

Manuscript Number: WM-18-3082R1

Title: Bioethanol production from industrial algae waste

Article Type: VSI:Biowaste-to-Biofuels

Section/Category: Thermal treatment

Keywords: industrial algae waste; bioethanol; acid hydrolysis;
fermentation; solid biomass

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Abstract: This paper presents a study carried out to determine the feasibility to obtain bioethanol from an industrial algae waste. The influence of some parameters on acid hydrolysis was studied. The results indicated that the overall hydrolysis ability is a function of the combined action of acid concentration, temperature and reaction time. The hydrolysates with higher amount of reducing sugars were used for ethanolic fermentation using *S. Cerevisiae*. The highest bioethanol yield 11.6 gEtOH/galgae was obtained at 70 min, a sulphuric acid concentration of 9% wt. and acid/dried algae ratio of 7. The present study showed that the industrial waste of *Euchema Spinosum* contains carbohydrates that can be converted into bioethanol with an efficiency of 75%. Moreover, the potential of the residue after acid hydrolysis process as sustainable solid fuel regarding its properties was analysed. The results were compared with those obtained from the literature and discussed according to international standards.

*Highlights

Bioethanol production from industrial waste of *Euchema Spinosum*

Algae waste contains carbohydrates that can be converted into bioethanol

The ethanol conversion efficiency was about 75% of theoretical

Subsequent study of the final residue after acid hydrolysis as solid biofuel

1 Bioethanol production from industrial algae waste

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8

9 ABSTRACT

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24 1. Introduction

25 The present growing energy demand, the limited fossil resources and the need to reduce
26 carbon dioxide emissions in atmosphere are promoting the use of renewable energies
27 (RE), protecting the climate, encouraging economic development and strengthen the
28 energy security (Suman, 2018; Gouveia, Fortes and Seixas, 2012).

29 One of renewable resource are biofuels, this kind of fuel can be defined as a liquid or
30 gaseous non-toxic fuel produced from various biological material with reduced
31 emission levels compared to diesel (Bildirici, 2017). Their utilization leads to a decrease
32 in the harmful emissions of CO_2 and hydrocarbons and, to the elimination of SO_x
33 emissions, with a consequent decrease in the greenhouse effects (Shuba and Kifle,

2018). It is for this reason that biofuels are positioned as a resource to replace current fossil fuels.

Moreover, in the 2°C Scenario (2DS) released by the IEA for transport, it is predicted that biofuel will provides until a 30.7% of total transport energy demand for the year 2060. In order to reach this objective, the production of biofuels should be increased by a factor of 10 (Frankl, 2018).

First and second generation biofuels cannot meet global demands in sustainable way (Adeniyi, Azimov and Burluka, 2018). The shortage of feedstock is the main disadvantage for these kind of biofuels. The third generation biofuels are referred to the bio-oil made from non-food feedstock and often derived from high-yield microalgae and macroalgae biomass (Sudhakar et al., 2018). These organisms have a high oil mass fraction thus showing a great potential for production of biofuels.

The processes such as anaerobic digestion, fermentation, transesterification, liquefaction and pyrolysis are able to convert algal biomass into biofuels, such as biogas, bioethanol, biodiesel and bio-oils (Chen et al., 2015)

Macroalgae and microalgae are catalogued as the most promising bio-feedstock for renewable fuel production (Vassilev and Vassileva, 2016). These organisms are feed with the energy provided of sun, water, nutrients and carbon dioxide (MamathaKumari et al., 2015). Moreover, they are easy to be cultivated on non-arable land using low quality water and they can grow without attention using only water not necessarily for human consumption (Mata, Martins and Caetano, 2010). Microalgae grow in any water habitat, even on wastewater diminishing the lack of freshwater (Hanifzadeh et al., 2018); whereas most macroalgae species can be found in the marine environment.

The advantages of macroalgae against microalgae are that they have similar characteristics as plants (Maceiras et al., 2011), making the harvesting process more

simply, a significant amount of biomass could be obtained due to their size, and the possibility of collecting the algae residue from beaches or even industry (Sudhakar et al., 2018).

The production of biofuels from macroalgae biomass receives less attention; however, there are different ways of biofuel production from biochemical and thermochemical processes. Several of these products, such as bioethanol, can be obtained through different conversion mechanisms: anaerobic digestion, fermentation, transesterification, liquefaction and pyrolysis processes (Chen et al., 2015).

The production of bioethanol from macroalgae is a good option due to their high carbohydrate content. For this conversion to take place, it is necessary to carry out a hydrolysis with a subsequent fermentation with bacteria or yeast (Sudhakar et al., 2018).

Macroalgae can be classified into three major groups based on their photosynthetic pigmentation variations: red (*Rhodophyta*) that grow in warm water (inter-tropical), brown (*Phaeophyta*) that grow under low-intermediate temperatures and green algae (*Chlorophyta*) that could grow in almost any kind of water temperature (Chen et al., 2015). From the red seaweed *Eucheuma Spinosum*, the agar is produced with the carrageenan from the algae. Agar is a viscous polysaccharide compound (70% agarose, 30% agarpectin) that comprises the cell walls of red algae. It is used for various applications, mainly as a food technology: thickener for soups, vegetable gelatines, ice cream and some desserts and as a beer-clarifying agent (Kim et al., 2013).

This paper studies the production of bioethanol of third generation from macroalgae residue of the industrial extraction of agar. The process begins with the pretreatment of the algae residue *Eucheuma Denticulatum (Spinosum)*, followed by an acid hydrolysis, whose variables will be optimized, to obtain the maximum possible quantity of reducing sugars. Then, these reducing sugars are fermented with yeast (*Saccharomyces*

cerevisiae) in order to obtain bioethanol. Finally, the biofuel is separated by distillation and characterized. Moreover, the residue after acid hydrolysis is analysed with the aim to be used as solid fuel.

2. Materials and Methods

2.1. Biomass

Eucheuma Denticulatum Spinosum macroalga was provided by CEAMSA (Spain). This alga is a waste of its industrial process where they remove the carrageenan to the algae. This waste contains a considerable amount of water (about 35%); therefore, the samples were dried at room temperature during 24 hours, reaching a level of humidity of 5%. Proximate analysis of the macroalgae waste consists of the determination of moisture content, organic matter, total Kjeldahl nitrogen, ammonium nitrogen, total organic carbon and minerals (Cd, Ca, Cr, Cu, Fe, Pb, Mg) was carried out. The moisture content of the dried samples was determined using a halogen moisture analyser RADWAG MA 110.R. The cellulose content of macroalgae was determined using the method proposed by Van Soest and Wine (1967). The analysis of organic matter was carried out ashing the waste at 600 °C. The total Kjeldahl nitrogen and ammonium nitrogen were measured by spectrophotometry. Total organic carbon was determined by combustion followed by infrared spectrometry. The minerals were analyzed by digestion and ICP determination.

2.2. Acid hydrolysis

In acid hydrolysis tests, 10 grams of macroalgae waste were mixed with H₂SO₄ at different acid concentrations (3, 5, 7, 9 wt. %) and different acid/dried algae ratio (7:1, 10:1, 13:1 v/w). The mixture was placed into in an autoclave at 121 °C at different reaction times (35, 70, 105 min). Then, the samples were cooled at room temperature

and centrifuged at 4000 rpm for 4 min. Finally, the obtained supernatant was analysed for reducing sugars.

With the aim to evaluate the effect of different variables on acid hydrolysis, some tests were conducted using an experimental design devised by George E. P. Box and Donald Behnken in 1960 (Box and Beknken, 1960). This method was used for the acid hydrolysis optimization using the following variables: sulphuric acid concentration (5–9 w/v), acid/dried algae ratio (7:1-13:1 v/w) and reaction time (35-105 min). For instance, the Box–Behnken design for three factors needs to include a centre point and two equidistant axial points. The experimental design matrix is shown in Table 1.

2.3. Fermentation

After hydrolysis and centrifugation, the obtained algal hydrolysate was subjected to fermentation with 0.5 grams of *Saccharomyces cerevisiae*. Previous this step, the pH of the samples was adjusted from 1.0 to 4.5 using sodium hydroxide. Fermentation process was performed at 30 °C for 24 h. Finally, samples were filtered, distilled and analysed for ethanol content.

2.4. Analytical methods

The glucose content of hydrolysed samples was determined by DNS method (Miller, 1959). The DNS reacts with the reducing sugars allowing a quantitative spectrophotometric measurement. Thus, the amount of reducing sugars present was measured at 540 nm using a UV VIS spectrophotometer SPEKOL 1500.

