



# Bioethanol Production from Marine Macroalgae Waste: Optimisation of Thermal acid Hydrolysis

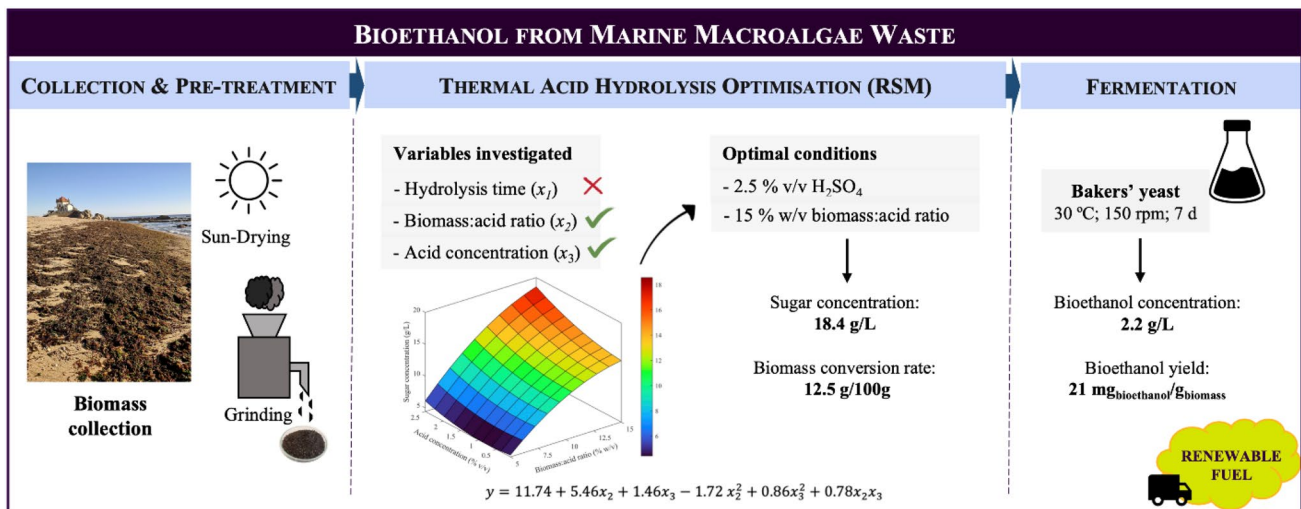
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## Abstract

Marine macroalgae waste, resulting from the accumulation of drifted algal biomass along the coastline, might be a relevant complementary raw material aiming sustainable bioethanol production. In the present study, the optimisation of thermal acid hydrolysis was performed using response surface methodology (RSM) considering the effect of three variables, namely, reaction time (10–60 min), acid concentration (0.1–2.5% (v/v) H<sub>2</sub>SO<sub>4</sub>) and biomass:acid ratio (5–15% (w/v)) on sugar concentration and yield. Under the best conditions, the resulting hydrolysates were fermented (7 days, 30 °C, 150 rpm, commercial yeast) to produce bioethanol. A statistically valid second-order model was obtained ( $r^2 = 0.9876$ ; Prob > F lower than 0.05), showing that sugar concentration is mostly influenced by the biomass:acid ratio while reaction time was not significant. The maximum predicted sugar concentration was 18.4 g/L, being obtained at 2.5% H<sub>2</sub>SO<sub>4</sub> concentration and 15% (w/v) biomass:acid ratio, corresponding to a sugars yield of 12.5 g/100 g (less 36% than that obtained using 10% (w/v)). At the best conditions, the hydrolysates were fermented to obtain a bioethanol concentration up to 2.4 g/L and a 21 mg<sub>bioethanol</sub>/g<sub>biomass</sub> yield, emphasizing the biomass potential for bioenergy production.

## Graphical Abstract



**Keywords** Biomass recovery · Ethanol production · Renewable energy · Response surface methodology · Hydrolysis

Extended author information available on the last page of the article

## Statement of Novelty

Studies on ethanol production from residual biomass of marine macroalgae are scarce, particularly concerning hydrolysis optimisation. Consequently, the present study assessed the utilisation of an unexplored waste—Marine Macroalgae Waste—for renewable energy production (bioethanol), by optimising the hydrolysis step (a critical phase) using Response Surface Methodology. The feasibility of ethanol production was further evaluated through fermentation of the hydrolysates obtained under the best conditions. The developed work plays a highly relevant role for the production of sustainable fuels, enabling waste treatment and recovery of bioresources, thereby contributing to positive environmental, social and economic impacts.

## Introduction

The production of renewable liquid fuels is of high relevance and bioethanol is considered one of the most promising solutions to replace fossil fuels such as gasoline [1–3]. Initial preparation of the raw materials by drying or shredding using different procedures is conventionally employed; however, the production of bioethanol comprises essentially three main stages: hydrolysis (conversion of polysaccharides to monosaccharides), fermentation (conversion of sugars into ethanol by microorganisms) and ethanol recovery [4]. The hydrolysis step is essential to obtain available sugars and different methods can be employed namely biological, chemical (alkali or acid) or thermal, also supported by physical/mechanical procedures [2, 4]. According to the literature, a reduction in pH together with an increase in temperature generally improves sugar release, also enhancing fermentation yield [2, 4].

