total suspensions for the first two conditions (18.5 mg-RS/g-biomass, over control), and also the effect was low in the case of the filtered solids. At higher temperatures, a slight improvement was observed, with an increase in production until around double at 220 °C. The gross production achieved in this study at that temperature was 160 mg-RS/g-biomass, while results obtained with hydrothermal pretreatment of brown algae in other studies are 120 mg-RS/g-biomass (Saravanan et al., 2018).

For the viability of the biorefinery processes, as important as the ratio of sugars extracted is the final concentration of sugars obtained in the hydrolysed broth. In this study, the higher final concentration achieved was almost 12 g/L, which is similar to those obtained in other works driven to polyhydroxyalkanoates production (Azizi et al., 2017). Hence, it is expected that EH after hydrothermal pretreatment has a potential viability.

After performing EH of filtered solids, it was observed that the rising of yields was higher than the one in the other case. This could indicate the existence of less by product inhibition when the sugars solubilised during pretreatment were removed.

3.2.2 Effect of microwave pretreatment on Enzyme Hydrolysis

In order to evaluate the total effect of pretreatment on the yield of sugars, the obtained sugars during pretreatment with the obtained sugars during hydrolysis (over the control without pretreatment) were represented in Figure 6.

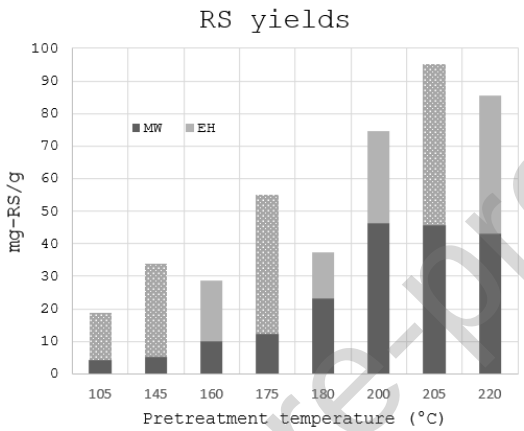


Fig 6: Total and partial RS yields of microwave assisted hydrothermal pretreatment (MW) followed by enzyme hydrolysis (EH).

As it can be seen, at low temperatures, hydrolysis yields were considerably higher than those of pretreatments, whilst, at higher temperatures, microwave provides a similar contribution to the RS yield, even exceeding EH. It is remarkable how the pretreatment yield decreases from 200-205 °C to 220 °C. As it has been mentioned above, sugars degradation process can begin to occur at that temperatures. Finally, due to the contribution of the hydrolysed sugars, total yields obtained with the hydrolysis of solids were notably higher than those obtained with the hydrolysis of suspensions are.

3.3 Dark fermentation

The results obtained in the experiments of DF, in terms of the hydrogen production and the Total Volatile Fatty Acidity (TVFA) production, are shown in Table 4. These values represent the production obtained in the fermentation of the algal biomass over the inoculum control.

Table 4: Total Volatile Fatty Acidity (TVFA)s and biohydrogen production during dark fermentation for different pretreatment conditions over inoculum control for a significance p<0.05.

Temperature (°C)	Hydrogen yield (mL-H_2/g)	TVFA yield (mg HAc/g)
22	1.09 ± 0.08	7.89 ± 0.06
160	0.74 ± 0.04	7.80 ± 0.03
180	1.17 ± 0.15	8.48 ± 0.01
200	6.15 ± 0.13	14.87 ± 0.06
220	4.18 ± 0.34	21.52 ± 0.2

3.3.1 Biohydrogen yield

In figure 7 can be seen how hydrogen production starts after the first 24 hours, which demonstrates that inoculum was fully active and suitable for the seaweed biomass degradation, without holding latency periods. Generally, hydrogen production occurred mostly in the first 3 days; however, the end of the trials was extended until day 9 in order to ensure the complete substrate degradation. All trials, including the non-pretreated biomass control (22 °C), produced significantly higher hydrogen quantities than the inoculum fermented without biomass. Thus, it can be suggested that fermentative microorganisms had successfully degraded the substrate.

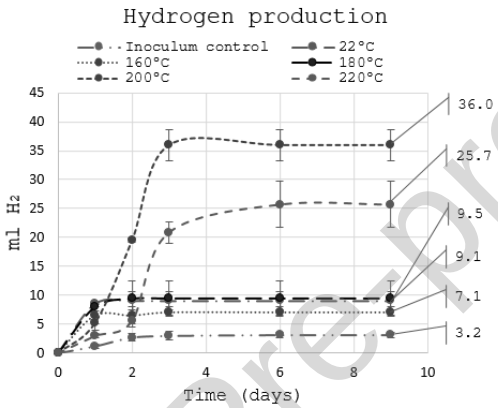


Fig 7: Hydrogen production during DF trials and its maximum value.