After fermentation, the ethanol content was measured using two techniques: potassium dichromate method (Williams and Reese, 1950) and refractometry. The potassium dichromate method reacts with ethanol in the presence of sulphuric acid forming acetic acid. After that, ethanol concentration is determined spectrophotometrically at 584 nm

using a UV VIS spectrophotometer SPEKOL 1500. For refractometry measurements, the refraction index was determined using a refractometer ABBE-REF 1. The yield of the hydrolysis was determined as the amount of extracted reducing sugars per total amount of theoretical glucose in the algae according the following equation:

$$\text{hydrolysis yield (\%)} = \frac{\text{extracted reducing sugars (g)}}{\text{theoretical glucose (g)}} \times 100$$

For a total conversion, the glucose theoretically obtained is the same that the amount of cellulose in the algae waste. Whereas, total reducing sugars are obtained by DNS method as follows:

$$\text{extracted reducing sugars (mg)} = \text{Concentration}_{DNS} \left(\frac{\text{mg}}{\text{ml}} \right) \times \text{Volume}_{test} (\text{ml})$$

Moreover, fermentation yield (Y) was expressed as obtained ethanol after distillation process per the amount of extracted reducing sugars

$$Y (\%) = \frac{\text{ethanol (g)}}{\text{extracted reducing sugars (g)}} \times 100$$

Additionally, fermentation efficiency correspond to the ratio of the produced ethanol over the theoretically produced ethanol (Sayed et al., 2018) as follows:

$$\text{Ethanol}_{o/t} (\%) = \frac{[\text{ethanol}]_{observed}}{[\text{ethanol}]_{theor}} \times 100$$

The bioethanol yield can be obtained using the following equation:

$$\text{bioethanol yield (g/g)} = \frac{\text{Volume}_{hydrolysate} (L) \times \text{Concentration}_{bioethanol} (g/L)}{10 \text{ g algae waste}}$$

Finally, the physical properties of the obtained residue after acid hydrolysis were tested. This analysis included moisture, calorific value, and elemental, inorganic compounds and immediate analysis according to the standards. The moisture content was determined according to UNE-EN 14774- 2. The analysis for determination of calorific value was performed in a Parr 1351 bomb calorimeter, following the standard UNE-EN 14918. The elemental analysis for determination of carbon, hydrogen, nitrogen and

sulfur in the residue was measured in a TruSpec Lecno CHNS 4084 according to UNE-EN 15104. The inorganic elements were analyzed using Wavelength Dispersive X-ray Fluorescence (WDXRF) in a S8 Tiger spectrometer. The immediate chemical analysis was based on standards to determine volatile materials (UNE-EN 15148) and ash content (UNE-EN 15403). Fixed carbon content (CF) was derived from the difference.

3. Results and Discussion

3.1. Biomass characterization

The moisture content was measured previous hydrolysis step for each test. The values varied from 4 to 6%. This value was similar to other species. A proximate of the waste algae used in this study is presented in table 2. It can be observed that the amount of organic matter is low compared with other red algae that show values from 55-65% (Rodrigues et al., 2015). This can be due to that the algae used in this research is a waste of an industrial process and they could lose organic matter in the process.

The macroalgae waste in this study was found to contain macro- and microminerals and heavy metals. It has been reported that the levels of elements in algae are variable and vary between species (Bonanno and Orlando-Bonaca, 2018) and macroalgae are able to accumulate levels of metals higher than the corresponding concentrations in surrounding waters (Al-Shwafi and Rushdi, 2008). Some researchers (Al-Shwafi and Rushdi, 2008; Rodrigues et al., 2015; Bonanno and Orlando-Bonaca, 2018) have studied different red algae obtaining the following results: the concentrations of Cd ranged from 0.17 to 93 µg/g, the levels of Cr varied from 0.7 to 12 µg/g, for Cu they varied from 0.2 to 17 µg/g, the concentrations of Fe ranged from 10.75 to 1610 µg/g and the levels of Pb varied from 0.35 to 2.80 µg/g. Few studies reported the concentrations of Ca and Mg in algae (Biancarosa et al., 2018; Hanifzadeh et al., 2018). The levels of minerals and heavy metals of *Eucheuma Denticulatum Spinosum* are among those values.

The cellulose content of the waste was about 37%. Moreover, the cellulose content of the *Eucheuma Denticulatum Spinosum* macroalgae without any treatment was also measured, obtaining a value of 77%. Thus, the industrial process to extract the carrageen diminishes the cellulose content. Nevertheless, other raw materials have been used to obtain bioethanol with similar cellulose content. Saha et al. (2008) studied bioethanol production from rice hulls with a cellulose content of 35%.

3.2. Optimization of acid hydrolysis

Different tests were done to study the effect of some variables on efficiency process based on the amount of reducing sugars in the algal hydrolysate. Obtained results showed that the amount of reducing sugars increases with the studied acid concentration (Fig. 1) and diminishes with the used acid/dried algae ratio (Fig. 2). Similar results were also reported by Borines et al. (2013) in experiments on acid hydrolysis of *Sargassum spp.*, where the reducing sugars yield increased with the acid concentration. Sunwoo et al. (2016) found that sugar concentration increased with acid concentration from 30 to 70 mM for *Kappaphycus alvarezii* and *Gracilaria verrucosa*.

Reaction time has also a great influence on reducing sugars. As shown in Fig. 3, the highest value of reducing sugars was obtained at 70 min. High reaction time in combination with high acid concentration and acid/dried algae ratio resulted to diminish the amount of reducing sugars, probably due to glucose decomposition under these conditions. Other species of red macroalgae showed similar results (Sunwoo et al., 2016).

All the obtained results were analyse using Box-Behnken method with the aim to stablsh the best conditions for the process. Figs. 4-6 show the influence of different conditions on the amount of reducing sugars (DNS). Fig. 4 reflects that an increase in hydrolysis time and acid/dried algae ratio increase the DNS value until a maximum (70

min and 10:1). Fig. 5 shows the influence of acid concentration and acid/dried algae ratio at 70 min, it can be observed a red region at high concentration level, indicating high amount of reducing sugars; whereas it is difficult to establish an optimum ratio. However, it seems that lower and higher values or ratios show similar values of reducing sugars. Taking into account that a higher value of ratio needs higher amount of acid for obtaining similar results, the value of 7 is considered suitable for the process. This finding is according with the results of Borines et al. (2013). Fig. 6 presents the 3D plot for DNS values with a fixed acid/dried algae ratio of 7:1 (v/w). This graph reveals that a maximum DNS is obtained at 70 min and high values of acid concentration. Based on these results, the predictive model for reducing sugars is the following:

$$DNS = 0,11859x - 0,0009x^2 + 0,72345y - 0,03745y^2 + 0,16256z - 0,01613z^2 - 0,02565xy + 0,0013xy^2 + 0,00019x^2y - 0,00001x^2y^2 + 0,00072xz$$

where DNS is the amount of reducing sugars (g/L), x the time (min), y the acid/dried algae ratio (v/w), z the acid concentration (% wt.)

These figures showed that the best analysed conditions in terms of reducing sugar yield were 70 min, a sulphuric acid concentration of 7% wt. and 7 mL acid per gram of algal waste. Additionally, another experiment (test number 16) increasing the acid concentration until 9% wt. was carried out to analyze its effect, obtaining that the amount of reducing sugars increased from 0.24 g/L (test 4.) to 0.30 g/L (test 16). Based on this result, the optimum conditions for acid hydrolysis of waste algae were found to be 70 min, a sulphuric acid concentration of 9% wt. and acid/dried algae ratio of 7.

Ashokkumar et al. (2017) obtaining an algal hydrolysate with 19.7% of reducing sugars after alkali hydrolysis of algal biomass (*Padina tetrastromatica*) collected from coastal region. Others authors (Borines et al., 2013) have carried out an enzymatic hydrolysis in macroalgae (*Sargassum spp.*) harvested in coastal waters, obtaining the highest amount

of glucose of 30 mg/g biomass after 96 hours and a cellulase loading of 50 FPU/g biomass. The present study show that macroalgae waste contains carbohydrates that can be extracted by acid hydrolysis.

3.3. Bioethanol production and characterization

After the study of the different conditions for hydrolysis, the tests with the highest value of DNS were undergone to fermentation process. The obtained results are collected in Table 3, also including the yields of the different processes. The obtained results showed that the test 2, with the highest amount of acid (70 min, acid concentration 5%, ratio 13:1) achieved higher yield of hydrolysis; however, test 16 (70 min, acid concentration 9%, ratio 7:1) obtained the highest amount of glucose (93.5 g/L) and bioethanol and fermentation yield. This can be due to that the test 2 has a great amount of acid resulting in higher amount of hydrolysate but with less amount of reducing sugars. Then, it could be said that the acid concentration has more effect on the process than the acid/dried algae ratio. This is according with the previous results obtained using Box-Behnken method.