The most used feedstocks for bioethanol production are terrestrial starch and sugar crops (e.g., sugar cane or corn); however, the presence of lignin (more difficult to break down), the cost of raw materials and the competition for arable land are relevant limitations to their use for biofuel production [1, 2]. The use of alternative non-terrestrial raw materials appears thus as a renewable and sustainable solution to meet the world energy demand for biofuels, where marine macroalgae (MM) can play a relevant role [1–3, 5].

The growing interest in the use of MM for bioethanol production is essentially due to their: (i) fast and easy growth in different environments (e.g., fresh water, saline water or municipal wastewater); (ii) high carbohydrate content (generally between 30 and 60% w/w) [2, 6] which

indicates fermentation viability; (iii) absence of lignin, increasing hydrolysis efficiency; and (iv) lack of competition for arable land or food [1, 2, 7].

The optimisation of the hydrolysis and fermentation steps is seen as one of the challenges in bioethanol production [8]. The hydrolysis step can be chemical or biological, namely including the use of acids (acidic pre-treatment), alkalis (alkali pre-treatment) or enzymes (enzymatic pre-treatment) [2]. Thermal acid treatment is the preferable and most commonly used method, mostly due to the lower costs associated, comparatively with biological treatments, using enzymes [5]. The success of acid hydrolysis is influenced by several parameters, such as acid type and concentration, temperature, biomass-to-acid ratio and reaction time [1, 5, 8]. Revised studies on bioethanol production from MM showed that thermal acid hydrolysis is mainly performed using: (i) temperatures from 115 to 121 °C; (ii) different acids (mostly H<sub>2</sub>SO<sub>4</sub> and HCl) with concentrations from 1 to 5% (v/v); (iii) biomass to acid ratios from 8 to 25% (w/v); and (iv) during 0.25–1.5 h [9–15]. The wide range of reported conditions shows that optimisation studies are relevant for each raw material (e.g., MM species).

Although other optimisation methods are reported in the literature in the field of bioethanol production [16], Response Surface Methodology (RSM), using a central composite design (CCD), is the most common and widely used tool in MM hydrolysis optimisation, considering several conditions [5, 7, 9–11, 17, 18]. With reduced experimental time, it identifies linear, quadratic and sometimes cubic effects between variables together with their interactions, and determines the best reaction conditions leading to an optimal release of sugars [5, 19]. Compared with other RSM designs, such as Box-Behnken design, CCD presents higher robustness and accuracy since it considers an embedded factorial design [20]. Kadimpati et al. [5] applied RSM for the optimisation of *Sargassum cinereum* hydrolysis (brown seaweed), showing an optimum biomass conversion of around 20% (w/w) at 104.4 °C, using H<sub>2</sub>SO<sub>4</sub> at 5.01% and 55.2 min as hydrolysis time. Borines et al. [9] used RSM to find the optimum conditions for reducing sugar yields from the acid hydrolysis of *Sargassum* spp.. The optimum conditions were found to be 1.84–2.27% (v/v) H<sub>2</sub>SO<sub>4</sub>, 115 °C and 86–90 min, corresponding to a reducing sugar yield between 3.75% and 4.50% (w/v). On the other hand, Cho et al. [10] found the optimal hydrolysis conditions at 121 °C, using 13% (w/v) biomass loading, sulfuric acid at 0.4% (v/v), during 60 min; for those conditions, the authors report a maximum monosaccharide concentration in the hydrolysate of 28.8 g/L, representing 42% (w/w) of carbohydrate conversion.

As the exploitation of MM to meet energy needs can be costly, energy production from waste biomass or integrated in a biorefinery approach should be an alternative to consider [2, 3]. Thus, the use of Marine Macroalgae Waste

(MMW; drifted MM accumulated on the beaches beyond normal ecological status) [2] for ethanol production is seen as a sustainable waste management solution (as opposed to abandonment or landfilling – the most practiced for MMW). Following such valorisation route, a contribution to fulfil the energy needs is established as well as the sustainable management of MMW, mitigating negative environmental impacts, health concerns and coastal degradation caused by inadequate practices [2], also contributing to implement a circular economy.

While a wide range of studies was already performed regarding ethanol production from MM, the use of residual biomass (MMW) is still understudied, namely concerning hydrolysis optimisation. To fill such gap, the present study describes the primary step towards the bioethanol production from MMW, namely the optimisation of hydrolysis through RSM, considering the influence of different variables (time, acid concentration and biomass:acid ratio) on sugar concentration. The feasibility of ethanol production was also preliminarily assessed by fermenting the hydrolysates obtained under the best hydrolysis conditions.

## Materials and Methods

### Sample Collection and Preparation

MMW, composed by a mixture of several species, was collected from a northern Portugal beach (Marbelo beach, Vila Nova de Gaia municipality, 41°6'23.616" N, 8°39'46.591" W) at the end of the summer season (September 2020). The biomass was manually collected during the low tide, on the sand, near the water (up to 2 m) and transported to the laboratory in plastic buckets.

The preparation of the biomass consisted in a thorough washing with tap water to remove sand, stones and other minor materials, in agreement with previous studies [21–23], sun-drying for a few days and grinding ( $\leq 1$  mm) in a Cutting Mill (Zipor, by contract) followed by a laboratory mill (20 s; 10 000 rpm; Grindomix GM 200, Retsch, Germany). The MMW biomass was stored at room temperature until use.

