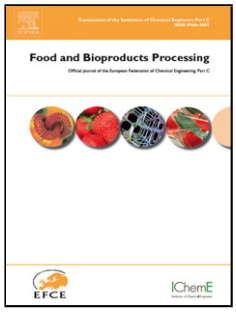

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E. Trigueros, M.T. Sanz, A. Filipigh, S. Beltrán, P. Riaño

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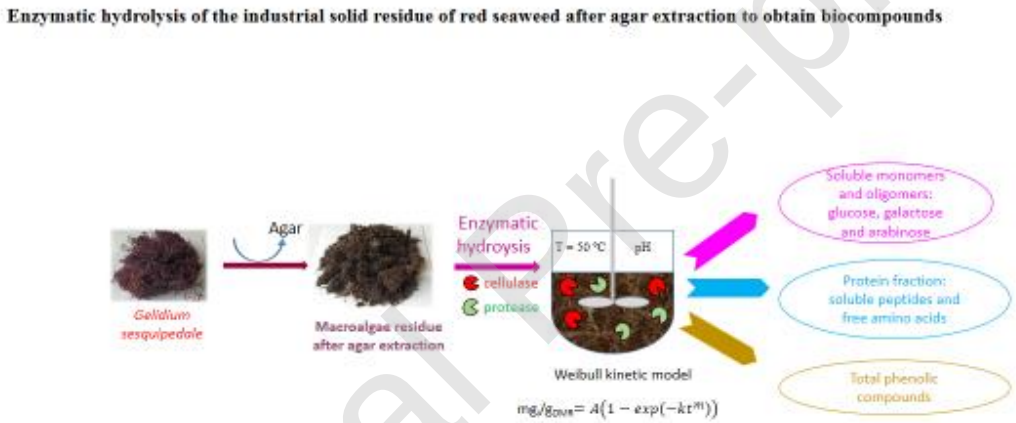
Enzymatic hydrolysis of the industrial solid residue of red seaweed after agar extraction: extracts characterization and modelling

E. Trigueros, M. T. Sanz∗, A. Filipigh, S. Beltrán, P. Riaño

Department of Biotechnology and Food Science (Chemical Engineering Section), University of Burgos, 09001 Burgos. Spain

Graphical abstract



Highlights

- EAE is a suitable technique to valorise the residue from algae after agar extraction
- Cell wall degradation by hydrolytic enzymes allows TPC release
- Cellulase presented higher hydrolytic capacity than other enzymes or their mixtures
- Carbohydrate fraction was released as monomers and oligomers

∗ Corresponding author. Tel.: +34 947 258810. Fax: ++34947258831. E-mail address tersanz@ubu.es

- Protease allowed the preferential release of hydrophobic amino acids

Abstract

The efficiency of enzymatic hydrolysis for the release of different biocompounds such as total phenolic compounds (TPC), sugars and proteins from the industrial solid residue of *G. sesquipedale* after agar extraction has been evaluated. Cellulase (0.25 % - 8 %, w/w, enzyme: solid residue, pH =5) has been proved to be an efficient enzyme to degrade the algae cell wall improving the release of the bound TPC with values up to 7.5 mg gallic acid equivalent/g dry macroalgae residue. Monomer and oligomer carbohydrates were released to the reaction medium, namely glucose, galactose and arabinose with higher yields by increasing cellulase concentration. Enzyme combinations with other hydrolytic enzymes, such as xylanase and protease, did not bring any improvement of the TPC and sugar yields. Protein was also released to the enzymatic medium with protein extraction yields around 30 %. The use of protease under basic conditions led to an increase in the release of the protein fraction and of the free amino acids content with a hydrophobic ratio higher than in the raw material. The kinetics of TPC and protein hydrolysis have been fitted to the power law and the Weibull models yielding the Weibull model the best fitting quality.

Keywords: hydrolytic enzymes, kinetic models, macroalgae industrial residue, TPC, carbohydrates, proteins

1. Introduction

Gelidium sesquipedale is a red algae that is widely used for agar production. The industrial process of agar extraction generates a solid residue that accounts for 15-40 % of the initial dry biomass (Álvarez-Viñas et al., 2019). This solid residue can be used for fodder and fertilizer although most of it is disposed of (Ferrera-Lorenzo et al., 2014). However, it presents a valuable

chemical composition with high protein content with all the essential amino acids, carbohydrates and with low insoluble lignin content (Trigueros et al., 2020). To incorporate commercially exploited macroalgae into a biorefinery context, valorization of this solid residue must be considered to reach the concept of zero residues.

Due to the complex chemical structure of macrolagae, it allows to produce different high added values compounds (Ruiz et al., 2015). The most essential step to break the cell wall of the algae to release biomolecules is the pretreatment technique. The pretreatment techniques available for the macroalgae can be classified into four main categories: physical, physicochemical, chemical and biological ones (Shobana et al., 2017).

One of the most attractive technologies for pretreatment of the biomass are those based on hydrothermal processes using different heating sources such as subcritical water (subW) and steam explosion. These techniques can be considered as green and clean technologies since are based on the use of water as extracting/hydrolytic agent. In steam explosion, the biomass is subjected to high pressure saturated steam (0.69-4.83 MPa) at a temperature of 160-260 ºC and then the pressure is suddenly reduced to let the water escape in an explosive way that causes explosion of the biomass (Baruah et al., 2018). SubW uses water a solvent at temperature above its boiling point, 100ºC, and below its critical point 374ºC and pressure high enough to keep water at its liquid state. At these conditions water provides an effective medium for acid- and base-catalyzed reactions. Therefore, an important advantage of these techniques is that the use of catalysis or chemicals is avoided to get biomass hydrolysis.

Recently, subW treatment was applied to the solid residue generated after agar extraction from *G. sesquipedale* (Trigueros et al., 2020). By using a semicontinuous fix-bed reactor configuration nearly 70% of the protein content was recovered with nearly 100 mg of free amino

acids/$g_{protein}$. SubW treatment also allowed the release of total phenolic compounds to the medium presenting a high antioxidant capacity.

On the other hand, the use of enzyme assisted extraction (EAE) is a promising technology that presents a great potential to improve the extraction efficiency of bioactive components (Sabeena et al., 2020). EAE offers several advantages over conventional extraction methods such as high yield and specificity, being an environmental friendly technology (Wijesinghe and Jeon, 2012). However, the cost of the enzyme is one of the technical limitations for processing large volumes of raw material (Puri et al., 2012). The use of hydrolytic enzymes helps to degrade the cell wall structure thereby enhancing the release of bioactive compounds since some of these compounds are either dispersed in the cell cytoplasm or retained in the cell wall by hydrogen or hydrophobic bonding (Fleurence et al., 1995). Furthermore, antioxidant properties of the hydrolysates are enhanced due to the breakdown of high molecular weight polysaccharides and proteins (Wang et al., 2010). In the literature, some studies can be found regarding the use of EAE to increase the extraction of bioactive compounds from different brown, red and green algae (Sabeena et al., 2020) (Hardouin et al., 2014) (Wang et al., 2010) (Vásquez et al., 2019). However, only a few studies deal with the use of EAE from the residue generated after phycocoloids extraction from macroalgae. Shukla and Jumar (Shukla et al., 2016) treated the leftover pulp after agar extraction of *Gracilaria Verrucosa* by enzyme hydrolysis to obtain reducing sugars that were later used as fermentation broth to produce ethanol by using *S. cerevisiae*.

The aim of this work was to study the potential of EAE, as an alternative to hydrothermal processes, to improve the release of biocompounds from the industrial solid residue remaining after agar extraction from *G. sesquipedale* by using cellulase, protease and xylanase as hydrolytic enzymes. The main goal of this article was to provide a detailed characterization of the enzymatic extracts, showing the ability of the hydrolytic enzymes to release different

biocompounds into the extraction medium. Extraction kinetics and yield of TPC, proteins were determined. The knowledge of the extraction curves is important to determine the time needed to obtain a certain extraction yield and analyse the characteristics of enzymatic extraction. The presence of other biocompounds in the final extract was also determined such as the presence of free amino acids and carbohydrates, namely, glucose, galactose and arabinose. To our knowledge the enzymatic extraction from the solid residue after agar extraction had not been previously reported in the literature and results would help to valorize this industrial solid waste.