In addition, the macroalgae waste could produce about 0.1 g bioethanol/g waste and 0.12 g bioethanol/g reducing sugars with an ethanol concentration of 36.6 g/L using acid hydrolysis and yeast fermentation. The ethanol conversion efficiency was about 75% of theoretical. These results are in a good agreement with other published papers (Eshaq et al., 2010).

3.4. Characterization of hydrolysis residue

The residues of acid hydrolysis were characterized by elemental, inorganic compounds and immediate analysis and their calorific value and moisture with the aim to study the possibility to be used as solid fuel. Table 4 collects the obtained results. With regards to elemental analysis, the nitrogen and sulphur content are in accordance with the UNE-

EN ISO 17225-6 (2014). Immediate analysis indicated that the moisture and ash content found in this residue are higher than the values of the standard. The fixed carbon and volatile matters are important parameters of biomass quality according to EN ISO 17225-1 (2014); nevertheless, they do not appear in the international standards. The fixed carbon is similar to wood pellets obtained by other authors (Roy et al., 2013); whereas the ash content is higher than the wood pellets. This value is related to the amount of inorganic elements. It can be observed in table 4, that there is a considerable number of inorganic compounds in the residue, increasing the ash content; so it would be suitable to remove these elements if the residue wanted to be used as solid biofuel. Finally, the calorific value is lower than the standard values. In a previous research (Maceiras et al., 2015), the pellets from different fresh macroalgae were characterized resulting in higher calorific values and volatile matter and similar results for fixed carbon and ash content. The high heating value is defined as the sum of the heat released when fuel is combusted and the heat of condensation of the water vapour formed inside a bomb calorimeter. However, in many processes this vapour is released to the atmosphere together with combustion gases. This could be the reason to obtain a lower heating value than fresh macroalgae.

4. Conclusions

In this research, the bioethanol production from industrial waste was investigated. The results indicated that the overall hydrolysis ability is a function of the combined action of acid concentration, temperature and reaction time. Higher acid concentrations resulted in general in higher hydrolysis rates. The highest reducing sugars yield was obtained at 70 min, an acid concentration of 9% wt. and ratio of 7. The hydrolysates were converted into bioethanol using *S. Cerevisiae* with a yield of 11.6 g/g and an efficiency of 75%. Moreover, the potential of the residue after acid hydrolysis process

as sustainable solid fuel regarding its properties was analysed. The results showed some differences compared to international standards. The main novelty of this research is the bioethanol production from industrial algae waste and the subsequent study of the final residue as solid biofuel.

Acknowledgements

The authors gratefully acknowledge support from Defense University Center and collaboration of Compañía Española de Algas Marina (CEAMSA).

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375

376 **Table 1.** Design matrix with values of independent variables.

Test number	Time (min)	Ratio (v/w)	Concentration (%)
1	35	7:1	5
2	70	13:1	5
3	35	10:1	7
4	70	7:1	7
5	105	10:1	7
6	70	7:1	3
7	70	13:1	3
8	105	10:1	3
9	35	10:1	3
10	35	13:1	5
11	70	10:1	5
12	70	10:1	5
13	70	10:1	5
14	105	13:1	5
15	105	7:1	5

377

378 **Table 2.** Characterization of industrial waste macroalgae.

Parameter	Value
Cellulose	37 %
Organic matter	13.2 %
Total Kjeldahl nitrogen	1180 mg/kg
Ammonium nitrogen	10.8 mg N/kg
Total organic carbon	2.89 %
Cd	1.46 µg/g
Ca	70100 µg/g
Cr	3.00 µg/g
Cu	3.30 µg/g
Fe	1210 µg/g
Pb	1.4 µg/g
Mg	5080 µg/g

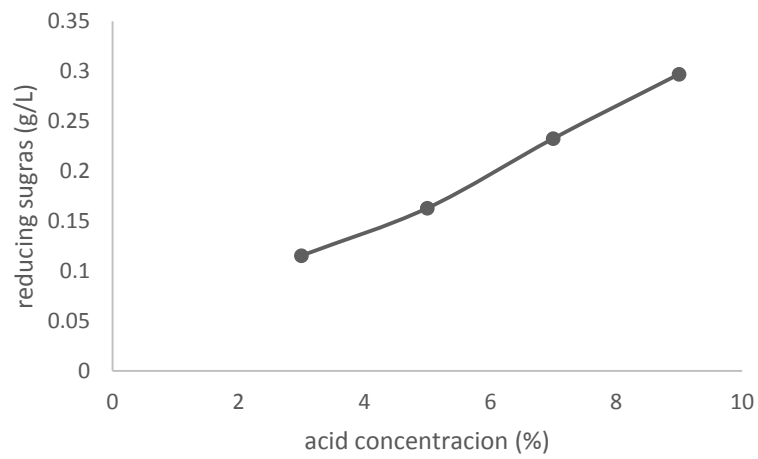
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Table 3. Yield of processes.

Test	Glucose (g/L)	Hydrolysis yield (%)	Fermentation yield (%)	Bioethanol yield (g/g)	Ethanol _{o/t} (%)
2	52.632	0.52	4.81	0.092	38.42
4	70.175	0.19	4.15	0.028	29.92
16	93.567	0.24	11.68	0.102	76.47

Acid hydrolysis residue properties	Value
Elemental analysis	
C	0.43%
H	0.02%
N	0.13%
S	0.20%
Inorganic elements	
Na ₂ O	3.49%
MgO	0.46%
Al ₂ O ₃	13.10%
SiO ₂	74.50%
P ₂ O ₅	963 ppm
BaO	0.22%
CaO	1.33%
TiO ₂	0.16%
SO ₃	0.59%
K ₂ O	5.03%
Fe ₂ O ₃	0.93%
SrO	173 ppm
Rb ₂ O	145 ppm
MnO	544 ppm
ZrO ₂	112 ppm
Immediate analysis	
Moisture	49.42%
Volatile materials	6.04%
Fixed carbon	3.77%
Ash content	40.77%
Calorific value	
Higher heating value (dry)	543.28 kcal/kg
Low heating value (dry)	245.71 kcal/kg
Higher heating value (wet)	389.61 kcal/kg
Low heating value (wet)	92.04 kcal/kg



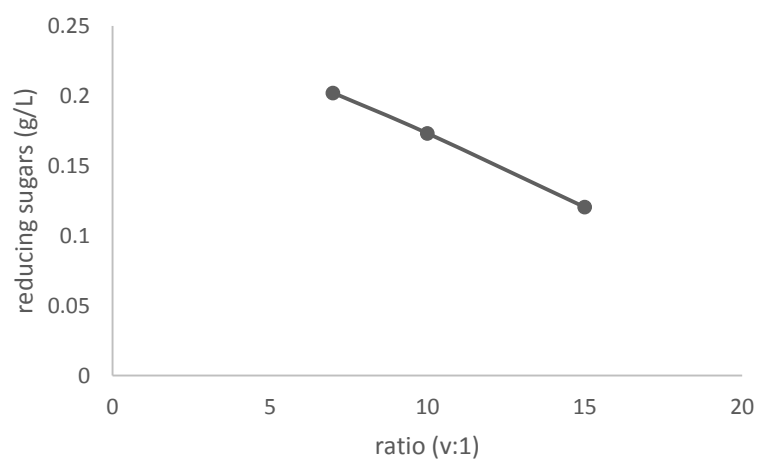
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387 Fig. 1. Effect of acid concentration on total reducing sugars

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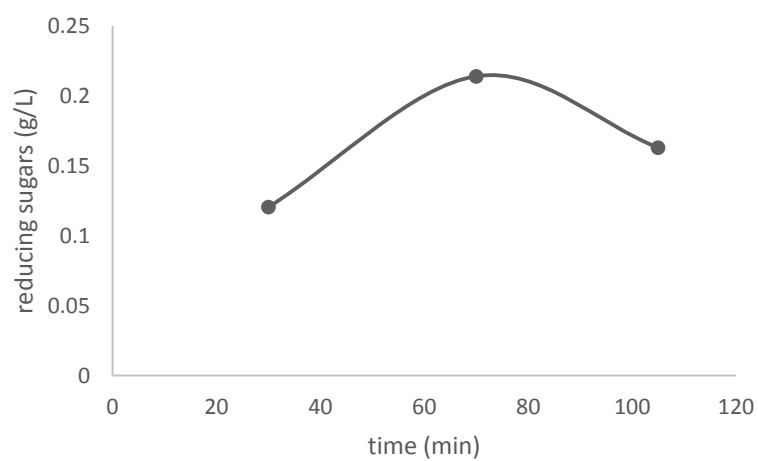
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391 Fig. 2. Effect of acid/dried algae ratio on total reducing sugars