Moreover, it is remarkable that hydrogen yields obtained with the 160 °C pretreatment were quite similar to those obtained with the non-pretreated control (22 °C). Thus, it suggests that microwave pretreatment under these conditions causes non-appreciable improvement of the DF process. However, when the pretreatment temperature was raised up to 200 - 220 °C, a major increase of production was observed. This increase was even greater at 200 °C than at 220 °C, which means that the former is better than the latter in order to maximise the hydrogen production. Thus, microwave pretreatment can increase the production obtained when compared to that obtained without pretreatment. The decrease in the hydrogen production at 220 °C is in accordance with the simulation carried out previously, where a concentration of TP greater than 1 g/L was obtained at 207 °C. Thus, inducing a much more pronounced inhibition of the hydrogen production for those conditions (Sharma & Melkania, 2017).

It is already mentioned that thermal pretreatments can cause a decomposition of monosaccharides to 5-hydroxymethyl furfural (5-HMF), which is an inhibitor of the hydrogen production (Sun et al., 2020). This is in agreement with the decrease of reducing sugars observed in pretreatments at 220 °C in Figure 3. However, according to some studies, this inhibitory effect starts to be appreciated from concentrations higher than 1 g/L (Sharma & Melkania, 2017; Sun et al., 2020). Since Figure 3 shows a difference in sugars concentration of less than 1 g/L between 200 and 220 °C, it could indicate that not as much of 5-HMF was produced, but sugar solubilisation at 220 °C was expected to be more than at 200 °C. Moreover, inhibition of hydrogen production could be contributed synergistically with the high concentration of TP.

3.3.2 Volatile Fatty Acids yields

As far as VFAs are concerned, a significant influence of pretreatment conditions is observed in Table 4. Total Volatile Fatty Acidity (TVFA) was computed as the sum of the acidity of each volatile acid pondered on its molecular weight and expressed in acetic acid units. Thus, it is observed that the TVFA obtained increases with pretreatment temperature in both cases after pretreatment and after dark fermentation.

Initial levels of TVFA before DF already shows an increase of 18 %, 37 %, 62 % and 45 % over the non-pretreated biomass for 160 °C, 180 °C, 200 °C and 220 °C respectively. This increases have be seen in other studies (Yin et al., 2019), due to the fact that at higher temperatures seaweed biomass degradation is greater. Consequently, a larger lysis of structural lipids as triglycerides occurs, producing glycerol and its derived fatty acids. Therefore, a temperature dependence of these TVFA values was observed, showing a maximum at 200 °C. This point may be also due to a possible VFAs degradation at high temperatures.

A temperature dependence can be also found in the case of final values of TVFA after DF. The increases of the final values were 7 %, 20 %, 67 % and 93 % over the non-pretreated control. In other words, a strong dependence of pretreatment temperature on the VFAs production during dark fermentation also exists. When comparing total yields of VFAs with other studies, the quantities obtained in the present study are considerably lower than others, but when comparing yields of the microwave pretreatment, they are quite similar or even higher (Pham et al., 2012; Yin et al., 2019). In this study, the maximum TVFA production was 46.3 mg/g-biomass.

3.3.3 Selection of pretreatment for dark fermentation

When selecting the best pretreatment for a DF, it is not only interesting to maximise the total VFAs production but also the VFAs distribution. Thus, acetic acid has been detected as the principal VFAs obtained, with proportions of 67 - 71 % after 9 days. This demonstrates that acetogenesis stage has been almost completely developed by acetogenic microorganisms. This fact, combined with the fact that the levels of the other VFAs with higher molecular weight were very low (less than 50 ppm in all cases), indicates that acetogenesis has not been inhibited.

Figure 8 shows the proportions of different VFAs after dark fermentation of *R. okamurae* when it was pretreated at different temperatures. As it can be observed, the propionic acid fraction increases at higher temperatures. This point is interesting for a subsequent polyhydroxyalkanoates production stage, because the odd acids (propionic and valeric) are involved in the production of polyhydroxyvalerate, which is more valuable than polyhydroxybutyrate due to its better mechanical characteristics (Stiger-Pouvreau et al., 2016). Then, despite 200 °C is the optimum pretreatment temperature to maximise the hydrogen production, when considering the VFAs production the better results has been observed at 220 °C. In addition, the propionic acid fraction increases at higher temperatures, which is other point in favor of the 220 °C pretreatment.