2. Materials and methods

2.1 Raw material

The raw material used in this work has been provided by Hispanagar (Burgos, Castilla y León, Spain) (https://www.hispanagar.com/es) and it consisted of the solid residue from *G. sesquipedale* after agar extraction. This raw material was oven dried at 45 ºC for 24 hours to preserve it and retard microbial growth during storage. This solid residue was called "macroalgae residue", MR. Final moisture content of this by-product was determined gravimetrically by weighing before and after drying in an oven at 105 ºC until constant weight resulting to be 5 ± 2 %. Sample moisture was used to express results per gram of dried MR (DMR).

Biomass characterization was performed according to the NREL protocols (Sluiter et al., 2010). Carbohydrates were quantified by high-performance liquid chromatography (HPLC) with a Bio-Rad Aminex-HPX-87 H column, a variable wavelength detector (VWD) and a refractive index detector (RID) using a mobile phase constituted by 0.005M sulphuric acid. The column detector was maintained at 40 ºC. Uronic acids were determined after acid hydrolysis according to the method of Filisetti-Cozzi (Filisetti-Cozzi and Carpita, 1991).

Protein content in the MR was obtained by the total nitrogen content as measured by the elemental analysis (Thermo Scientific Model Flash 2000) by using the nitrogen factor (NF). The NF was determined according to the amino acid profile of the MR determined after acid and basis hydrolysis. After hydrolysis, the amino acid profile was determined by using the EZ:faast Phenomenex® procedure (Trigueros et al., 2020).

2.2 Hydrolytic enzymes.

Cellulase, 1,4-(1,3:1,4)-β-D-Glucan 4-glucanohydrolase, EC 3.2.1.4, from *Aspergillus niger* was provided by Sigma-Aldrich. Xylanase, a mixture of xylanase endo-1,4β (EC 3.2.1.8) and xylanase endo-1,3β (EC 3.2.1.32) from *Trichoderma Longibrachiatum* and a protease, proteinase EC 3.2.1.6, from *Bacillus subtilis* were supplied by Biocon.

2.3 Enzymatic hydrolysis

7.5 g of the MR were introduced into a 500 mL jacketed reactor provided with magnetic agitation together with 150 mL of the reaction medium. pH was adjusted by adding HCl or NaOH. Enzyme treatment was carried out at constant temperature of 50 ºC. When the temperature was reached, the enzyme was added to the mixture and hydrolysis was carried out for more than 24 h. Samples were withdrawn at regular time intervals to plot the corresponding extraction curves. To stop the hydrolytic reaction, the enzyme was inactivated by heating the withdrawn samples at 100 ºC for 5 min and immediately were cooled in ice and kept in the

refrigerator until analysis. Samples were centrifuged (Thermo Scientific Sorvall ST16) and filtered through a Whatman nº 542 filter before analysis.

The cellulase concentration, expressed as enzyme:DMR ratio (w/w), was varied in the mass percent range from 0.25 % to 8 %. A control sample at the same extraction temperature but with no enzyme added to the extraction medium was also carried out. Other hydrolytic enzymes such as xylanase and protease were also tested separately and the effect of the association with cellulase was also determined.

2.4. Characterization of the enzymatic liquid extracts

2.4.1. Extraction yield.

1.5 mL of the final enzymatic extract was filtered through a 0.22 µm filter and dried at 105ºC to remove the solvent until constant weight. Extraction yield was calculated as the ratio between the residual weight of the dry extract obtained after removing the solvent by evaporation, by considering the initial volume of the solvent, and the weight of the DMR charged into the enzymatic reactor.

2.4.2. Determination of total phenolic compounds.

TPC were evaluated according to the method of Folin Ciocalteu (Singleton et al., 1999). A calibration curve was prepared with standard solutions of gallic acid by following the same colorimetric method and results were expressed as extraction yield, defined as mg of gallic acid equivalent (GAE) per gram of DMR.

2.4.3 Determination of carbohydrates and derived compounds

Identification and quantification of carbohydrates were performed by HPLC as described in section 2.1. Monosaccharides and derived products was directly analyzed in the liquid enzymatic hydrolysates previously filtered through 0.22 µm pore size (Scharlab syringe filter). Total sugars were determined after hydrolysis of the sample according to the Laboratory Analytical Procedure

(LAP) (Sluiter et al., 2010) to hydrolyze the oligomer sugars. Sugar yield was calculated as monomer yield and oligomer yield (Sánchez-Bastardo et al., 2017):

$$Monomer\ yield\ (\%) = \frac{Monomeric\ sugar\ in\ the\ enzymatic\ hydrolysate}{Sugar\ in\ the\ raw\ material} \cdot 100 \quad [1]$$

$$Total\ yield\ (\%) = \frac{Monomeric\ and\ oligomeric\ sugar\ in\ the\ enzymatic\ hydrolysate}{Sugar\ in\ the\ raw\ material} \cdot 100 \quad [2]$$

2.4.4 Protein determination

Total protein content in the enzymatic extracts was determined by using the kit DC™ (Bio-Rad Laboratories, Hércules-CA, USA) based on the Lowry protocol (Lowry et al., 1951). This assay was chosen since, according to the literature, the Lowry method is recommended to measure protein content in seaweeds and allows a better quantification compared to other methods (Barbarino and Lourenço, 2005). A calibration curve was done by using bovine serum albumin as standard. The protein extraction yield was calculated as:

$$Protein\ yield\ (\%) = \frac{protein\ in\ the\ enzymatic\ hydrolysate}{protein\ in\ the\ raw\ material} \cdot 100 \quad [3]$$

2.4.5 Free amino acids and hydrolysis degree

Free amino acids in the enzymatic extracts were determined by using the EZ:faast Phenomenex® kit (see section 2.1). The percentage of hydrolysed peptide bonds, the hydrolysis degree (DH), was evaluated by the ninhydrin reaction method according to the Sigma Aldrich protocol. 2 mL of sample were gently mixed with 1 mL of ninhydrin reagent solution and placed into a boiling water bath for 10 min. Samples were cooled and 5 mL of 95 % ethanol were added. Absorbance was measured at 570 nm. A calibration curve was obtained by using different standards of a leucine solution that was daily prepared (Friedman, 2004). The DH was evaluated as (Adler-Nissen et al., 1983):

$$DH = h/h_{tot} \cdot 100\ (\%) \quad [4]$$

where h is the number of equivalent peptide bonds hydrolysed, expressed as meq/g protein and h_{tot} is the total amount of milimols of individual amino acids per gram in the unhydrolysed protein that can be evaluated from the amino acid profile.

2.5 Modelling of the extraction curves

Modelling of the extraction curves is a useful tool to optimize the extraction process reducing the cost of the process. The use of empirical models helps to simplify the study of complex systems such as the extraction of biocompounds from plant materials (González-Centeno et al., 2015). In this work, two empirical models that have been previously proposed to model the extraction and recovery of compounds from different types of solid matrix were used to fit the experimental data obtained from the extraction and try to elucidate the extraction mechanism: the power law and the Weibull models (Alonso-Riaño et al., 2020) (Kitanović et al., 2008). The power law model can be described by the following equation:

$$Extraction\ yield, mg_{biocompound}/g_{DMR} = Bt^n \quad [5]$$

where t is the extraction time (s), B is a constant incorporating the characteristics of the particle-active substance system and n is the diffusional exponent.

The Weibull's model was expressed as:

$$Extraction\ yield,\ mg_{biocompound}/g_{DMR} = A\left(1 - exp(-kt^m)\right) \quad [6]$$

where t is the extraction time (s), A, k and m are the kinetic parameters.

A deep analysis of the kinetic parameters for both models would help to determine the dependence on operating conditions leading to a better desing, simulation, optimization and control of further industrial processes (González-Centeno et al., 2015).

To estimate the kinetic parameters, non-linear regression was performed by using the Marquardt algorithm (Statgraphics X64). Experimental results were then compared with those of the model

prediction through the values of the Root Mean Square Deviation (RMSD) between experimental and calculated extraction yields:

$$RMSD = \sqrt{\frac{\sum_{i=1}^{n}\left(Yield_{exp} - Yield_{calc}\right)^2}{n}} \cdot 100 \quad [7]$$

where n is the number of experimental data points in each kinetic curve.