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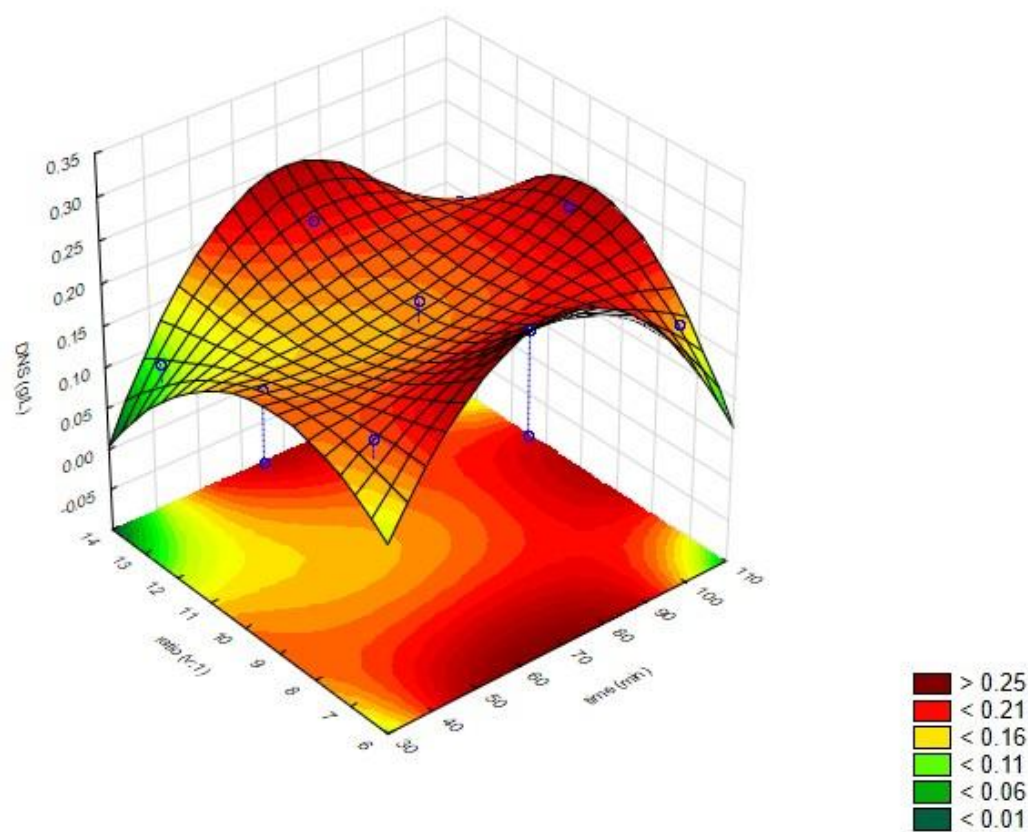
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396 Fig. 3. Effect of reaction time on total reducing sugars

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401 Fig. 4. Effect of acid/dried algae ratio and time on total reducing sugars at a fixed acid
402 concentration of 7%.

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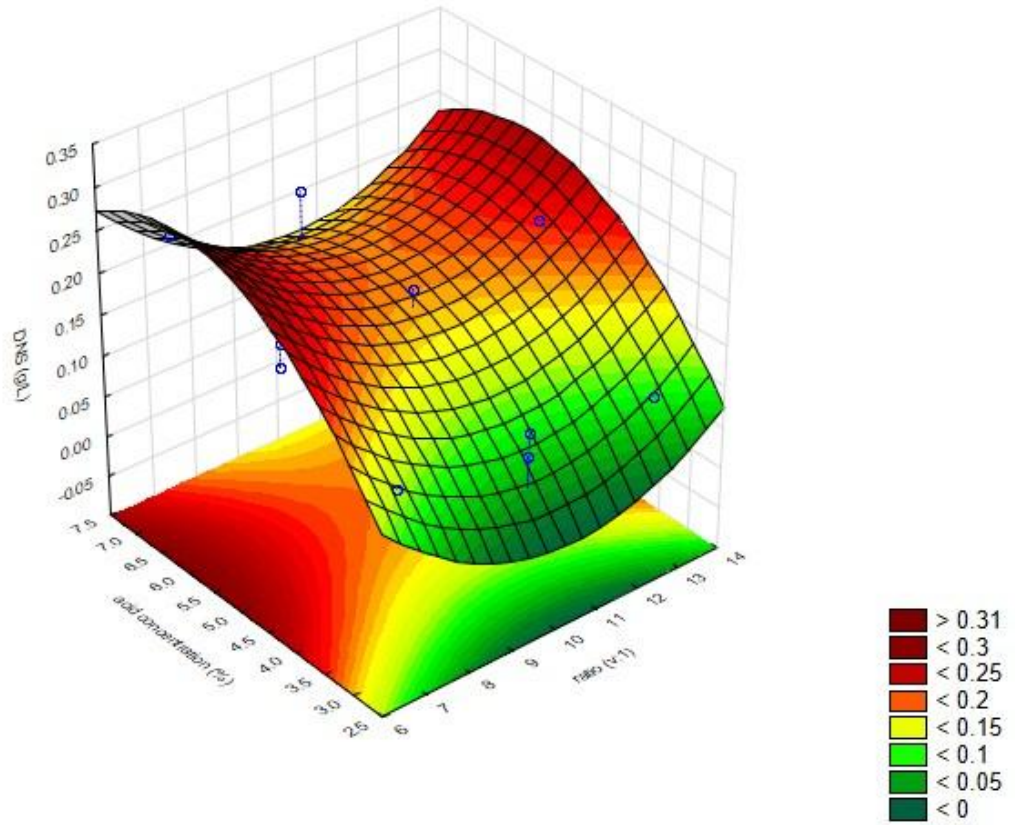
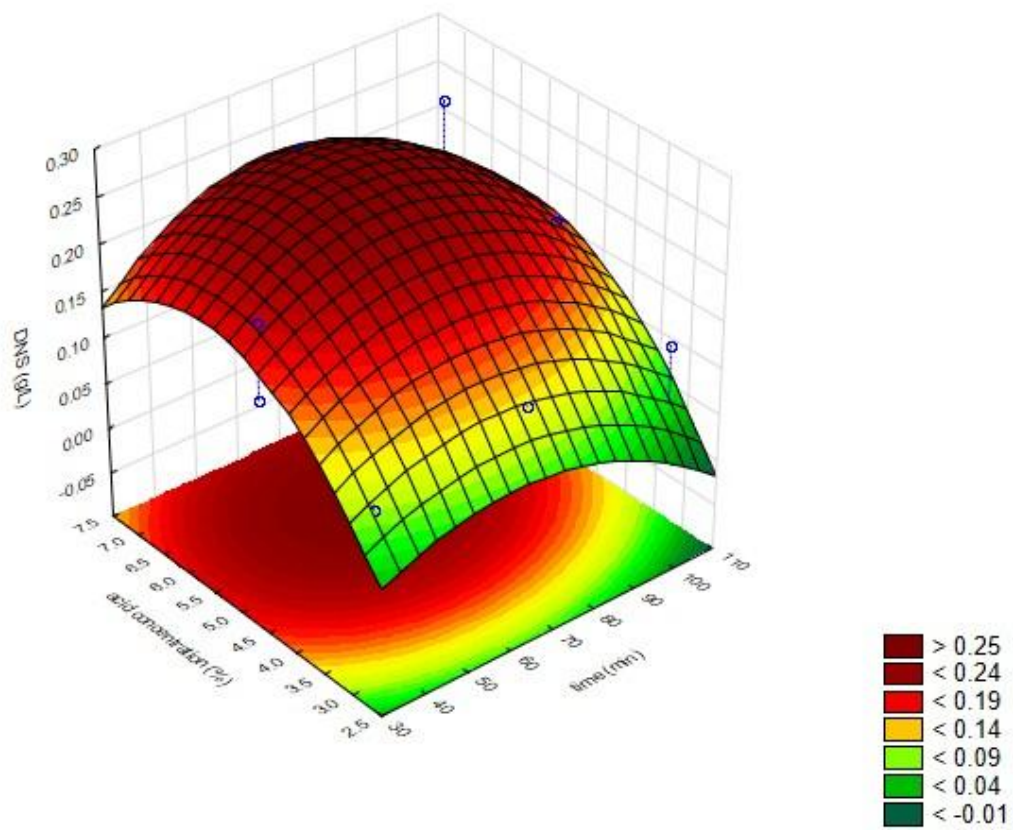


Fig. 5. Effect of acid/dried algae ratio and acid concentration on total reducing sugars at a fixed time of 70 min.