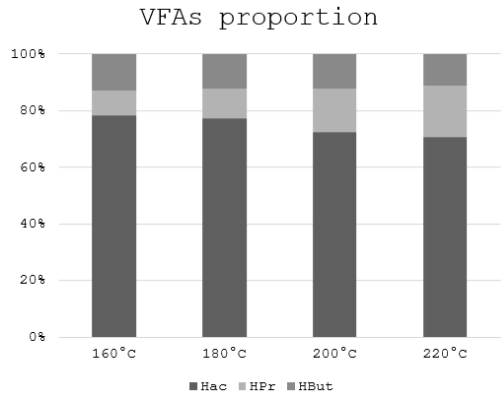


Fig 8: Distribution of VFAs obtained after dark fermentation of R. okamurae pretreated at different temperatures.

3.4 Techno-economic analysis

In the first place, regarding to the use of *R. okamurae* for enzyme hydrolysis after microwave-assisted hydrothermal pretreatment, it is observed that sugars yields depend strongly on the pretreatment temperature, varying from 2 % to almost 10 % of the processed biomass. According to Figure 6, during pretreatment at 205 °C (optimum conditions) a 4.5 % of the initial biomass is already solubilised in form of RS. Moreover, after the EH stage, RS yield increases in other 5 %. Thus, taking into consideration that the EU reference price for sugar on the regulated market is around 0,4 k€/t-sugar (European Parliament and Council of the European Union, 2013), the obtaining income by the sugar yielded can be around 40 €/t-biomass.

On the other hand, although microwave irradiation is more efficient energetically than conventional heating systems (Hoang et al., 2021; Xia, Cheng, & Song, 2013), it would be necessary to supply 3.8 MWh/t-biomass during the pretreatment at 205 °C of a biomass suspension of 40 g/L. Later, the maintenance of that suspension at 50 °C for at least 24 h during the EH stage would require another energy input of approximately 1.5 MWh/t-biomass. Hence, the whole process would initially involve about 5 MWh/t-biomass. However, considering 90 % of average efficiency in the industrial thermal machines (T. Yuan et al., 2019; Zhang et al., 2019), and considering 90 % of the average energy saving by energy integration of the industrial heat streams (Pejpichestakula et al., 2013; Habibi et al., 2018), the total energy input can be reduced to 0.5 MWh/t-biomass by operation the process in an optimized continuous mode. Consequently, the industrial energy costs of this process could be around 40 €/t-biomass, based on the average price of industrial energy in Spain in 2019 (80.4 €/MWh) (Ministry for Ecological transition, 2019).

Nevertheless, the main cost of this process is associated to the enzymes for the EH stage, as it would be necessary to add about 300 L of enzyme cocktail (CellicCTec2) per ton of processed biomass. This specialized biocatalyst represents a cost of around 500 k€/t-biomass (Sigma-Aldrich, 2022). However, an efficient use of enzymes in the industrial process can reduce the enzymes cost drastically to 100 €/t-biomass (Klein-Marcuschamer et al., 2012). In any case, the economical balance of the overall process would make it not competitive in terms of sugar

production. A way to revert this situation consist in producing the enzymes in the process itself and thus rid this economical charge (Marzo et al., 2021).

Concerning the option of DF as the central process, the maximum VFA yield is about 20 kg-HAc/t-biomass. Taking into account that the price of these short-chain acids is around 0.5-1.2 €/kg (Sigma-Aldrich, 2022), the income associated to the VFAs production could be as high as 20 €/t-biomass.

On the other hand, acidogenic reactor works at 55 °C for a HRT of 6 days, which means that the energy input of this process would be about 6 times higher than EH. Consequently, it is needed an energy supply of 9 MWh/t-biomass. Taking into account the contribution of pretreatment at 220 °C (3.9 MWh/t-biomass), the total energy duty would reach 12.9 MWh/t-biomass. Basing on the same considerations as in the previous case, the energy cost of the process could go up to around 110 €/t-biomass. Notwithstanding, in this case no other comparable process cost would have to be added (no enzymes).

In addition, it should be mentioned the biohydrogen yield is about 6 L-H_2/t-biomass (0.5 kg-H_2/t-biomass). Then, considering the calorific value of biohydrogen (around 120 MJ/kg), it could reach an energy production of around 2 kWh/t-biomass. It is clear that the energy saving from hydrogen production would be negligible, compared to the energy needs of the whole process.