2.6 Statistical analysis

Statistical analyses were conducted using the software Statgraphics X64. The results were presented as a mean ± standard deviation of at least three replicates. To confirm significant differences, the Fisher's least significant differences method at p-value ≤ 0.05 was applied.

3. Results and discussion

3.1 Macroalgae solid residue characterization

Table 1 presents the chemical characterization of *G. sesquipedale* solid residue after agar extraction (Trigueros et al., 2020). Chemical composition would depend on the industrial extraction process as well as other intrinsic factors such as origin and season of the collected macroalgae due to different stages of macroalgae development. Polysaccharides accounted for a mass percent of 41 ± 2 % in a dry basis. This fraction consisted mainly of glucans (23.4 ± 0.9 %) and galactans (11.9 ± 0.5 %) with small amounts of arabinans, 2.9 ± 0.2 % and 3.8 ± 0.1 % of uronic acids. The higher content of glucans compared to galactans is due to the efficient reduction of the galactans content during the agar industrial extraction process. Similar results were reported in the literature by Kim et al. (2015) for *Gelidium amansii* after autoclaving treatment of the macroalgae. These authors determined that the main sugars were galactose and glucose, 30.3 ± 0.6 and 21.6 ± 0.4 (mass percent % in a dry basis) in the raw *Gelidium amansii*; however, after autoclaving, due to efficient reduction of the galactan content, the relative content of glucose increased and galactose decreased. After 40 min of autoclaving, these authors

determined 13.5 ± 0.3 and 39.6 ± 1.2 (mass percent % in a dry basis) for galactose and glucose, respectively. Lipid content was low, less than 1%. The lipid content agreed with the range reported in the literature between 0.3 and 3.6 g/100 g dry seaweed for different red, brown and green algae (Rodrigues et al., 2015). Extractives represented 11.5 ± 0.9 %, proteins 21 ± 1 % and ashes 22 ± 2 % of DMR composition. The relative high protein content of the solid residue after agar extraction agreed with the fact that red algae have the highest protein content among the three types of macroalgae (Postma et al., 2018) with all essential amino acids, being worthly its valorization. Insoluble lignin accounted for 3 ± 1 %, as corresponds to third generation biomass.

Table 1. Chemical composition of macroalgae residue, MR, expressed as mass percent % ± SD in a dry basis.

Compound	Composition, %
Extractives	11.5 ± 0.9
Polysaccharides	41.0 ± 2.0
Glucans	23.4 ± 0.9
Galactans	10.9 ± 0.5
Arabinans	2.9 ± 0.2
Uronic acids	3.8 ± 0.1
Lignin	11.7 ± 1.1
Soluble	8.7 ± 0.1
Insoluble	3.0 ± 1.0
Proteins*	21 ± 1
Lipids	0.87 ± 0.09
Ashes	22 ± 2

*Protein includes the protein content in the extractive fraction (2.6 %).
NF = 4.9 (see Table 6 for amino acid profile)

3.2 TPC release by cellulase hydrolysis

Figure 1 shows the time course of TPC release at different cellulase loading from 0.25 to 8 % (mass percent %, enzyme:MR ratio) at pH=5. This pH was initially selected since in the literature has been reported as the optimum pH for cellulase activity (Shukla et al., 2016). By increasing the enzyme loading, the initial TPC release rate and the final extraction yield increased. A high cellulase loading led to a more effective cell wall degradation and consequently an improved release of the bound phenolic compounds. Hydrolytic extraction curves indicated that more than 70 % of final TPC release took place in the first 3 h (10800 s) of enzymatic treatment, but 24 h of treatment were needed to reach the final extraction equilibrium. Vásques et al. (Vásquez et al., 2019) also reported long hydrolysis time due to the complexity of the algae matrix, between 12 h and 18 h for red and brown algae, respectively. Figure 1 also shows the TPC release when using water as solvent at its natural pH. The low values of TPC extraction yield indicated that the non-enzymatic TPC release is much lower in the experimental conditions evaluated due to the phenolic compounds bound to the cell wall that limits its extraction efficiency (Sabeena et al., 2020).

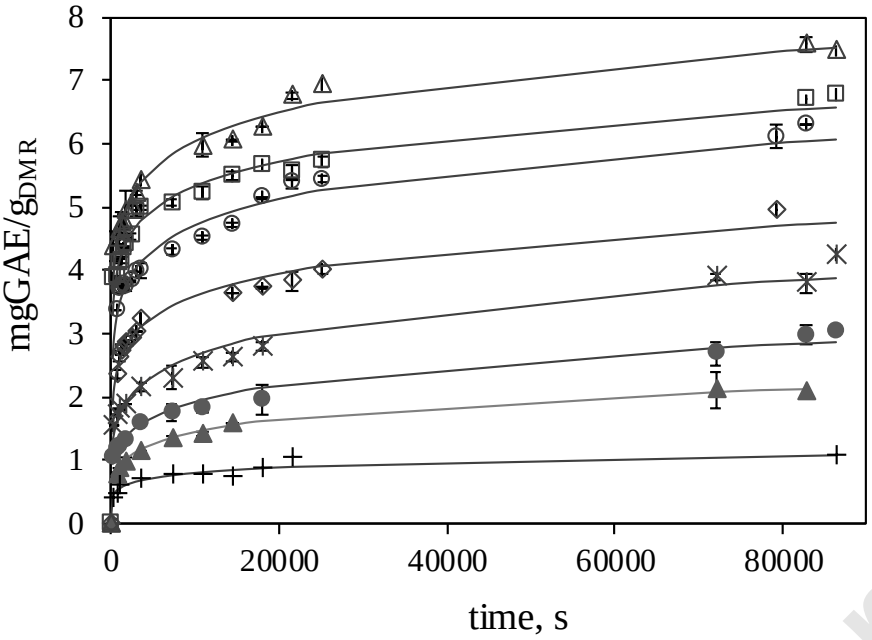


Figure 1. TPC extraction kinetics at 50ºC and pH = 5 at different cellulase loadings (▲ 0.25 %, ● 0.5 %, ∗ 1%, ◇ 2%, ○ 4%, □ 6%, △ 8%) and by using water as solvent at its natural pH, (+). The continuous lines represent the Weibull model (Table 3).

To evaluate the effect of the pH of the hydrolysis medium, enzymatic hydrolysis was carried out at 6 % cellulase loading in water as reaction medium at its natural pH. Results are presented in Figure 2, where it can be observed that half TPC extraction yield was reached at pH of 5. Gligor et al. (Gligor et al., 2019) described that an acid pH influences the catalytic activity by modifying the protein configuration and binding capacity to the substrates, concluding that enzymes usually require acidic pH values. The hydrolytic effect of an acid extraction medium was evaluated by performing the kinetics at pH of 5 in the absence of enzyme (see also Figure 2). Similar results were obtained as when using 6 % of cellulase in water. This can be due to the fact that an acidic extraction led to hydrogen bonds destabilization resulting in increased cellular wall plasticity (Gligor et al., 2019).

The maximum TPC obtained in this work, as determined by the Folin Ciocalteu method, was lower than the values obtained by subW treatment of this solid residue. In previous work, by

applying subW (Trigueros et al., 2020), 26 mg GAE/g_{DMR} were obtained in a semicontinuous fix-bed reactor at 200ºC and residence time of 18.7 min. However, at this temperature, Maillard reactions products between sugars and amino acids would interfere in the analysis, providing higher TPC values.