414

415 Fig. 6. Effect of acid concentration and time on total reducing sugars at a fixed

416 acid/dried algae ratio of 7:1.

417

1 Bioethanol production from industrial algae waste

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8

9 ABSTRACT

10 This paper presents a study carried out to determine the feasibility to obtain bioethanol
11 from an industrial algae waste. The influence of some parameters on acid hydrolysis
12 was studied. The results indicated that the overall hydrolysis ability is a function of the
13 combined action of acid concentration, temperature and reaction time. The hydrolysates
14 with higher amount of reducing sugars were used for ethanolic fermentation using *S.*
15 *Cerevisiae*. The highest bioethanol yield $11.6 \text{ g}_{\text{EtOH}}/\text{g}_{\text{algae}}$ was obtained at 70 min, a
16 sulphuric acid concentration of 9% wt. and acid/dried algae ratio of 7. The present study
17 showed that the industrial waste of *Euchema Spinosum* contains carbohydrates that can
18 be converted into bioethanol with an efficiency of 75%. Moreover, the potential of the
19 residue after acid hydrolysis process as sustainable solid fuel regarding its properties
20 was analysed. The results were compared with those obtained from the literature and
21 discussed according to international standards.

22 Keywords: industrial algae waste, bioethanol, acid hydrolysis, fermentation, solid
23 biomass

24 1. Introduction

25 The present growing energy demand, the limited fossil resources and the need to reduce
26 carbon dioxide emissions in atmosphere are promoting the use of renewable energies
27 (RE), protecting the climate, encouraging economic development and strengthen the
28 energy security (Suman, 2018; Gouveia, Fortes and Seixas, 2012).

29 One of renewable resource are biofuels, this kind of fuel can be defined as a liquid or
30 gaseous non-toxic fuel produced from various biological material with reduced
31 emission levels compared to diesel (Bildirici, 2017). Their utilization leads to a decrease
32 in the harmful emissions of CO_2 and hydrocarbons and, to the elimination of SO_x
33 emissions, with a consequent decrease in the greenhouse effects (Shuba and Kifle,

2018). It is for this reason that biofuels are positioned as a resource to replace current fossil fuels.

Moreover, in the 2°C Scenario (2DS) released by the IEA for transport, it is predicted that biofuel will provides until a 30.7% of total transport energy demand for the year 2060. In order to reach this objective, the production of biofuels should be increased by a factor of 10 (Frankl, 2018).

First and second generation biofuels cannot meet global demands in sustainable way (Adeniyi, Azimov and Burluka, 2018). The shortage of feedstock is the main disadvantage for these kind of biofuels. The third generation biofuels are referred to the bio-oil made from non-food feedstock and often derived from high-yield microalgae and macroalgae biomass (Sudhakar et al., 2018). These organisms have a high oil mass fraction thus showing a great potential for production of biofuels.

The processes such as anaerobic digestion, fermentation, transesterification, liquefaction and pyrolysis are able to convert algal biomass into biofuels, such as biogas, bioethanol, biodiesel and bio-oils (Chen et al., 2015)

Macroalgae and microalgae are catalogued as the most promising bio-feedstock for renewable fuel production (Vassilev and Vassileva, 2016). These organisms are feed with the energy provided of sun, water, nutrients and carbon dioxide (MamathaKumari et al., 2015). Moreover, they are easy to be cultivated on non-arable land using low quality water and they can grow without attention using only water not necessarily for human consumption (Mata, Martins and Caetano, 2010). Microalgae grow in any water habitat, even on wastewater diminishing the lack of freshwater (Hanifzadeh et al., 2018); whereas most macroalgae species can be found in the marine environment. The advantages of macroalgae against microalgae are that they have similar characteristics as plants (Maceiras et al., 2011), making the harvesting process more

simply, a significant amount of biomass could be obtained due to their size, and the possibility of collecting the algae residue from beaches or even industry (Sudhakar et al., 2018).

The production of biofuels from macroalgae biomass receives less attention; however, there are different ways of biofuel production from biochemical and thermochemical processes. Several of these products, such as bioethanol, can be obtained through different conversion mechanisms: anaerobic digestion, fermentation, transesterification, liquefaction and pyrolysis processes (Chen et al., 2015).

The production of bioethanol from macroalgae is a good option due to their high carbohydrate content. For this conversion to take place, it is necessary to carry out a hydrolysis with a subsequent fermentation with bacteria or yeast (Sudhakar et al., 2018).

Macroalgae can be classified into three major groups based on their photosynthetic pigmentation variations: red (*Rhodophyta*) that grow in warm water (inter-tropical), brown (*Phaeophyta*) that grow under low-intermediate temperatures and green algae (*Chlorophyta*) that could grow in almost any kind of water temperature (Chen et al., 2015). From the red seaweed *Eucheuma Spinosum*, the agar is produced with the carrageenan from the algae. Agar is a viscous polysaccharide compound (70% agarose, 30% agarpectin) that comprises the cell walls of red algae. It is used for various applications, mainly as a food technology: thickener for soups, vegetable gelatines, ice cream and some desserts and as a beer-clarifying agent (Kim et al., 2013).

This paper studies the production of bioethanol of third generation from macroalgae residue of the industrial extraction of agar. The process begins with the pretreatment of the algae residue *Eucheuma Denticulatum (Spinosum)*, followed by an acid hydrolysis, whose variables will be optimized, to obtain the maximum possible quantity of reducing sugars. Then, these reducing sugars are fermented with yeast (*Saccharomyces*

cerevisiae) in order to obtain bioethanol. Finally, the biofuel is separated by distillation and characterized. Moreover, the residue after acid hydrolysis is analysed with the aim to be used as solid fuel.

2. Materials and Methods

2.1. Biomass

Eucheuma Denticulatum Spinosum macroalga was provided by CEAMSA (Spain). This alga is a waste of its industrial process where they remove the carrageenan to the algae. This waste contains a considerable amount of water (about 35%); therefore, the samples were dried at room temperature during 24 hours, reaching a level of humidity of 5%.

Proximate analysis of the macroalgae waste consists of the determination of moisture content, organic matter, total Kjeldahl nitrogen, ammonium nitrogen, total organic carbon and minerals (Cd, Ca, Cr, Cu, Fe, Pb, Mg) was carried out. The moisture content of the dried samples was determined using a halogen moisture analyser RADWAG MA 110.R. The cellulose content of macroalgae was determined using the method proposed by Van Soest and Wine (1967). The analysis of organic matter was carried out ashing the waste at 600 °C. The total Kjeldahl nitrogen and ammonium nitrogen were measured by spectrophotometry. Total organic carbon was determined by combustion followed by infrared spectrometry. The minerals were analyzed by digestion and ICP determination.

2.2. Acid hydrolysis

In acid hydrolysis tests, 10 grams of macroalgae waste were mixed with H₂SO₄ at different acid concentrations (3, 5, 7, 9 wt. %) and different acid/dried algae ratio (7:1, 10:1, 13:1 v/w). The mixture was placed into in an autoclave at 121 °C at different reaction times (35, 70, 105 min). Then, the samples were cooled at room temperature

and centrifuged at 4000 rpm for 4 min. Finally, the obtained supernatant was analysed for reducing sugars.

With the aim to evaluate the effect of different variables on acid hydrolysis, some tests were conducted using an experimental design devised by George E. P. Box and Donald Behnken in 1960 (Box and Beknken, 1960). This method was used for the acid hydrolysis optimization using the following variables: sulphuric acid concentration (5–9 w/v), acid/dried algae ratio (7:1-13:1 v/w) and reaction time (35-105 min). For instance, the Box–Behnken design for three factors needs to include a centre point and two equidistant axial points. The experimental design matrix is shown in Table 1.

2.3. Fermentation

After hydrolysis and centrifugation, the obtained algal hydrolysate was subjected to fermentation with 0.5 grams of *Saccharomyces cerevisiae*. Previous this step, the pH of the samples was adjusted from 1.0 to 4.5 using sodium hydroxide. Fermentation process was performed at 30 °C for 24 h. Finally, samples were filtered, distilled and analysed for ethanol content.

2.4. Analytical methods

The glucose content of hydrolysed samples was determined by DNS method (Miller, 1959). The DNS reacts with the reducing sugars allowing a quantitative spectrophotometric measurement. Thus, the amount of reducing sugars present was measured at 540 nm using a UV VIS spectrophotometer SPEKOL 1500.