### Preliminary Studies: Definition of Reaction Conditions

The conditions and respective ranges selected in the preliminary assays were based on the reported literature [9–15]. To understand the influence of temperature and acid concentration in hydrolysate sugars concentration (first set of experiments), preliminary studies were conducted in a water bath at  $\sim 90$  °C or in a thermoreactor (1 h at 100 °C, followed by 30 min at 148 °C; Thermoreaktor TR 300, Merck), using

3 g of biomass in a  $\text{H}_2\text{SO}_4$  solution (1:40 (w/v)) at different concentrations (2.5, 3 and 4% (v/v)). The reactions were conducted for 90 min.

A second set of experiments was performed using the best conditions from the first set (considering the use of the water bath or the thermoreactor), varying the hydrolysis time (30–90 min), the amount of biomass (biomass:acid ratio from 1:10 to 1:100 (w/v)), and the concentration of acid (1 and 7% v/v).

All experiments were conducted in duplicate. These preliminary studies allowed to establish the range of variables to optimise the hydrolysis step considering technical aspects, which included the study of autoclave-assisted hydrolysis.

### Thermal Acid Hydrolysis: Experimental Design and Laboratory Assays

JMP software was used for experimental planning, following a RSM using a CCD. A design of three factors at three levels ( $-1$ ,  $0$  and  $1$ ) was conducted. The dimensionless variables were defined as  $x_1$ ,  $x_2$  and  $x_3$ , corresponding to hydrolysis time, biomass:acid ratio and acid concentration, respectively. The central point (coded by 0) chosen for experimental design was: 35 min autoclaving time, 1.3% (v/v) acid concentration and 10% (w/v) biomass:acid ratio. Following the experimental planning, 32 experimental runs were conducted, and the response variable was defined as the concentration of sugars in the hydrolysate (g/L). The experimental runs include all conditions in duplicate, whereas four experiments were conducted at the central point.

The results allowed to relate the sugars concentration (the dependent variable,  $y$ ) with the independent variables through a polynomial model. Considering the results from each assay, a polynomial regression equation was determined to predict the sugar content. The obtained model only considered the statistically significant regression parameters ( $p < 0.05$ ). The performance of the model in training (data used to obtain the model parameters) and test (data used to evaluate the predictive performance of the achieved model) sets was assessed by the coefficient of determination,  $r^2$ , and the root mean square error (RMSE).

The critical points (maximum and minimum values) of the model were determined by JMP software; however, the best hydrolysis conditions (considering variable constraints), corresponding to the maximum value given by the achieved model, were determined using the Microsoft Excel Solver.

The biomass was further subjected to thermal acid hydrolysis with  $\text{H}_2\text{SO}_4$  in an autoclave (121 °C; Uniclave 88, AJC, Portugal). According to the described methodology, different  $\text{H}_2\text{SO}_4$  concentrations (0.1, 1.3 and 2.5% (v/v)), biomass:acid ratios (5, 10 and 15% (w/v)), and autoclaving time (10, 35 and 60 min) were evaluated.

## Sugar Quantification

After hydrolysis, the hydrolysate samples were cooled down to room temperature, and the supernatant (upper brown liquid–hydrolysate) was centrifuged at 4000 rpm for 10 min (HERMLE Z 200 A). For the present study, only the total sugar content was determined. Determination was performed spectrophotometrically (UV mini-1240, UV–VIS Spectrophotometer, Shimadzu) at 490 nm through the phenol–sulfuric acid method described by Connan [24], but using 0.25 ml of the sample instead of 0.5 ml. Glucose was used as standard. Determinations were performed in triplicate, and the results were expressed in g/L.

The biomass conversion yield ( $\text{g}_{\text{sugar}}/100\text{g}_{\text{biomass}}$ ) was obtained considering the amount of MMW used in the hydrolysis and the final sugars obtained.

## Fermentation of the Hydrolysates and Ethanol Quantification

The hydrolysates obtained at the best hydrolysis conditions were subjected to a fermentation process to produce bioethanol.

Prior to fermentation, the samples were neutralised (pH adjustment to 4.5–5,  $[\text{NaOH}] = 5 \text{ M}$ ). The fermentation was carried out in 250 mL Erlenmeyer flasks during 7 days in an orbital incubator (Agitorb 200IC), at 30 °C and 150 rpm, using 12 g of a commercial yeast (baker's yeast, *Saccharomyces cerevisiae*), according to the literature [25].

After fermentation, the supernatant was centrifuged at 5000 rpm for 10 min (HERMLE Z 200 A) and analysed for sugar (according to previously described methodology, to evaluate yield of sugar conversion) and bioethanol concentration.

Bioethanol concentration in the fermented hydrolysates was determined by gas chromatography (GC-2010 Plus, Shimadzu). The work conditions, developed at the laboratory, were: an automatic sampler (injection volume of 1.0  $\mu\text{L}$ ) and a split/splitless injector (AOC-20i Shimadzu, Japan) operating with a 1:5 split ratio at 250 °C, a SGE BP20 (WAX) column (30 m  $\times$  0.25 mm inner diameter and 0.25  $\mu\text{m}$  film thickness), being the detector at 250 °C. Helium was the gas carrier (30.0 mL/min), and separation was achieved with the following temperature program: 40 °C held for 4 min, followed by a temperature rise at 50 °C per min up to 240 °C, with a total running time of 8 min. Cyclohexanone was used as internal standard. The results are presented as concentration (g/L) and yield ( $\text{mg}_{\text{bioethanol}}/\text{g}_{\text{biomass}}$ ).