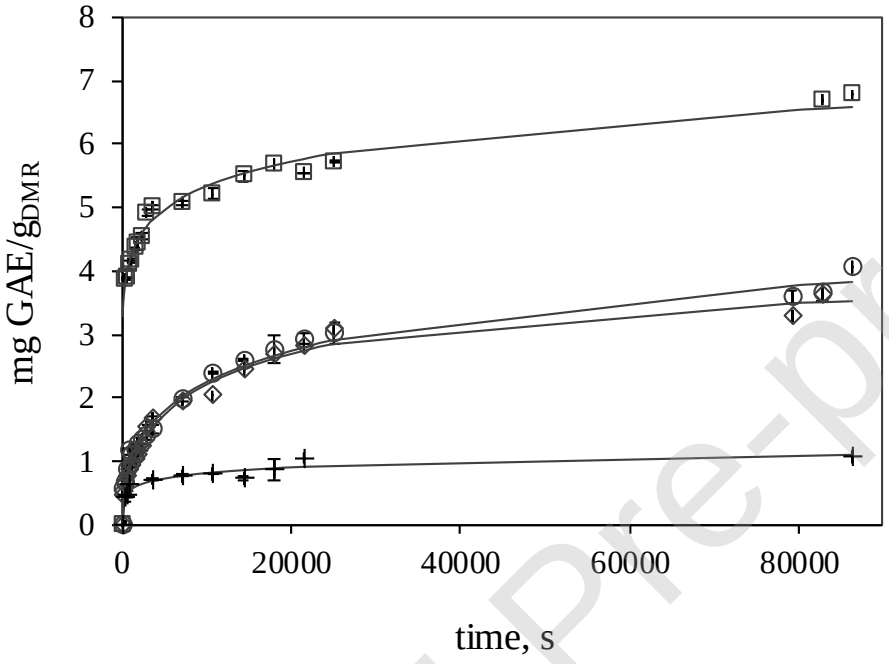


Figure 2. TPC extraction kinetics at 50ºC (□ 6% cellulase pH = 5; ◇ 6 % cellulase in water, ○ no cellulase pH = 5, + water as solvent). The continuous lines represent the Weibull model (Table 3).

The total yield of enzymatic extracts after 24 h ranged from 11.2 ± 0.9 % to 73 ± 2 % at 0.25 and 8 % (w/w) cellulase loading respectively (see Table 2). These extraction yields were higher than the value obtained when using water as extraction solvent at its natural pH, 6.1 ± 0.3 %. Similar to TPC release, by increasing enzyme loading, higher extraction yields were obtained due to the breakdown of the cell wall of the algae and the release of the compounds into the extraction medium (Sabeena et al., 2020). Other extraction yields reported in the literature for enzymatic

extracts ranged from 36.0 to 95.1 % for different brown seaweeds by using commercial enzymes (Sabeena et al., 2020).

Table 2. Total extraction yield obtained at different cellulase loadings at pH = 5 and for the extraction controls (water, 6% cellulase in water and no enzyme added to medium at pH =5).

Extraction medium	Extraction yield, %
Water (control)	6.1 ± 0.3^{a}
0.25 % cellulase	11.2 ± 0.9^{b}
0.5 % cellulase	15.5 ± 0.8^{c}
1 % cellulase	22.5 ± 0.7^{d}
2 % cellulase	39.5 ± 0.9^{e}
4 % cellulase	47.0 ± 1.0^{f}
6 % cellulase	60.0 ± 1.0^{g}
8 % cellulase	73.0 ± 2.0^{h}
6 % cellulase in water (control)	18.0 ± 1.0^{c}
No enzyme, pH = 5 (control)	18.0 ± 2.0^{c}

Values with different letters are significantly different when applying the Fisher's least significant differences (LSD) method at p-value ≤ 0.05

3.3 Enzymatic hydrolysis by other hydrolytic enzymes and their combination with cellulase.

To ensure a complete cell wall breakage, the association of cellulase with other hydrolytic enzymes, such as xylanase and protease was studied. First, the hydrolytic effect of xylanase and protease by acting alone was studied by performing the enzymatic treatment for an enzyme loading of 6 % mass percent and pH=5 (Figure 3). It can be observed that cellulase was the most

effective enzyme on weakening the cell wall structure favouring faster TPC release. This result agrees with the recent literature that describes cellulases as enzymes with a high hydrolytic capacity as well as a vast substrate specificity (Gligor et al., 2019). Among the three enzymes, xylanase led to the lowest TPC yield probably due to the structure of the cell wall of the MR since no xylanes were determined in the chemical composition. For the protease, slower extraction kinetics but similar final extraction yield was reached as for cellulase, probably due to the breakdown of the complexes formed between some phenolic compounds and proteins similar to phlorotannins naturally found in brown algae (Sánchez-Camargo et al., 2016). To further analyse the good results obtained by protease, the effect of pH on the protease activity was evaluated in the pH range from 5 to 8, since some proteases can exhibit high activity at high pH value (Faulds et al., 2008). The results are presented in Figure 4 where it can be observed that the hydrolytic activity of the protease used in this work increased as the pH of reaction medium increased.

TPC extraction curve was also determined for different enzyme combinations: two binary enzyme combinations: 3% cellulase + 3% protease and 3% cellulase + 3% xylanase and a ternary combination 2% cellulase + 2% protease + 2% xylanase (Figure 3). None of the combinations led to better results than for cellulase. The lowest yield was obtained for the combination of xylanase and cellulase. In this regard, different results have been found in the literature regarding the effect of enzyme combinations. Rakariyatahm (Rakariyatham et al., 2019) found that the TPC extraction yield from Longan (*Dimocarpus longan*) peel by combining cellulase, α-amylase, protease and β-glucosidase significantly increased the extraction yield. Hosni et al. (Hosni et al., 2013) reported opposite results for the extraction of essential oils from two different species, *Thymus capitatus* and *Rosmarinus officinalis* leaves. A combination of cellulase and hemicellulase led to a better extraction yield for *T. capitatus* while for *R.*

officinalis, lower extraction yield was obtained than for the enzyme acting alone. Boulila et al. (Boulila et al., 2015) recovered bioactive compounds from bay leaves (*Laurus nobilis L.*) by enzymatic treatment with cellulase, hemicellulase, xylanase and the ternary mixture. These authors found that, when enzymes were combined, the extraction yield was reduced suggesting a competitive adsorption to the cell wall polysaccharides that led to steric hindrance to bind the enzymes to the substrate decreasing the breakdown of the cell wall components.

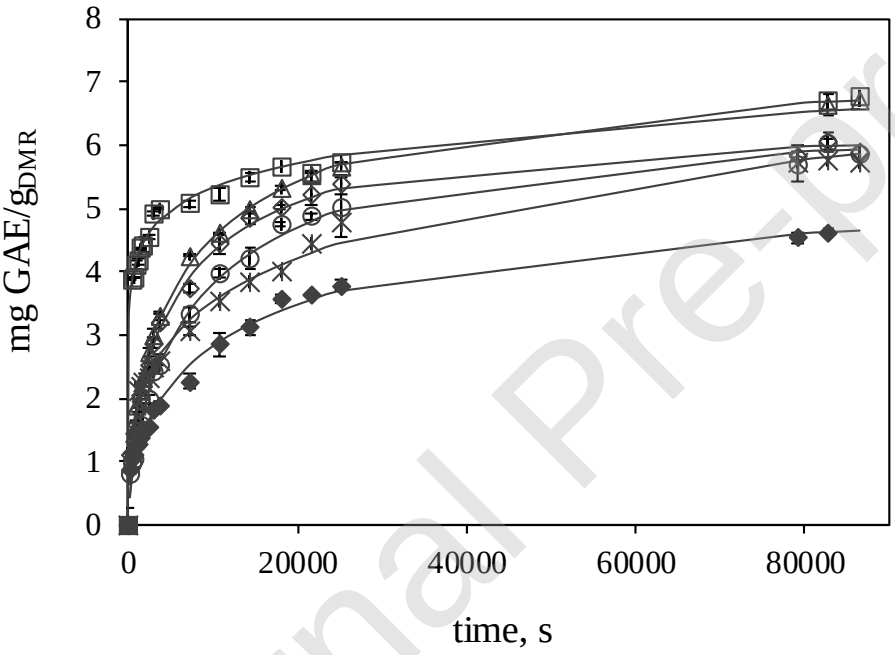


Figure 3. TPC extraction kinetics at 50ºC and pH = 5: □ 6% cellulase, △ 6% protease, ∗ 6% xylanase, ◇ 3 % cellulase + 3 % protease, ◆ 3 % cellulase + 3 % xylanase, ○ 2 % cellulase + 2 % protease + 2 % xylanase. The continuous lines represent the Weibull model (Table 3).