After fermentation, the ethanol content was measured using two techniques: potassium dichromate method (Williams and Reese, 1950) and refractometry. The potassium dichromate method reacts with ethanol in the presence of sulphuric acid forming acetic acid. After that, ethanol concentration is determined spectrophotometrically at 584 nm

using a UV VIS spectrophotometer SPEKOL 1500. For refractometry measurements, the refraction index was determined using a refractometer ABBE-REF 1. The yield of the hydrolysis was determined as the amount of extracted reducing sugars per total amount of theoretical glucose in the algae according the following equation:

$$\text{hydrolysis yield (\%)} = \frac{\text{extracted reducing sugars (g)}}{\text{theoretical glucose (g)}} \times 100$$

For a total conversion, the glucose theoretically obtained is the same that the amount of cellulose in the algae waste. Whereas, total reducing sugars are obtained by DNS method as follows:

$$\text{extracted reducing sugars (mg)} = \text{Concentration}_{DNS} \left(\frac{\text{mg}}{\text{ml}} \right) \times \text{Volume}_{test} (\text{ml})$$

Moreover, fermentation yield (Y) was expressed as obtained ethanol after distillation process per the amount of extracted reducing sugars

$$Y (\%) = \frac{\text{ethanol (g)}}{\text{extracted reducing sugars (g)}} \times 100$$

Additionally, fermentation efficiency correspond to the ratio of the produced ethanol over the theoretically produced ethanol (Sayed et al., 2018) as follows:

$$\text{Ethanol}_{o/t} (\%) = \frac{[\text{ethanol}]_{observed}}{[\text{ethanol}]_{theor}} \times 100$$

The bioethanol yield can be obtained using the following equation:

$$\text{bioethanol yield (g/g)} = \frac{\text{Volume}_{hydrolysate} (L) \times \text{Concentration}_{bioethanol} (g/L)}{10 \text{ g algae waste}}$$

Finally, the physical properties of the obtained residue after acid hydrolysis were tested. This analysis included moisture, calorific value, and elemental, inorganic compounds and immediate analysis according to the standards. The moisture content was determined according to UNE-EN 14774- 2. The analysis for determination of calorific value was performed in a Parr 1351 bomb calorimeter, following the standard UNE-EN 14918. The elemental analysis for determination of carbon, hydrogen, nitrogen and

sulfur in the residue was measured in a TruSpec Lecno CHNS 4084 according to UNE-EN 15104. The inorganic elements were analyzed using Wavelength Dispersive X-ray Fluorescence (WDXRF) in a S8 Tiger spectrometer. The immediate chemical analysis was based on standards to determine volatile materials (UNE-EN 15148) and ash content (UNE-EN 15403). Fixed carbon content (CF) was derived from the difference.

3. Results and Discussion

3.1. Biomass characterization

The moisture content was measured previous hydrolysis step for each test. The values varied from 4 to 6%. This value was similar to other species. A proximate of the waste algae used in this study is presented in table 2. It can be observed that the amount of organic matter is low compared with other red algae that show values from 55-65% (Rodrigues et al., 2015). This can be due to that the algae used in this research is a waste of an industrial process and they could lose organic matter in the process.

The macroalgae waste in this study was found to contain macro- and microminerals and heavy metals. It has been reported that the levels of elements in algae are variable and vary between species (Bonanno and Orlando-Bonaca, 2018) and macroalgae are able to accumulate levels of metals higher than the corresponding concentrations in surrounding waters (Al-Shwafi and Rushdi, 2008). Some researchers (Al-Shwafi and Rushdi, 2008; Rodrigues et al., 2015; Bonanno and Orlando-Bonaca, 2018) have studied different red algae obtaining the following results: the concentrations of Cd ranged from 0.17 to 93 µg/g, the levels of Cr varied from 0.7 to 12 µg/g, for Cu they varied from 0.2 to 17 µg/g, the concentrations of Fe ranged from 10.75 to 1610 µg/g and the levels of Pb varied from 0.35 to 2.80 µg/g. Few studies reported the concentrations of Ca and Mg in algae (Biancarosa et al., 2018; Hanifzadeh et al., 2018). The levels of minerals and heavy metals of *Eucheuma Denticulatum Spinosum* are among those values.

The cellulose content of the waste was about 37%. Moreover, the cellulose content of the *Eucheuma Denticulatum Spinosum* macroalgae without any treatment was also measured, obtaining a value of 77%. Thus, the industrial process to extract the carrageen diminishes the cellulose content. Nevertheless, other raw materials have been used to obtain bioethanol with similar cellulose content. Saha et al. (2008) studied bioethanol production from rice hulls with a cellulose content of 35%.

3.2. Optimization of acid hydrolysis

Different tests were done to study the effect of some variables on efficiency process based on the amount of reducing sugars in the algal hydrolysate. Obtained results showed that the amount of reducing sugars increases with the studied acid concentration (Fig. 1) and diminishes with the used acid/dried algae ratio (Fig. 2). Similar results were also reported by Borines et al. (2013) in experiments on acid hydrolysis of *Sargassum spp.*, where the reducing sugars yield increased with the acid concentration. Sunwoo et al. (2016) found that sugar concentration increased with acid concentration from 30 to 70 mM for *Kappaphycus alvarezii* and *Gracilaria verrucosa*.

Reaction time has also a great influence on reducing sugars. As shown in Fig. 3, the highest value of reducing sugars was obtained at 70 min. High reaction time in combination with high acid concentration and acid/dried algae ratio resulted to diminish the amount of reducing sugars, probably due to glucose decomposition under these conditions. Other species of red macroalgae showed similar results (Sunwoo et al., 2016).

All the obtained results were analyse using Box-Behnken method with the aim to stablsh the best conditions for the process. Figs. 4-6 show the influence of different conditions on the amount of reducing sugars (DNS). Fig. 4 reflects that an increase in hydrolysis time and acid/dried algae ratio increase the DNS value until a maximum (70

min and 10:1). Fig. 5 shows the influence of acid concentration and acid/dried algae ratio at 70 min, it can be observed a red region at high concentration level, indicating high amount of reducing sugars; whereas it is difficult to establish an optimum ratio. However, it seems that lower and higher values or ratios show similar values of reducing sugars. Taking into account that a higher value of ratio needs higher amount of acid for obtaining similar results, the value of 7 is considered suitable for the process. This finding is according with the results of Borines et al. (2013). Fig. 6 presents the 3D plot for DNS values with a fixed acid/dried algae ratio of 7:1 (v/w). This graph reveals that a maximum DNS is obtained at 70 min and high values of acid concentration. Based on these results, the predictive model for reducing sugars is the following:

$$DNS = 0,11859x - 0,0009x^2 + 0,72345y - 0,03745y^2 + 0,16256z - 0,01613z^2 - 0,02565xy + 0,0013xy^2 + 0,00019x^2y - 0,00001x^2y^2 + 0,00072xz$$

where DNS is the amount of reducing sugars (g/L), x the time (min), y the acid/dried algae ratio (v/w), z the acid concentration (% wt.)

These figures showed that the best analysed conditions in terms of reducing sugar yield were 70 min, a sulphuric acid concentration of 7% wt. and 7 mL acid per gram of algal waste. Additionally, another experiment (test number 16) increasing the acid concentration until 9% wt. was carried out to analyze its effect, obtaining that the amount of reducing sugars increased from 0.24 g/L (test 4.) to 0.30 g/L (test 16). Based on this result, the optimum conditions for acid hydrolysis of waste algae were found to be 70 min, a sulphuric acid concentration of 9% wt. and acid/dried algae ratio of 7.

Ashokkumar et al. (2017) obtaining an algal hydrolysate with 19.7% of reducing sugars after alkali hydrolysis of algal biomass (*Padina tetrastromatica*) collected from coastal region. Others authors (Borines et al., 2013) have carried out an enzymatic hydrolysis in macroalgae (*Sargassum spp.*) harvested in coastal waters, obtaining the highest amount

of glucose of 30 mg/g biomass after 96 hours and a cellulase loading of 50 FPU/g biomass. The present study show that macroalgae waste contains carbohydrates that can be extracted by acid hydrolysis.

3.3. Bioethanol production and characterization

After the study of the different conditions for hydrolysis, the tests with the highest value of DNS were undergone to fermentation process. The obtained results are collected in Table 3, also including the yields of the different processes. The obtained results showed that the test 2, with the highest amount of acid (70 min, acid concentration 5%, ratio 13:1) achieved higher yield of hydrolysis; however, test 16 (70 min, acid concentration 9%, ratio 7:1) obtained the highest amount of glucose (93.5 g/L) and bioethanol and fermentation yield. This can be due to that the test 2 has a great amount of acid resulting in higher amount of hydrolysate but with less amount of reducing sugars. Then, it could be said that the acid concentration has more effect on the process than the acid/dried algae ratio. This is according with the previous results obtained using Box-Behnken method.