## Results and Discussion

*Saccorhiza polyschides* (brown algae) was the predominant species in MMW. It is known, by previous studies using biomass from the same collection, that this biomass has a high total carbohydrate content, of around 70% (w/w) [23, 26]. Also, the carbohydrate fraction is mostly comprised by fibres (~63%; 50% of insoluble fibres), being the remaining fraction (~37% of carbohydrates) composed essentially by sugars and other polysaccharides [26, 27].

### Preliminary Results

Table 1 presents the results of the preliminary assays conducted in the water bath (~90 °C) and in the thermoreactor (100–148 °C). These experiments were initially performed to understand the influence of the temperature and acid concentration on the sugar concentration and biomass conversion yield.

The first set of experiments (Table 1) showed that the temperature clearly influences the hydrolysis step. For all studied acid concentrations, the use of a water bath (~90 °C) results in lower sugar concentrations (~2 g/L) and biomass conversion yields (~9 g/100 g). Using the thermoreactor (1 h at 100 °C followed by 30 min at 148 °C), it was observed an increase in both sugar concentration and biomass conversion yield, being the results about two-fold (~4 g/L and ~16 g/100 g, respectively) compared with the results at 90 °C using the water bath.

Regarding acid concentration, there are no differences at the studied temperatures, which can indicate that the concentration established is higher than that required for the process at those conditions. Since a higher sugar release from the biomass was observed (4.2 g/L, corresponding to a conversion yield of 16.6 g/100 g of biomass) at the lower studied acid concentration (2.5%), it can be inferred that higher concentrations are not needed.

The second set of experiments (Table 1) confirmed that changes in the acid concentration does not influence the sugar release even when varying from 1 to 7% (v/v) being the results ~4 g/L and ~16 g/100 g in both cases, respectively.

Concerning the biomass amount (biomass:acid ratio), this variable affects the sugar concentration, since it is observed that, keeping constant the other variables, higher MMW amounts (1:10) lead to a sugar concentration of about eightfold than that obtained using a biomass:acid ratio of 1:100. However, the biomass conversion yield decreased around 22% (from 16.9 to 13.2 g/100 g), possibly due to mass transfer limitations (excess of biomass). Also, at times lower than 90 min it is possible to observe



**Table 1** Preliminary studied conditions to understand hydrolysis behaviour at different temperatures (90–148 °C) and acid concentrations (1–7% (v/v))

	T (°C)	[H <sub>2</sub> SO <sub>4</sub> ] (% v/v)	Time (min)	MMW:acid ratio (w/v)	Sugar concentration (g/L)	Biomass conversion yield (g/100 g)
First set	~90	2.5	90	1:40	2.2 ± 0.2	8.7 ± 0.6
	~90	3	90	1:40	2.3 ± 0.2	8.9 ± 1.0
	~90	4	90	1:40	2.3 ± 0.2	9.3 ± 0.9
	100–148	2.5	90	1:40	<b>4.2 ± 0.3</b>	<b>16.6 ± 0.3</b>
	100–148	3	90	1:40	3.85 ± 0.09	15.2 ± 0.4
	100–148	4	90	1:40	4.0 ± 0.2	15.7 ± 0.9
Second set	100–148	1	90	1:40	4.0 ± 0.1	15.6 ± 0.3
	100–148	7	90	1:40	4 ± 1	16 ± 4
	100–148	2.5	90	1:100	1.73 ± 0.05	<b>16.9 ± 0.4</b>
	100–148	2.5	90	1:10	<b>13.3 ± 0.6</b>	13.2 ± 0.6
	100–148	2.5	30	1:40	2.6 ± 0.2	10.4 ± 0.9
	100–148	2.5	60	1:40	3.00 ± 0.04	11.8 ± 0.1

Highlighted results (bold) correspond to the maximum sugar concentrations (g/L) and biomass conversion yield (g/100 g)

T ~ 90 °C corresponds to the temperature of the water bath. Temperature range of 100 – 148 °C corresponds to the temperature of the thermoreactor

Values of sugar concentration and biomass conversion yield are expressed as mean value ± standard deviation

a sugar concentration decrease of about 33% (on average), reflecting that time is a relevant variable under the studied conditions.

The preliminary results showed that the best conditions were higher reaction temperatures (100–148 °C) and time (90 min), 2.5% (v/v) acid concentration and 10% (w/v) biomass:acid ratio, which led to obtain 13.3 g/L sugars concentration, not compromising the biomass conversion yield (13.2 g/100 g).

Hebbale and Ramachandra [7] evaluated the influence of reaction temperature (30–120 °C), reaction time (30–120 min), H<sub>2</sub>SO<sub>4</sub> concentration (0.5–1%) and biomass:acid ratio (1–9% (w/v)), in the concentration of reducing sugars from *Ulva intestinalis* and *Ulva lactuca* (green algae). The authors verified that the reducing sugars increased gradually with the increase of H<sub>2</sub>SO<sub>4</sub> concentration and with the reaction temperature. The maximum concentration of reducing sugars was obtained after 45 min and 5% (w/v) was considered the optimum biomass:acid ratio, with reducing sugar concentration decreasing for higher values. Considering the optimal conditions, the authors reported a reducing sugar yield of around 20 g/100 g [7] for both species. Kadimpati et al. [5] evaluated the influence of biomass loading (3–10% (w/v)), H<sub>2</sub>SO<sub>4</sub> concentration (0–10%) in the hydrolysis of *Sargassum cinereum* (brown seaweed), keeping constant the temperature (100 °C) and the reaction time (60 min). The results reveal that the sugar release from the seaweed depends on both parameters, increasing with the raising of acid concentration from 0 to 4%, and with the biomass amount until 7% (w/v). At those

conditions, the biomass conversion yield into reducing sugars was 17.6% [5].