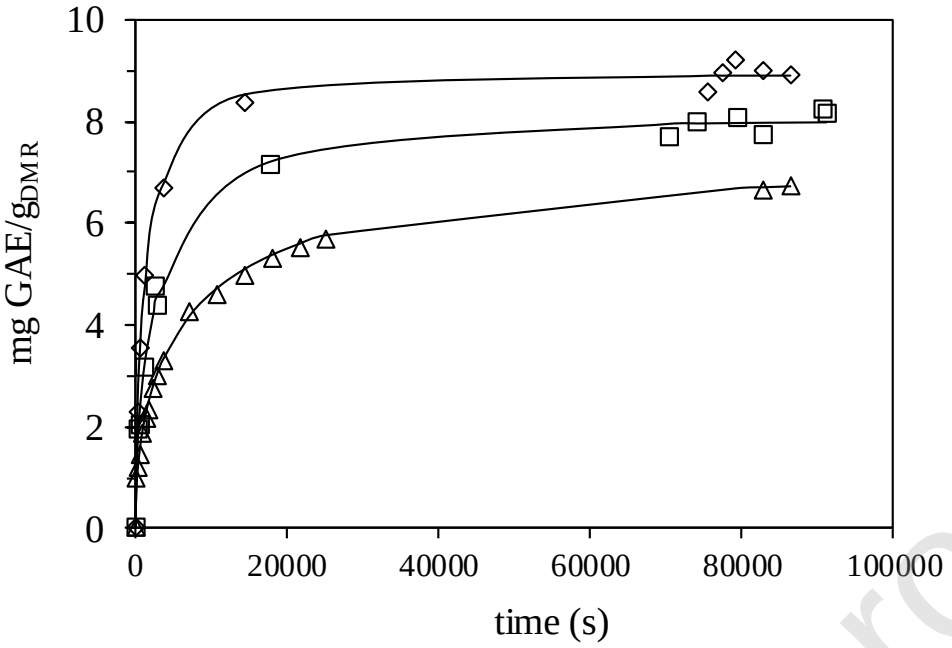


Figure 4. TPC extraction kinetics at 50ºC by using 6 % protease at different pH values (△ pH = 5, □ pH = 6, ◇ pH = 8). The continuous lines represent the Weibull model (Table 3).

3.4 Kinetic models

TPC extraction curves were fitted to the power law and the Weibull models and the corresponding parameters of the models have been listed in Table 3. According to the low values of the RMSD, both models fitted the extraction kinetics quite well, ranging from 0.032 to 0.650 and from 0.025 to 0.240 for the power law and the Weibull models, respectively.

For the power law model, the diffusional exponent, n, was lower than the unity, as reported for other plant materials (Kitanović et al., 2008). An observation of parameters listed in Table 3 indicates that the type of enzyme and its concentration had a great effect on initial rate and final extraction yield. By observing the parameters reported in Table 4, for the experiments carried out by cellulase as biocatalyst, the parameters of the power law model were correlated with the cellulase concentration, in the range from 0.25 to 8 % (mass percent) of catalys loading:

$B = 0.255\ [\text{Cellulase}] + 0.281\ (R^2 = 0.945)$ [8]

$n = -0.029 \ln [Cellulase] + 0.169$ ($R^2 = 0.892$) [9]

In the same way, for protease, a correlation was stablished between the pH of the medium in the range from 5 to 8:

$B = 0.369 [pH] - 1.460$ ($R^2 = 0.997$) [10]

$n = -0.2135 \ln [pH] + 0.600$ ($R^2 = 0.960$) [11]

Regarding the Weibull model, the trend observed for the k constant is justified by considering that his parameter is related with the extraction rate. The A constant increased as the final extraction yield also increased since it represents the maximum extraction yield at infinite time. The shape parameter, m, indicates the shape of the extraction curves. In this work, values of $m < 1$ indicates upward concavity. For the experiments carried out with cellulase different realtionships have been found for the parameters and the cellulase concentration, in the range from 0.25 to 8 % mass percent of cellulase loading:.

$A = 2.772 \ln [Cellulase] + 8.524$ ($R^2 = 0.9622$) [12]

$k = 0.016 [Cellulase] + 0.0416$ ($R^2 = 0.9007$) [13]

$m = -0.0365 \ln [Cellulase] + 0.2070$ ($R^2 = 0.9531$) [14]

Regarding the effect of pH for protease, a relationship between the Weibull parameters and the pH of the medium was also determined, in the pH range from 5 to 8:

$A = 3.968 \ln [pH] + 0.719$ ($R^2 = 0.973$) [15]

$k = 0.001 pH + 0.004$ ($R^2 = 1.000$) [16]

$m = 0.1453 \ln [pH] + 0.286$ ($R^2 = 0.914$) [17]

The Weibull model has been represented in Figures 1-4, where it can be observed the good fitting to the experimental data. This model presented slightly lower RMSD values than the power law model; furthermore, we consider the power law model less realistic since does not approach to a limit with time.

Table 3. Kinetic parameters for the power law and the Weibull models for TPC enzyme assisted extraction.

Enzyme concentration	Power law			Weibull			
	B	n	RMSD	A	k	m	RMSD
0.25 % C	0.232	0.169	0.032	3.717	0.045	0.259	0.025
0.5 % C	0.312	0.196	0.098	7.949	0.035	0.225	0.110
1 % C	0.470	0.187	0.156	8.580	0.047	0.224	0.181
2 % C	1.023	0.136	0.103	10.188	0.093	0.168	0.114
4 % C	1.355	0.137	0.175	12.325	0.113	0.157	0.171
6 % C	2.054	0.103	0.122	13.062	0.153	0.134	0.133
8 % C	2.075	0.115	0.168	14.579	0.148	0.140	0.153
No enzyme, water*	0.196	0.154	0.067	1.802	0.088	0.208	0.068
No enzyme, pH= 5	0.162	0.281	0.159	5.055	0.014	0.407	0.111
6 % C in water*	0.145	0.287	0.196	3.860	0.010	0.488	0.114
6 % X	0.351	0.249	0.039	10.009	0.022	0.326	0.141
6 % P	0.364	0.264	0.40	6.996	0.009	0.512	0.097
3% C+ 3% P	0.383	0.249	0.455	6.092	0.008	0.551	0.153
3% C+ 3 % X	0.189	0.287	0.233	5.129	0.008	0.505	0.195
2% C+ 2% X+2% P	0.215	0.301	0.428	6.141	0.005	0.582	0.139
6 % P (pH = 6)	0.787	0.206	0.562	8.007	0.0097	0.5584	0.240
6 % P (pH = 8)	1.482	0.161	0.650	8.901	0.012	0.583	0.192

Unless specified experiments were carried out at pH =5. C: cellulase, X: xylanase, P: protease
(*): natural water pH