In addition, the macroalgae waste could produce about 0.1 g bioethanol/g waste and 0.12 g bioethanol/g reducing sugars with an ethanol concentration of 36.6 g/L using acid hydrolysis and yeast fermentation. The ethanol conversion efficiency was about 75% of theoretical. These results are in a good agreement with other published papers (Eshaq et al., 2010).

3.4. Characterization of hydrolysis residue

The residues of acid hydrolysis were characterized by elemental, inorganic compounds and immediate analysis and their calorific value and moisture with the aim to study the possibility to be used as solid fuel. Table 4 collects the obtained results. With regards to elemental analysis, the nitrogen and sulphur content are in accordance with the UNE-

EN ISO 17225-6 (2014). Immediate analysis indicated that the moisture and ash content found in this residue are higher than the values of the standard. The fixed carbon and volatile matters are important parameters of biomass quality according to EN ISO 17225-1 (2014); nevertheless, they do not appear in the international standards. The fixed carbon is similar to wood pellets obtained by other authors (Roy et al., 2013); whereas the ash content is higher than the wood pellets. This value is related to the amount of inorganic elements. It can be observed in table 4, that there is a considerable number of inorganic compounds in the residue, increasing the ash content; so it would be suitable to remove these elements if the residue wanted to be used as solid biofuel. Finally, the calorific value is lower than the standard values. In a previous research (Maceiras et al., 2015), the pellets from different fresh macroalgae were characterized resulting in higher calorific values and volatile matter and similar results for fixed carbon and ash content. The high heating value is defined as the sum of the heat released when fuel is combusted and the heat of condensation of the water vapour formed inside a bomb calorimeter. However, in many processes this vapour is released to the atmosphere together with combustion gases. This could be the reason to obtain a lower heating value than fresh macroalgae.

4. Conclusions

In this research, the bioethanol production from industrial waste was investigated. The results indicated that the overall hydrolysis ability is a function of the combined action of acid concentration, temperature and reaction time. Higher acid concentrations resulted in general in higher hydrolysis rates. The highest reducing sugars yield was obtained at 70 min, an acid concentration of 9% wt. and ratio of 7. The hydrolysates were converted into bioethanol using *S. Cerevisiae* with a yield of 11.6 g/g and an efficiency of 75%. Moreover, the potential of the residue after acid hydrolysis process

as sustainable solid fuel regarding its properties was analysed. The results showed some differences compared to international standards. The main novelty of this research is the bioethanol production from industrial algae waste and the subsequent study of the final residue as solid biofuel.

Acknowledgements

The authors gratefully acknowledge support from Defense University Center and collaboration of Compañía Española de Algas Marina (CEAMSA).

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375

376 **Table 1.** Design matrix with values of independent variables.

Test number	Time (min)	Ratio (v/w)	Concentration (%)
1	35	7:1	5
2	70	13:1	5
3	35	10:1	7
4	70	7:1	7
5	105	10:1	7
6	70	7:1	3
7	70	13:1	3
8	105	10:1	3
9	35	10:1	3
10	35	13:1	5
11	70	10:1	5
12	70	10:1	5
13	70	10:1	5
14	105	13:1	5
15	105	7:1	5

377

378 **Table 2.** Characterization of industrial waste macroalgae.

Parameter	Value
Cellulose	37 %
Organic matter	13.2 %
Total Kjeldahl nitrogen	1180 mg/kg
Ammonium nitrogen	10.8 mg N/kg
Total organic carbon	2.89 %
Cd	1.46 µg/g
Ca	70100 µg/g
Cr	3.00 µg/g
Cu	3.30 µg/g
Fe	1210 µg/g
Pb	1.4 µg/g
Mg	5080 µg/g

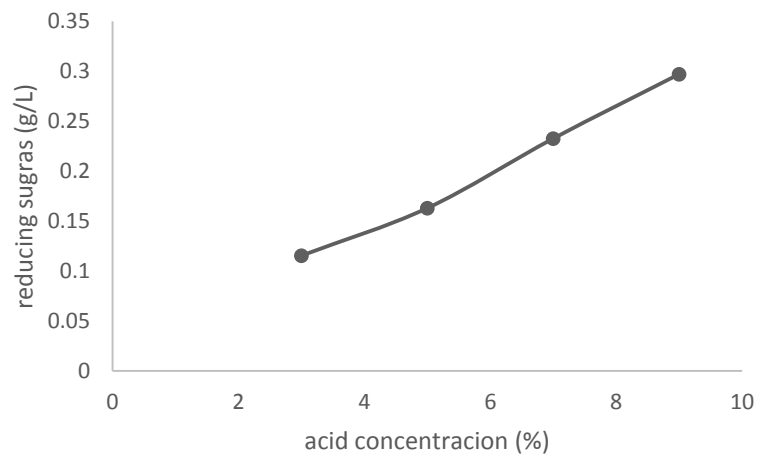
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Table 3. Yield of processes.

Test	Glucose (g/L)	Hydrolysis yield (%)	Fermentation yield (%)	Bioethanol yield (g/g)	Ethanol _{o/t} (%)
2	52.632	0.52	4.81	0.092	38.42
4	70.175	0.19	4.15	0.028	29.92
16	93.567	0.24	11.68	0.102	76.47

Acid hydrolysis residue properties	Value
Elemental analysis	
C	0.43%
H	0.02%
N	0.13%
S	0.20%
Inorganic elements	
Na ₂ O	3.49%
MgO	0.46%
Al ₂ O ₃	13.10%
SiO ₂	74.50%
P ₂ O ₅	963 ppm
BaO	0.22%
CaO	1.33%
TiO ₂	0.16%
SO ₃	0.59%
K ₂ O	5.03%
Fe ₂ O ₃	0.93%
SrO	173 ppm
Rb ₂ O	145 ppm
MnO	544 ppm
ZrO ₂	112 ppm
Immediate analysis	
Moisture	49.42%
Volatile materials	6.04%
Fixed carbon	3.77%
Ash content	40.77%
Calorific value	
Higher heating value (dry)	543.28 kcal/kg
Low heating value (dry)	245.71 kcal/kg
Higher heating value (wet)	389.61 kcal/kg
Low heating value (wet)	92.04 kcal/kg



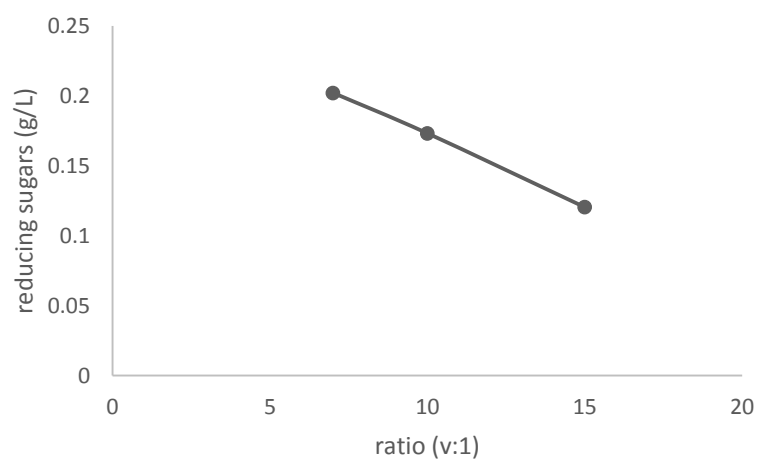
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387 Fig. 1. Effect of acid concentration on total reducing sugars

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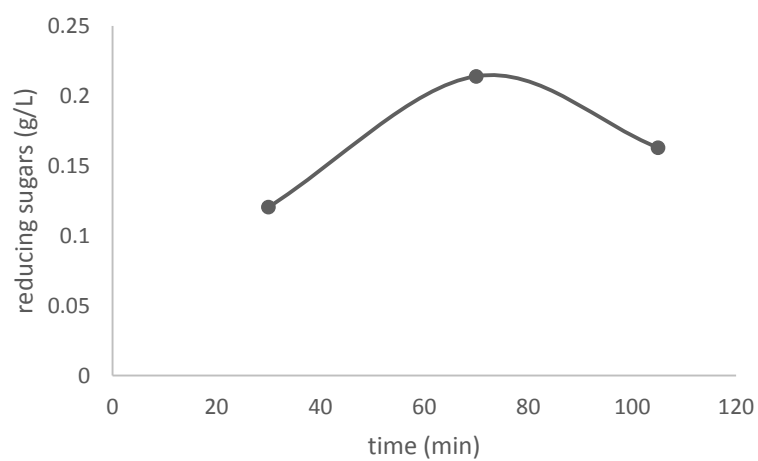
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391 Fig. 2. Effect of acid/dried algae ratio on total reducing sugars