Similarly to the present study, it is possible to verify that the temperature influences the sugar release, and the biomass amount affects the sugar concentration up to a certain limit depending on the other variables. Although the conversion yields in the revised studies are in the same order of magnitude of those obtained in the present study using the thermoreactor (~ 17 g/100 g), a direct comparison between the results cannot be performed since different species were used; thus, the evaluation of the influence of the reaction conditions in sugar concentration/yield should be carried for each kind of raw material.

Considering such results and to improve the sugar concentration without compromising the biomass conversion yield, the design of experiments was performed using more extreme temperature conditions (autoclave), varying the other parameters. The studied acid concentrations were reduced (0.1–2.5%) according to the results obtained. The amount of biomass (MMW) was increased (5–15% (w/v)) to balance the biomass:acid ratio, aiming to achieve optimum biomass conversion yields.

## Model Fitting

The results of the obtained experimental planning, using a CCD, are presented in Table 2.

The response variable (y) was fitted to a second-order model, aiming to correlate it with the independent variables ( $x_1$ ,  $x_2$  and  $x_3$ ). The fitting resulted in a model that considers

**Table 2** Results of experimental planning using central composite design

Run	Autoclaving time (min)	Biomass:acid ratio (% w/v)	[H <sub>2</sub> SO <sub>4</sub> ] (% v/v)	$x_1$	$x_2$	$x_3$	$y$ (g/L)
1	10	15	2.5	-1	1	1	17.7±0.9
2	35	10	1.3	0	0	0	11.3±0.6
3	10	15	2.5	-1	1	1	17.8±0.7
4	35	10	1.3	0	0	0	10.6±0.3
5	35	10	0.1	0	0	-1	9.8±0.2
6	10	15	0.1	-1	1	-1	15±1
7	60	5	0.1	1	-1	-1	5.5±0.1
8	10	5	2.5	-1	-1	1	5.7±0.2
9	35	10	2.5	0	0	1	16±1
10	35	5	1.3	0	-1	0	5.6±0.4
11	60	15	0.1	1	1	-1	14.2±0.2
12	60	5	0.1	1	-1	-1	5.6±0.2
13	60	5	2.5	1	-1	1	4.4±0.3
14	60	15	2.5	1	1	1	18.80±0.04
15	60	15	2.5	1	1	1	17.6±0.5
16	10	5	2.5	-1	-1	1	6.2±0.3
17	10	10	1.3	-1	0	0	12.2±0.4
18	60	10	1.3	1	0	0	11.5±0.2
19	60	5	2.5	1	-1	1	5.5±0.4
20	35	10	1.3	0	0	0	10.7±0.2
21	10	10	1.3	-1	0	0	12.1±0.6
22	10	15	0.1	-1	1	-1	14±1
23	35	10	2.5	0	0	1	17±1
24	35	15	1.3	0	1	0	15.8±0.5
25	35	10	0.1	0	0	-1	10.2±0.3
26	35	5	1.3	0	-1	0	5.3±0.5
27	35	15	1.3	0	1	0	16.2±0.3
28	35	10	1.3	0	0	0	11.2±0.8
29	10	5	0.1	-1	-1	-1	4.2±0.1
30	60	15	0.1	1	1	-1	14.0±0.2
31	60	10	1.3	1	0	0	11.7±0.2
32	10	5	0.1	-1	-1	-1	4.68±0.09

Design of experiments considers 2 replicates for each condition and 4 replicates for central point (0, 0, 0)  
 $y$  values are presented as mean value ± standard deviation (3 replicates)

Variables: Autoclaving time ( $x_1$ ): 10 min (-1), 35 min (0), 60 min (1); Biomass:acid ratio ( $x_2$ ): 5% (-1), 10% (0), 15% (1); [H<sub>2</sub>SO<sub>4</sub>] ( $x_3$ ): 0.1% (-1), 1.3% (0), 2.5% (1);  $y$ - response: sugar concentration (g/L)

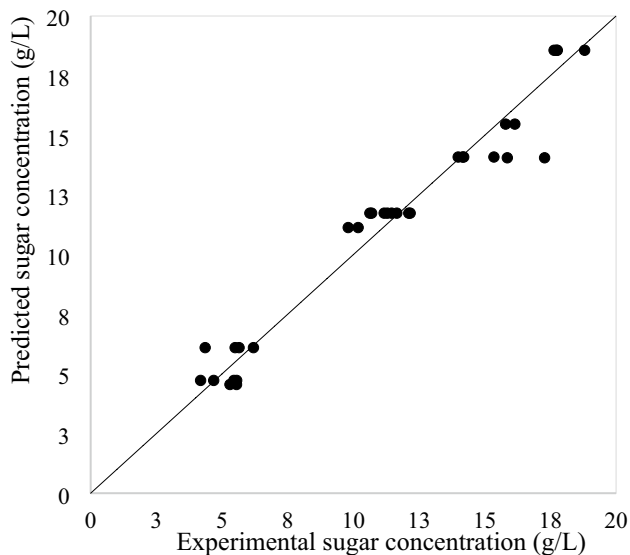
the linear, quadratic and interaction effects between variables. Preliminary fitting for the sugar concentration resulted in a predictive model with a determination coefficient ( $r^2$ ) of 0.9945, suggesting a very good adjustment. However, the estimated model parameters, which include the variable time (linear ( $x_1$ ), quadratic ( $x_1^2$ ) and interactions ( $x_1x_2$ ,  $x_1x_3$ )) were not significant ( $p > 0.05$ ), and for that reason, the model was not considered valid.