3.5. Sugar release

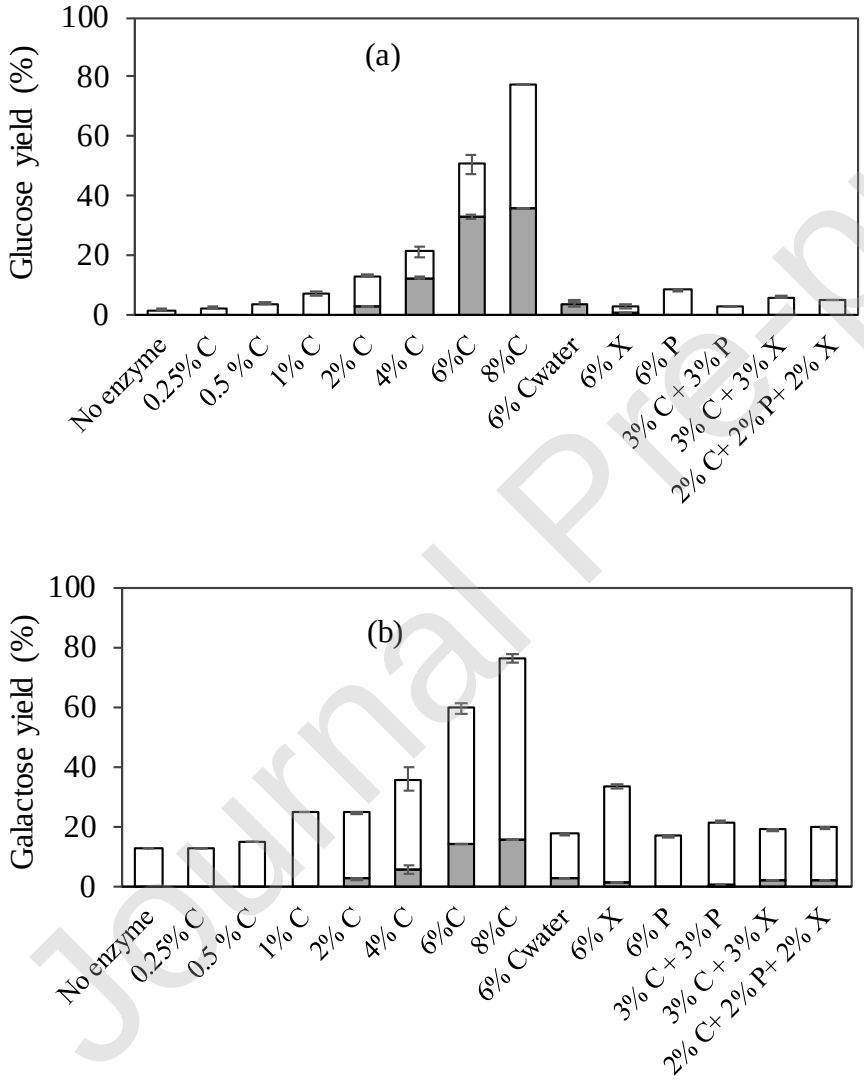
Cellulase has been found an efficient enzyme to release TPC bound to the cell wall algae due to its hydrolytic action on cell wall increasing its permeability. The disruption of cell wall algae would led to the release of the polysaccharide fraction present in the MR into the extraction medium. Therefore, monomers and oligomers of glucose, galactose and arabinose were determined. The results of the different sugars released from the carbohydrate fraction of the MR after 24 h of enzymatic treatment are presented in Figures 5 (a-c). The results have been presented as sugar yields for monomers and oligomers. By increasing the cellulase concentration, the sugar release for glucose, galactose and arabinose also increased. The lowest yield was obtained when using water as solvent with no enzyme added to the medium since no hydrolytic effect took place. At 6% cellulase in water at its natural pH, lower sugars yields were obtained than when pH was adjusted to 5. These results followed the same trend as the TPC release and the increase in the extraction yield as the cellulase concentration increased due to the breakdown of the cell walls of the algae and the presence of the polysaccharide fraction in the enzymatic extracts.

In case of the glucose, about half of the sugar was released into the extraction medium as monomeric glucose, while the rest was solubilized as oligomers. Regarding galactose and arabinose, less than 20 % of its content in the substrate was released as monomers and the rest as oligomers. In any case, the presence of monomers and oligomers support that these type of enzymes attack randomly the internal sites of the amorphous region of the polysaccharide chain producting small oligosaccharides of different length, as it has been described in the literature (Nadar et al., 2018). The higher percentage of glucose as monomer compared to the other sugars, indicated that the cellulase used in this work was more selective towards small chains of glucans

to produce glucose as monomer compared to the other oligomer fractions of galactans and arabinans.

In Figures 5a-c, the results obtained for the other hydrolytic enzymes, protease and xylanase, and their combinations with cellulase have been also presented. At the same enzyme concentration, 6 % (w/w), the best results, in terms of sugar release, were obtained for cellulase. This can be related to the raw material composition being glucans the main component and cellulase would act preferentially over this fraction helping the release of the other polysaccharide componets. For all the enzymes or their combinations, the highest sugar yields were achieved by arabinose followed by galactose and glucose. For instance, at the highest cellulase dose essayed in this work, 8%, the sugar yields were 95 % for arabinose, 76 % for galactose and 77 % for glucose. The lower yields obtained for both hexoses (galactose and glucose) compared to arabinose could be due to monomeric sugar degradation to other compounds via enzymatic conversion of hexoses (see Figure 6). When using the cellulase, the major sugar degradation compound was lactic acid that accounted up to 100 mg/g_{DMR}. Lactic acid bears a hydroxyl group and an acid function, being able to undergo numerous chemical conversions to useful products. Succinic, acetic, formic and glucuronic acids were also determined as well as small amount of ethanol. Acetic acid was one the major compounds formed when using the other enzymes or their combinations with cellulase, proving that the presence of other enzymes exert a competitive effect on the enzymatic transformation routes. Degradation products due to dehydration reaction of hexoses such as furfural and 5-hydroxymethylfurfural (HMF) and other degradation products as pyruvaldehyde were not determined in the enzymatic extracts. These products have been reported as degradation products from the polysaccharide fraction of biomass by hydrothermal process (Brunner, 2009).

It must be also highlighted that production of organic acids from sugars would decrease the pH of the medium. As a result, the reaction rate would decrease and even deactivation of the enzyme would be accelerated (Wasewar et al., 2003). Furthermore, in the literature, it has been described that enzymatic reaction suffer not only substrate inhibition but also competitive inhibition by lactic acid (Wasewar et al., 2003).



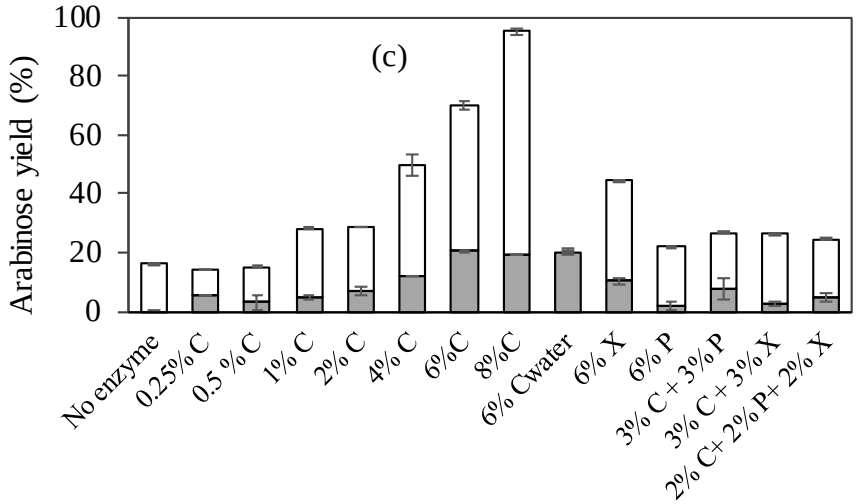


Figure 5. Sugars enzymatic yield at 50ºC after 24 h (a) Glucose yield (b) Galactose yield (c) Arabinose yield (□ monomer, ■ oligomer) by using different hydrolytic enzymes.

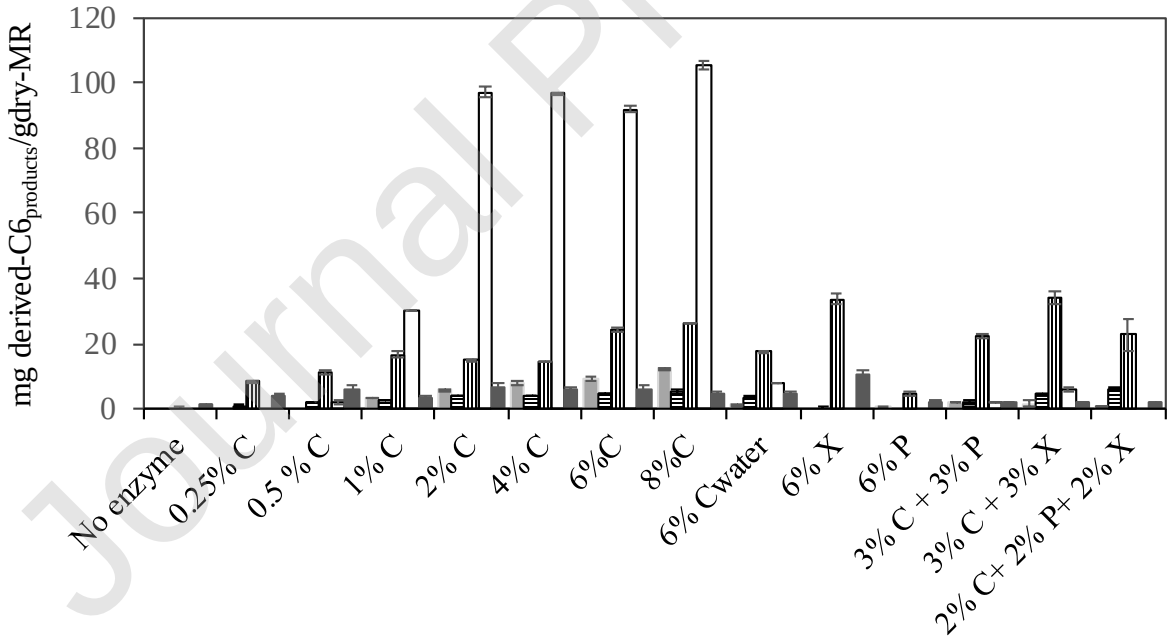


Figure 6. Concentration in the enzymatic extracts of sugars degradation products ■ succinic acid ▤ formic acid ▥ acetic acid, □ lactic acid, ■ ethanol.