From the point of view of the raw material of the process (*R. okamurae*), its massive coastal arrivals and blooms occurs in specific seasonal periods, so its generation is discontinuous. This reason recommends its use only as a co-substrate of other more regular raw material for biorefinery propose; i.e., agricultural wastes, animal manures, etc. Despite of this, the obtained results could be interesting to estimate the possibilities of other brown seaweeds that were chemically similar. In addition, any effort in the direction of including wastes into a circular economy scheme would be desirable, even when some benefits of this strategy are only environmentally quantifiable.

4. Conclusions

The main conclusions are:

- The microwave irradiation pretreatment is effective for the solubilisation of organic matter in the brown algal biomass of *R. okamurae*, specifically from 180 to 220 °C, becoming higher as the temperature increases.
- Reducing sugars (RS) solubilisation yield is maximum at 200 °C and decline at 220 °C while Total Polyphenols (TP) solubilisation yield increases gradually as temperature rises until 220 °C.
- The best pretreatment temperature to obtain the highest RS production by enzyme hydrolysis (EH) is 220 °C, obtaining 160 mg-RS/g-biomass, even when a certain proportion of sugars where degraded during the pretreatment due to the high temperature.
- In the case of the dark fermentation (DF), the highest yield in hydrogen production was obtained at 200 °C (6.6 mL-H_2/g-biomass), while the maximum TVFA production was obtained at 220 °C (46.3 mg/g-biomass). The decline in the hydrogen production of the pretreated biomass at 220 °C is due to the high accumulation of TP at this pretreatment temperature.

For all these reasons, it could be affirmed that microwave pretreatments can benefit the production of different compounds (RS and VFA) for its later exploitation as biorefinery bricks in bioprocesses from the brown algae *Rugulopteryx okamurae.*

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CRediT authorship contribution statement

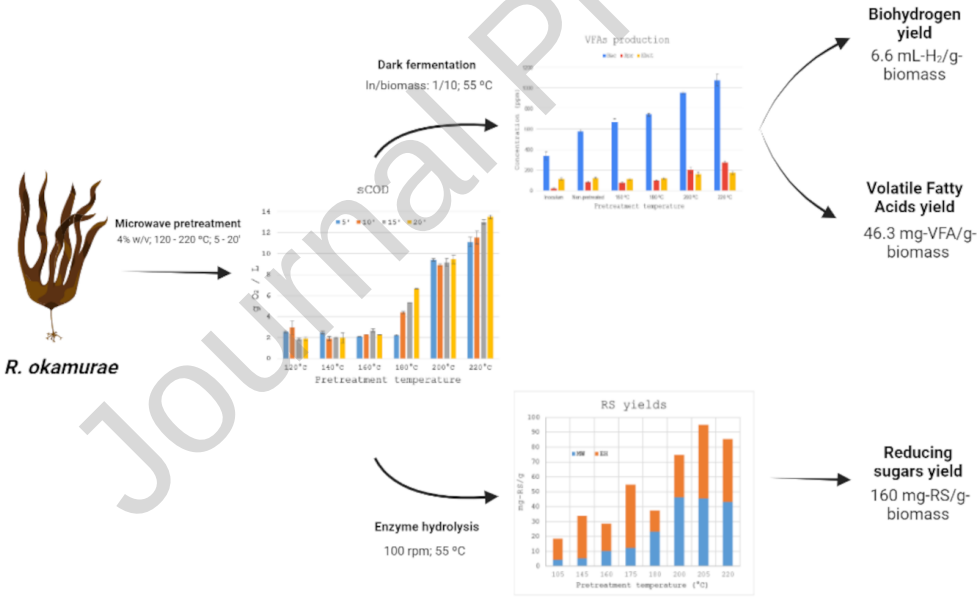
All the authors have equally contributed to the methodology, software, validation, formal analysis, investigation, data curation, writing—original draft preparation, writing—review and editing and visualization. **I. Caro** and **C.J. Álvarez-Gallego** have contributed additionally to conceptualization, resources, supervision, project administration and funding.

Declaration of Competing Interest

☒The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Graphical abstract



Highlights

- The best microwave-assisted solubilisation was obtained at 220 °C and 20 min.
- Reducing sugars production by enzyme hydrolysis was improved (35 %).
- VFAs production by dark fermentation was improved (173 %).
- H_2 production was partially inhibited in 220 °C thermally pretreated seaweed.