Therefore, a reconstructed model (considering statistically valid parameters;  $p < 0.05$  as defined in “[Thermal Acid Hydrolysis: Experimental Design and Laboratory Assays](#)”) was used to fit experimental data. The quadratic effect of

the acid concentration was also included in the model due to being statistically valid at a slightly lower confidence level (confidence level > 93%;  $p$  value = 0.0667). The regression model showed a  $r^2$  of 0.9876 and a RMSE value of 0.98 g/L (Prob > F lower than 0.05). The final equation (dimensionless variables) is given by Eq. 1.

$$y = 11.74 + 5.46x_2 + 1.46x_3 - 1.72x_2^2 + 0.86x_3^2 + 0.78x_2x_3 \quad (1)$$

Figure 1 shows the experimental results versus the results predicted by the model (Eq. 1). This figure shows that the model represents a relatively good description



**Fig. 1** Predicted versus experimental results concerning sugars concentration ( $r^2=0.96$ ). Line corresponds to zero error between experimental and predicted values ( $y=x$ )

of the experimental data regarding sugar concentration ( $r^2=0.96$ ;  $p < 0.0001$ ).

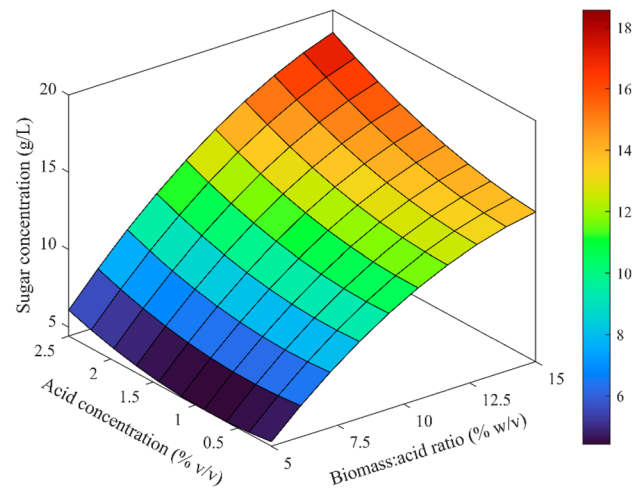
The modelling results showed that the most significant effect was the biomass:acid ratio linear effect, followed by the linear effect of acid concentration. The other effects showed similar and lower significance.

The determination of critical points using JMP resulted in a saddle point ( $x_2 = 1.26$ ;  $x_3 = -1.42$ ), which is a stationary point (not an extreme) outside the data range; this point corresponds to a sugar concentration of 14.1 g/L. Using Microsoft Excel Solver, under the range of studied conditions, the maximum was found to be  $x_2 = 1$  (15 g) and  $x_3 = 1$  (2.5%  $H_2SO_4$ ), corresponding to a sugar concentration of 18.4 g/L. Figure 2 represents the conditions that lead to such maximum sugar concentration.

It can be observed that there is a clear influence of the MMW amount (biomass:acid ratio) and acid concentration in the sugar release, which is traduced by higher sugar concentrations.

The minimum sugar concentration estimated was around 5 g/L, when the minimum amount of biomass was used (biomass:acid ratio of 5% (w/v)), and the acid concentration was either 0.1 or 1.3%. For higher acid concentrations (up to 2.5%), even for the lower amount of biomass, it was observed, however, an increase in the sugar concentration.

Figure 2 shows that the best hydrolysis conditions were obtained at the maximum biomass:acid ratio (15% (w/v)) using an acid concentration of 2.5% (v/v), which was corroborated by the maximum of the model function (determined using Microsoft Excel Solver).



**Fig. 2** Response surface for sugar concentration (g/L) under different conditions studied (biomass:acid ratio: 5–15% w/v; acid concentration 0.1–2.5% (v/v))

It is not possible to perform a deep comparison with results from the literature, since the studies using MM consider different methodologies to report the sugar content, and only one study was found focusing on the hydrolysis of MMW (from Gwangalli beach, Korea) [11]; such study is thus presented in more detail hereafter. Sunwoo et al. [11] evaluated the influence of biomass loading (6–10% (w/v)), acid concentration (1.1–2.4%) and hydrolysis time (60–120 min) on monosaccharides concentration in the hydrolysate (similar range of conditions evaluated in the present study). Using RSM, the following optimal conditions were obtained: 8% (w/v) biomass loading, 1.53%  $H_2SO_4$ , and 90 min at 121 °C. Such conditions allowed to obtain 9.7 g/L of monosaccharides (including glucose, galactose, and mannitol), corresponding to 30% of the total carbohydrates content [11]. As in the present study, the linear effect of biomass:acid ratio in the polynomial equation was the parameter that mostly affected sugar concentration. However, the predictive model had a lower determination coefficient (0.97). In the present study, considering the best conditions given by the model, the maximum sugar concentration (18.4 g/L) corresponds to around 18% of the total carbohydrates, considering a MMW carbohydrate content of 70% [23, 26]. Such difference should be related to the differences in biomass composition since the MM used in the study by Sunwoo et al. [11] had the predominance of green and red species.