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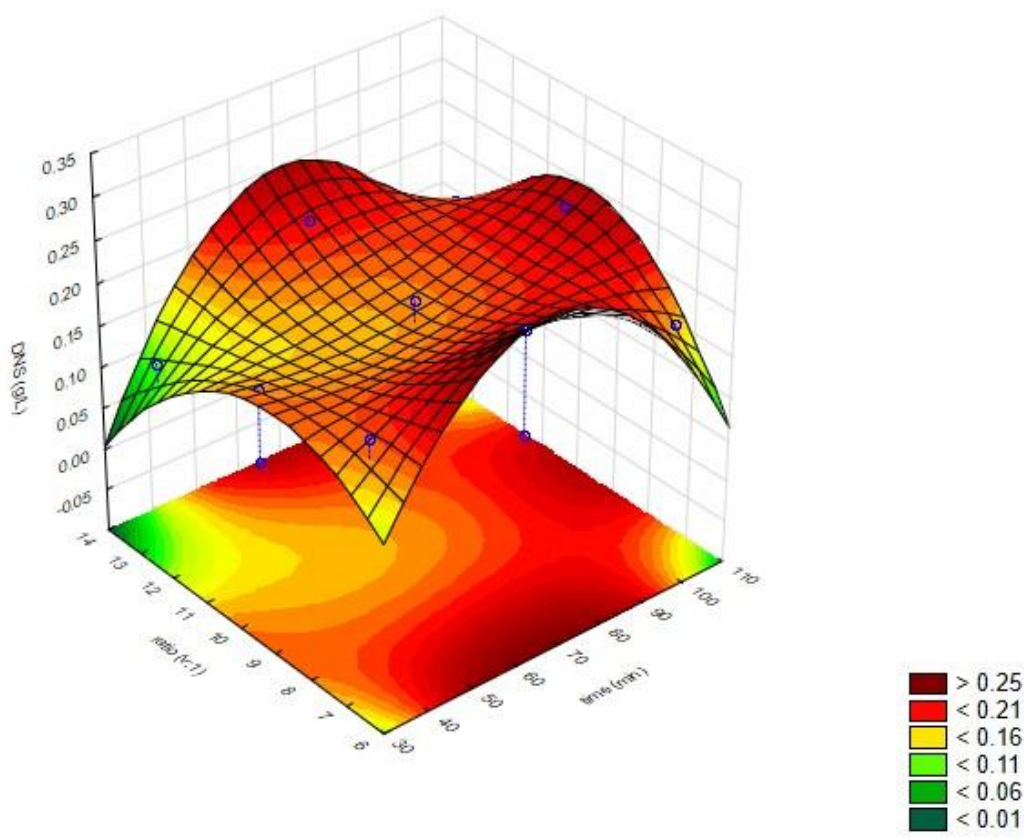
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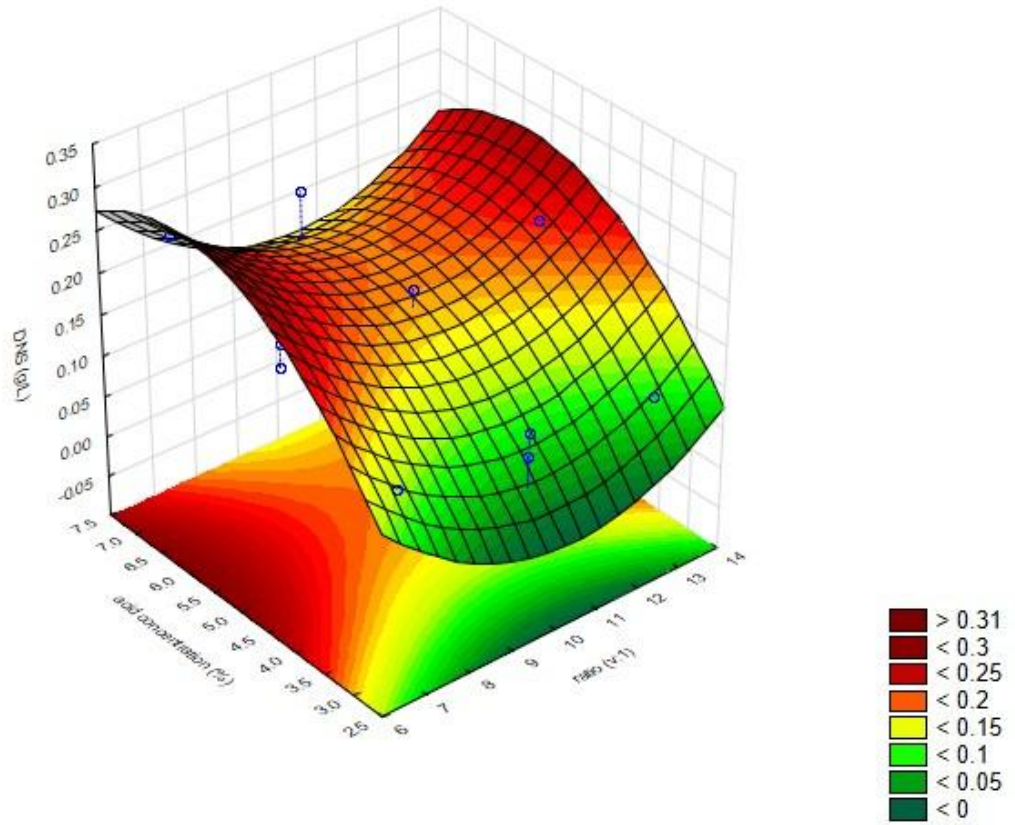
396 Fig. 3. Effect of reaction time on total reducing sugars

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400
401 Fig. 4. Effect of acid/dried algae ratio and time on total reducing sugars at a fixed acid
402 concentration of 7%.



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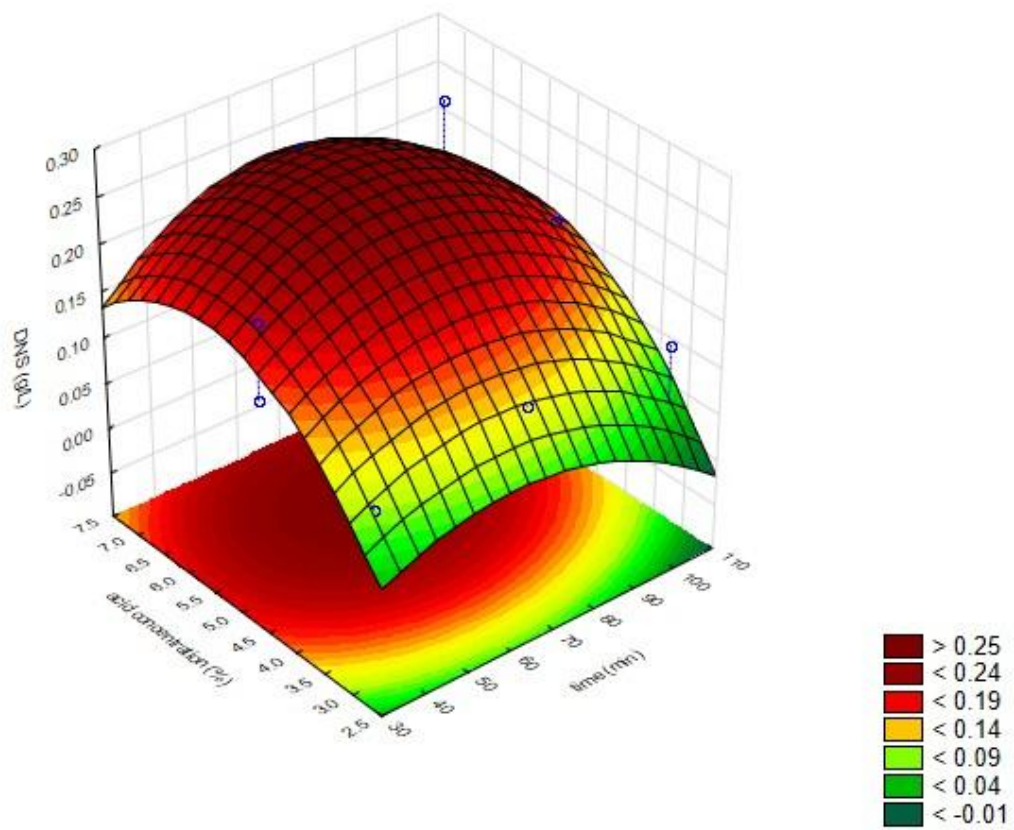
409 Fig. 5. Effect of acid/dried algae ratio and acid concentration on total reducing sugars at

410 a fixed time of 70 min.

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415 Fig. 6. Effect of acid concentration and time on total reducing sugars at a fixed

416 acid/dried algae ratio of 7:1.

417