3.6. Protein fraction release

Table 4 lists the total protein content released in the extraction medium, expressed as mg protein/g_{DMR} and as extraction yield (Eq. 3) for the different enzymes and their combinations. By increasing the cellulase dose, the protein extraction yield also increased; although at concentration higher than 4 % (w/w) no significative differences were observed. This result agrees with the literature, that also reported the use of nonspecific carbohydrases for protein extraction from seaweeds (Vásquez et al., 2019).

Table 4. Specific protein yield, mg protein/g_{DMR} and protein extraction yield for the different enzymes and their combinations at pH = 5.

Enzyme	mg protein/g_{DMR}	Extraction yield, %
0.25 % C	21.5 ± 0.1^{a}	10.2 ± 0.1^{a}
0.5 % C	21.8 ± 0.4^{a}	10.4 ± 0.2^{a}
1 % C	24.3 ± 0.1^{b}	11.6 ± 0.1^{b}
2 % C	53.6 ± 0.4^{e}	25.5 ± 0.2^{e}
4 % C	62.0 ± 2.0^{g}	29.3 ± 0.9^{g}
6 % C	$62.0 \pm 1.0^{g,}$	29.5 ± 0.6^{g}
8 % C	$63.0 \pm 1.0^{g,h}$	$29.9 \pm 0.4^{g,h}$
6 % X	50.9 ± 0.5^{d}	24.2 ± 0.3^{d}
6 % P	59.5 ± 0.6^{f}	28.3 ± 0.3^{f}
3 C + 3 % P	64.2 ± 0.5^{h}	30.6 ± 0.2^{h}
3 % C + 3 % X	46.0 ± 1.0^{c}	21.8 ± 0.5^{c}
3 % C + 2 % P + 2 % X	59.2 ± 0.4^{f}	28.2 ± 0.2^{f}

C: cellulase, X: xylanase, P: protease
Values with different letters in each column are significantly different when applying the Fisher's least significant differences (LSD) method at p-value ≤ 0.05

The highest protein extraction yield was determined for the binary enzyme mixture with cellulase and protease. In any case, the slightly higher content of protein determined in the medium in the presence of cellulase compared to protease, at the same enzyme concentracion, 6 %, could be due to the interference of the non-protein compounds released into the medium that also absorb at the same wavelength in the spectrophotometric method such as phenolic compounds and sugars. These compounds were produced in a bigger extent due to the action of the cellulase (see Figure 1 and 5), that would result in an overestimation of the protein content in the extract (Olivares-molina and Fernández, 2016).

Vásquez et al. (Vásquez et al., 2019) reported a protein extraction yield of 36.1 % for the red algae *Chondracanthus chamissoi* after 12 h of enzymatic treatment with cellulase and a ratio of 1:10 enzyme:seaweed. These authors reported that, according to literature, conventional methods for protein extraction from seaweed, such as chemical or physical extraction , show protein extraction yields between 24 and 59% for different seaweeds (Bleakley and Hayes, 2017). In this work, by using enzymes, protein extraction yields of the same order were obtained compared to conventional methods but using milder conditions. It must be highlighted that the protein recovery yield achieved by subW treatment of this solid residue was nearly 70 % in a semicontinuous fix-bed reactor configuration at 200 ºC and 18.7 min of residence time. At these conditions, the ionic product of water increases that induced lower pH values that favour biomass hydrolysis (Trigueros et al., 2020).

The effect of pH on protein release by the protease has been studied by performing kinetics at different pH values, 5, 6 and 8 (Figure 7). It can be observed that the protein release was faster with higher yields under basic conditions. At pH=8, nearly 80 mg protein/g_{DMR} was obtained, which represented a 38 % of protein extraction yield. Protein extraction kinetics at the different pH values were also fitted to the Weibull and the power law models (Table 5). For protein

release, the Weibull model yielded much lower RMSD than the power law model, thus, it has been chosen for being represented in Figure 7. Regarding the Weibull model, an increase in the pH led to higher values of the A parameter, according to the highest extraction yield.

Table 5. Kinetic parameters for the power law and the Weibull models for protein release by using 6 % protease at different pH values.

	Power law			Weibull			
	B	n	RMSD	A	k	n	RMSD
pH = 5	3.90	0.240	2.7	63.3	0.0220	0.418	1.3
pH = 6	5.89	0.214	8.7	75.04	0.0075	0.575	3.2
pH = 8	18.44	0.111	14.8	79.92	0.0238	0.509	1.5

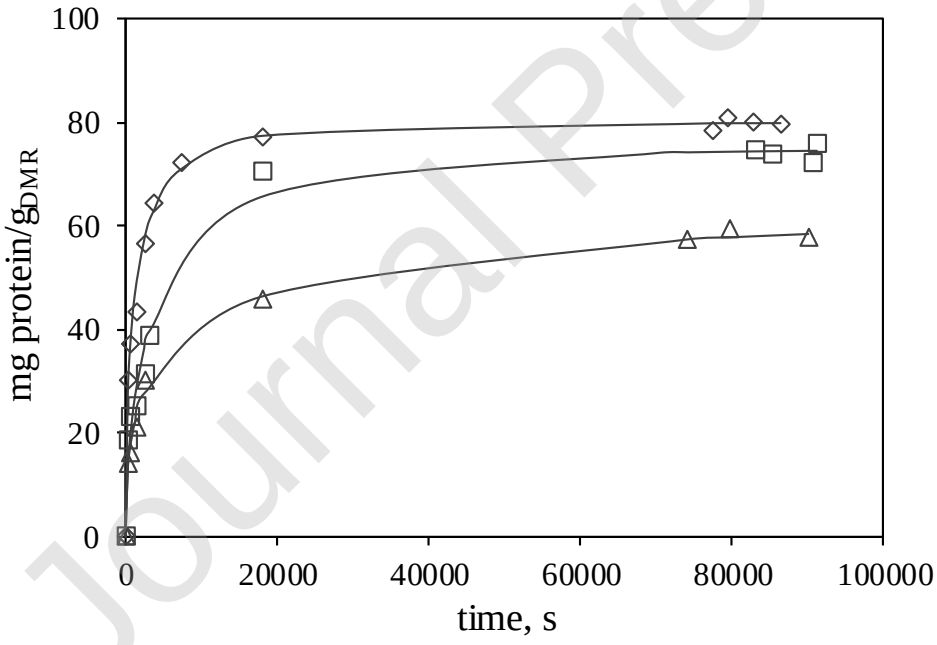


Figure 7. Protein extraction kinetics at 50 ºC by using 6 % protease at different pH values (△ 5, □ 6, ◇ 8). The continuous lines represent the Weibull model (Table 5).