To validate the model, additional assays were conducted and the results compared to the ones predicted by the model (Table 3).

The comparison of the experimental results with the predicted by the model (Table 3) results in a good fitting ( $r^2=0.89$ ; RMSE=1.62 g/L), which corroborates the model

**Table 3** Validation of predicted vs experimental values

Autoclaving time (min)	Biomass:acid ratio (% w/v)	[H <sub>2</sub> SO <sub>4</sub> ] (% v/v)	Predicted sugar concentration (g/L)	Experimental sugar concentration (g/L)
10	5	1.3	4.56	6.5±0.2
10	10	0.1	11.13	9.2±0.6
10	10	2.5	14.06	12.8±0.6
10	15	1.3	15.47	17.2±0.3
35	5	0.1	4.73	5.5±0.3
35	5	2.5	6.10	8±1
35	15	0.1	14.09	13.6±0.3
35	15	2.5	18.56	19±2
60	5	1.3	4.56	5.8±0.5
60	10	0.1	11.13	10.1±0.2
60	10	2.5	14.06	11.7±0.1
60	15	1.3	15.47	18±2

quality to predict sugar concentration in the range of the studied variables.

### Critical Analysis

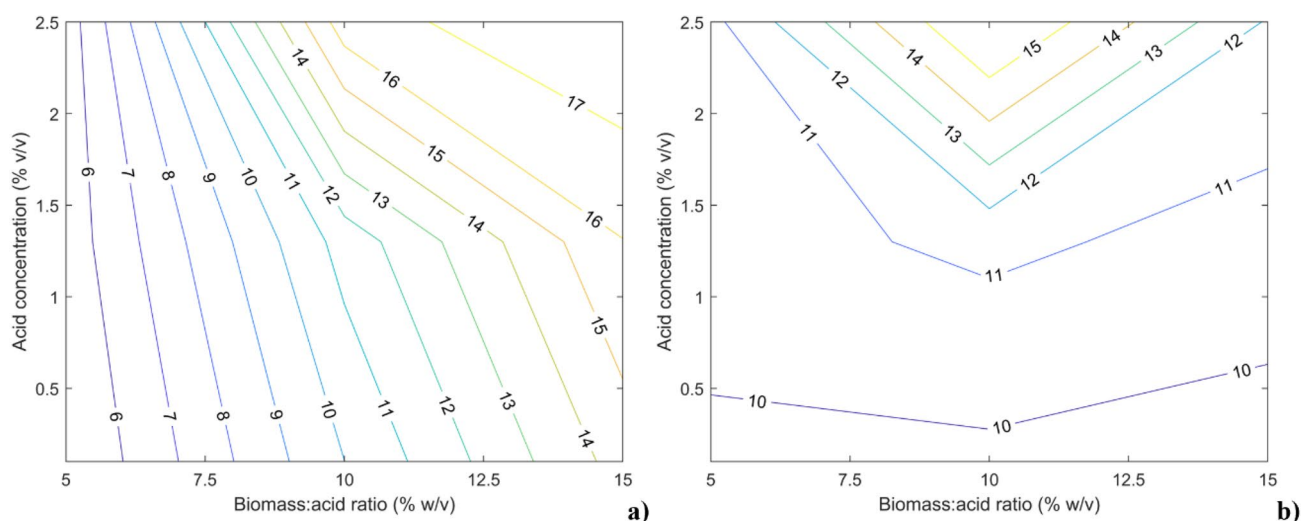
The conditions that offer an optimum sugar concentration were those considering 15% biomass:acid ratio and 2.5% acid concentration (v/v). The analysis of the model fitting (“**Model Fitting**”) showed that, under the studied conditions (5–15% (w/v) biomass:acid ratio; 0.1–2.5% (v/v) acid concentration; and 30–90 min reaction time), the time is not a significant variable. Thus, it is advised to follow the shorter time of 10 min.

In addition, a complete and critical analysis should be carried out before the upscaling of the process. As the objective is to obtain a higher sugar concentration (to be further

consumed by the yeast in the fermentation step) but without compromising the biomass conversion yield (i.e., g of sugars per 100 g of biomass), the best conditions should be those that combine such response variables. In addition to the sugar concentration and yields variables, further studies should consider the characterisation of the hydrolysates in terms of potential inhibitors.

Figure 3 shows the experimental results concerning the influence of acid concentration and biomass:acid ratio in sugar concentration (Fig. 3a) and in biomass conversion yield (Fig. 3b).

The experimental data reveal that higher sugar concentrations are obtained when the higher acid concentration (2.5% v/v) and biomass:acid ratio (15% (w/v) are considered (Fig. 3a), as previously described by the model; such conditions led to a sugar concentration of around 18 g/L,



**Fig. 3** Influence of acid concentration (% v/v) and biomass:acid ratio (% w/v) on: **a**) sugar concentration (g/L) and **b**) biomass conversion yield (g/100 g)



and a biomass conversion yield of around 12 g/100 g. However, if the goal is to maximise the biomass conversion yield (Fig. 3b), the biomass:acid ratio should be reduced to 10% (w/v). In this case, although sugar concentration in the hydrolysate decreased (16.6 g/L), the biomass conversion yield increased by around 36% (16.3 g/100 g) compared to the use of higher biomass amounts. Such biomass conversion yields are in the same order of magnitude of those reported in the literature (~20 g/100 g) [5, 7].

Considering that the aim is not only to obtain a hydrolysate with high sugar concentration, but also to maximise MMW recovery both parameters should be considered. Thus, since the use of 10% (w/v) biomass:acid ratio allows to obtain a higher biomass conversion yield, the optimum hydrolysis conditions established for further bioethanol production through fermentation processes are: 10 min of hydrolysis time, 10 or 15% (w/v) biomass:acid ratio depending on the purpose, and 2.5% H<sub>2</sub>SO<sub>4</sub> concentration.

## Ethanol Production

At Table 4 are presented the results obtained from the fermentation of the hydrolysates at the best hydrolysis conditions (10 min of hydrolysis time, 2.5% H<sub>2</sub>SO<sub>4</sub> concentration, and 15 or 10% (w/v) biomass:acid ratio), together with those referenced by the literature concerning other MM species/brown seaweeds.

The results of the present study (Table 4) show that an increase in the biomass:acid ratio led to a slight increase in the bioethanol concentration since more sugars are available to be consumed by the yeast. However, the obtained results are very close and thus the differences are not considered relevant. On the contrary, the bioethanol yield decreased with the increase of the biomass:acid ratio, indicating that the yeast does not consume all the available sugars. In fact, the used yeast was able to grow in algal hydrolysates, but at the end of the fermentation step, considering the final

total sugars concentration, only around 47 and 32% of sugars were consumed, for the condition using 10 and 15% (w/v) biomass:acid ratio, respectively. In that sense, the condition considering a biomass:acid ratio of 10% (w/v) appears to be the most appropriate for bioethanol production from MMW, allowing both a higher biomass conversion yield to sugars and a higher bioethanol yield.

No studies were found considering the fermentation of MMW to obtain bioethanol. Comparing with the use of MM (Table 4), the results of ethanol concentration obtained are in the same order of magnitude (2.74–4 g/L) [4, 9, 28]. Although being within the range reported in the literature for other brown algae, a direct comparison of the results cannot be made since it concerns different macroalgae species, as well as different hydrolysis and fermentation conditions. The results are highly satisfactory, considering that a commercial yeast was used and that the optimisation of the fermentation process was not conducted. Thus, there is margin for improvements in future studies.

## Conclusion

The optimisation study of marine macroalgae waste hydrolysis allowed to obtain a significant second-order model ( $r^2=0.9876$ ; Prob > F lower than 0.05), describing the influence of hydrolysis time, biomass:acid ratio and acid concentration, isolated and combined, on the sugar concentration in the hydrolysates. The model shows that the biomass:acid ratio is the parameter which mostly influences sugar concentration while reaction time was not a significant variable. The optimal conditions to maximise sugar concentration (~18 g/L) were obtained using 15% (w/v) of biomass and H<sub>2</sub>SO<sub>4</sub> at 2.5%. Under such conditions, the biomass to sugars conversion yield (12.5 g/100 g) was, however, compromised by 36% when compared with the use of only 10% (w/v).

**Table 4** Bioethanol concentration (g/L) and yield (mg<sub>bioethanol</sub>/g<sub>biomass</sub>) in the hydrolysates obtained at the best hydrolysis conditions (10 min of autoclaving time and H<sub>2</sub>SO<sub>4</sub> at 2.5% (v/v)) and comparison with literature

	Fermentation conditions	Bioethanol concentration (g/L)	Bioethanol yield (mg <sub>bioethanol</sub> /g <sub>biomass</sub> )	References
Present study: MMW; 10% w/v	7 days, 30 °C, 150 rpm, commercial yeast	2.2 ± 0.1	21 ± 1	–
Present study: MMW; 15% w/v		2.37 ± 0.04	15.8 ± 0.2	–
<i>Sargassum</i> sp. (brown algae)	48 h, 40 °C, <i>Saccharomyces cerevisiae</i>	2.79	81*	[9]
<i>Saccharina latissima</i> (brown algae)	72 h, 32 °C, <i>U. laminarinase</i> and Ethanol Red yeast	4	–	[4]
<i>Ulva lactuca</i> (green algae)	72 h, 30 °C, <i>Saccharomyces cerevisiae</i>	2.8	–	[25]

Results of the present study are presented as mean value ± standard deviation

MMW: Marine Macroalgae Waste

\*An average bioethanol conversion of 81% is reported

The best conditions to ferment the obtained hydrolysates imply the use of a biomass:acid ratio of 10% (w/v) and lead to a bioethanol concentration of 2.2 g/L, corresponding to a yield of 21 mg<sub>bioethanol</sub>/g<sub>biomass</sub>, in the range of the literature. Although with margin for improvements (both in hydrolysis and fermentation), the results show that marine macroalgae waste can be a potential feedstock for bioethanol production.

**Author Contributions** All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by SP and JO. The first draft of the manuscript was written by SP and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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**Data Availability** The datasets generated during and/or analysed during the current study are available from the corresponding authors on reasonable request.

## Declarations

**Competing interest** The authors have no relevant financial or non-financial interests to disclose.

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