The individual free amino acid profile in the enzymatic extracts after 24 h at the different pH values has been listed in Table 6. The hydrolysis yield for each individual amino acid has been evaluated taking into account the amino acid profile in the MR (see also Table 6). Regarding the amino acid profile of the MR, the major amino acid was aspartic acid, 97 ± 8 $mg/g_{prot\text{-}DMR}$, followed by alanine and glutamic acid. Glutamic and aspartic acids were found to be the main amino acids for many algae, including red algae (Lourenço et al., 2002). The amount of amino acids released increased with the pH of the medium, with the highest content at pH=8, 93 ± 4 mg $aa/g_{protein}$. The total hydrolysis yields were 7 ± 1, 11 ± 2 and 11± 2 (%), at pH values of 5, 6 and 8, respectively.This result agrees with the hydrolysis degree (Eq. 4, h_{tot} = 7.24) that also increased by increasing pH of the medium although no significant differences were observed at pH values of 6 and 8 (Table 6). The highest content of individual amino acids in the enzymatic hydrolysates corresponds to leucine, valine, alanine, isoleucine and phenylalanine. According to the literature, hydrophobic amino acids includes phenylalanine, leucine, isoleucine, tyrosine, tryptophan, valine, methionine, and proline (Widyarani et al., 2016). Therefore, high selectivity towards hydrophobic amino acids has been obtained by using this protease. Hydrophobic selectivity has been evaluated as the amount of free hydrophobic amino acids relative to the total liberated free amino acids on molar-base (Table 6). It can be observed that the hydrophobic ratio increased in the hydrolysates compared to the MR, reaching values around 0.62-0.64. According to the amino acid profile in the enzymatic hydrolysates, in general, higher yields were obtained for hydrophobic amino acids (see Figure 8). The highest yield corresponds to tryptophan, due to its small content in the raw material, although concentration in the enzymatic hydrolysates was only 1.3 ± 0.1 $mg/g_{protein}$. It can be highlighted that the production of mixtures rich in hydrophobic amino acids is an interesting process based on their potential application as in food and feed.

Conclusions

To incorporate macroalgae into the biorefinery concept, valorization of industrial wastes generated during industrial processing should be considered. It has been shown that EAE provides a mild and efficient technology to valorize the industrial solid waste residue after agar extraction from *G sesquipedale*. The use of hydrolytic enzymes allowed the release into the reaction medium of phenolic compounds bound to the cell wall of the macroalgae, as well as the release of the polysaccharide fraction as monomers and oligomers and the hydrolysis of the protein fraction with a higher content of hydrophobic amino acids compared to the raw material. Cellulase has been found to provide the highest hydrolytic capacity to break down the cell wall algae releasing different bioactive compounds such as TPC, proteins and the polysaccharide fraction. It was not observed a competitive adsorption effect to the cell wall polysaccharides by increasing the amount of cellulase in the medium, but the optimum enzyme dose must also take into account economic factors as the enzyme cost. Further works shoud be performed to determine the extraction curves of the polysaccharide fraction and sugar degradation products.

Table 6. Amino acid profile of macroalgae residue, MR, and of the protease enzymatic extracts, expressed as $mg/g_{protein-MR}$. Extraction yield percentage (yield, %) of individual amino acid. Hydrolysis degree (DH) and hydrophobic molar selectivity (HPB/TAA).

	MR	pH =5		pH = 6		pH = 8	
	$mg/g_{prot-DMR}$	$mg/g_{prot-DMR}$	Yield, %	mg/g_{prot}	Yield, %	mg/g_{prot}	Yield, %
Ala	94 ± 8	8.4 ± 0.1	9.0 ± 0.9	11.0 ± 0.5	11.7 ± 1.5	11.7 ± 0.3	12.4 ± 1.4
Gly	41 ± 4	2.0 ± 0.1	4.8 ± 0.8	3.1 ± 0.3	7.5 ± 1.5	3.2 ± 0.1	7.8 ± 1.0
Val	67 ± 7	7.6 ± 0.3	11.0 ± 2.0	12.7 ± 1.3	18.9 ± 4.0	13.7 ± 0.2	20.4 ± 2.6
Leu	78 ± 8	11.6 ± 0.5	14.9 ± 2.0	14.8 ± 1.6	19.0 ± 3.9	15.6± 0.2	20.0 ± 2.2
Ile	45 ± 5	6.4 ± 1.3	14.2 ± 4.7	10.1 ± 0.3	22.5 ± 3.5	10.7 ± 0.1	23.7 ± 3.2
Thr	36 ± 2	1.8 ± 0.1	5.1 ± 0.4	3.5 ± 0.3	9.9 ± 1.3	3.2 ± 0.2	8.8 ± 1.0
Ser	39 ± 6	--	--	--	--	--	--
Pro	73 ± 4	1.6 ± 0.3	2.3 ± 0.6	3.5 ± 0.1	4.8 ± 0.4	4.8 ± 0.2	6.6 ± 0.6
Asp	97 ± 8	--	--	--	--	--	--
Met	8 ± 1	0.8 ± 0.1	9.5 ± 2.7	1.5 ± 0.1	19.0 ± 4.0	1.4 ± 0.1	17.8 ± 4.2
Glu	79 ± 5	2.9 ± 0.5	3.6 ± 0.9	8.3 ± 2.0	10.5 ± 3.2	8.5 ± 1.5	10.7 ± 2.5
Phe	51 ± 6	7.3 ± 0.3	14.3 ± 2.3	9.2 ± 0.8	18.0 ± 3.7	9.8 ± 0.1	19.2 ± 2.4
Lys	57 ± 7	1.5 ± 0.1	2.7 ± 0.4	1.63 ± 0.04	2.9 ± 0.4	1.9 ± 0.3	3.3 ± 0.9
His	16 ± 2	0.50 ± 0.02	3.0 ± 0.4	0.9± 0.2	5.9 ± 1.7	1.1 ± 0.3	7.1 ± 2.4
Tyr	35 ± 6	4.4 ± 0.7	12.7 ± 4.1	5.4 ± 0.5	15.5 ± 4.0	6.0 ± 0.3	17.3 ± 3.6
Trp	3.0 ± 0.2	1.1 ± 0.2	35.9 ± 9.0	1.2 ± 0.1	41.3 ± 6.8	1.3 ± 0.1	42.2 ± 5.7
EAA	361 ± 39	39 ± 3	11 ± 2	56 ± 5	15 ± 3	59 ± 2	16 ± 2
Total	819 ± 80	58 ± 5	7 ± 1	87 ± 8	11 ± 2	93 ± 4	11 ± 2
HPB/TAA	0.4 ± 0.1	0.64 ± 0.01		0.62 ± 0.01		0.62 ± 0.01	
DH		4.2 ± 0.8[a]		7.70 ± 0.05[b]		9.3 ± 0.8[b]	

Values with different letters are significantly different when applying the Fisher's least significant differences (LSD) method at p-value ≤ 0.05

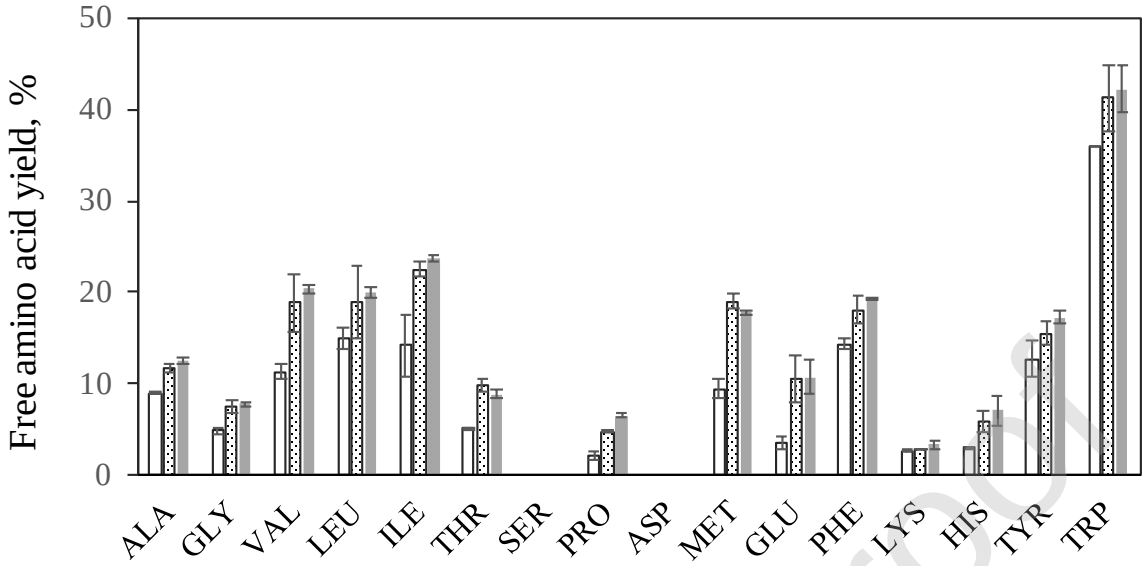


Figure 8. Free amino acid yield in the protease enzymatic extracts at different pH values after 24 h (pH =5 □; pH = 6 ▩; pH = 8 ■).

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Declarations of interest: none

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