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Rugulopteryx okamurae: Assessment of its potential as a source of monosaccharides for obtaining bio-products

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ABSTRACT

Beach-cast seaweed of the invasive brown macroalga *Rugulopteryx okamurae* was used in this study as raw material to obtain fermentable sugars, which can be converted into high added-value products. The dietary fibre composition of this macroalgae was determined and compared to other brown and red macroalgae, showing one of the highest proportions of dietary fibre (27.3 %) and cellulose (13.6 %). Therefore, the enzymatic hydrolysis of *R. okamurae* could lead to obtaining hydrolysates with a high concentration of reducing sugars. The main hydrolysis variables (biomass loading, enzyme dose and stirring rates) and the operation mode (fed-batch versus batch) were evaluated to maximize the sugar concentration. Thus, a maximum total reducing sugar concentration of 13.7 g/L was obtained at the optimum conditions: biomass loading of 10 % (w/v), 50 FPU/g biomass, 250 rpm and operating in batch mode. In addition, a kinetic model has been developed to describe the enzymatic hydrolysis of biomass. The model, unlike first-order kinetics, includes a specific term considering the enzyme diffusion through the solid biomass. The proposed kinetic model leads to better fitting of experimental data than the first-order model, especially for long incubation times.

1. Introduction

Invasive macroalgae represent more than 40 % of the invasive alien species in the European Union and are considered one of the main threats to the ecosystem biodiversity in coastal regions. These macroalgae are accumulated in large quantities on the coast, causing relevant environmental and social problems and affecting the local economy. *Rugulopteryx okamurae* is one of the most damaging seaweed, as it has a high rate of spread, causing the alteration of marine habitats and the displacement of native species. Currently, this species takes up over 85 % of the Natura 2000 network sites from 10 to 30 m depth on rocky seafloors [1–3].

In Spain, *R. okamurae* was first found in the Strait of Gibraltar (Tarifa and Ceuta) in the autumn of 2015, and by 2016 it had colonised most of the rocky bottoms of the Strait's shoreline. An example of the changes in the marine habitat is being observed in Ceuta, where *R. okamurae* has seriously affected the local benthic communities, which have been displaced. Thus, the competition for space is affecting *Astroides calycularis* (orange coral), one coral species threatened by the algal invasion [4]. Furthermore, according to data collected at the end of summer 2016, 15.5 km of the coastline of Ceuta was severely affected, and 5000 tonnes of seaweed deposits had to be removed in that area. This problem implies high costs derived from beach restoration and even the closure of emblematic beaches in the middle of the tourist beach season. Moreover, fishing is also affected by the accumulation of algae in fishing nets, causing significant economic losses to a productive sector, which is of great importance to the local economy [4,5]. According to the Spanish Ministry for Ecological Transition and the Demographic Challenge (MITECO), the expansion of the brown seaweed *R. okamurae* in 2019 affected the coasts of the Bay of Cadiz, Malaga, Chafarinas Islands, Granada and Almeria and hence, it is already on the EU list of alien species of concern.

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A promising strategy to reduce the impact of invasive macroalgae could be its utilization to produce high value-added products. In this regard, both the environmental problems and the non-renewable resource depletion have encouraged the research for using this biomass as an alternative raw material. That is an approach engaged in the circular economy and permits to obtain of valuable bioproducts through biorefinery processes. In addition, macroalgae biomass presents clear economic advantages over other types of biomasses, given that they do not require freshwater and arable land for their cultivation and, hence, they do not compete with conventional food sources. Furthermore, macroalgae have a high carbohydrate content and a low content of recalcitrant material, so they have recently received much attention for high added-value compounds production, such as liquids biofuels, biogas, succinic acid and polyhydroxyalkanoates, among others [6–9]. Nevertheless, in the biorefinery framework, for the global valorization of different species of brown algae is highly recommended the prior extraction of high added-value compounds, such as biopolymer (i.e. alginate, fucoidan, etc.) and bioactive compounds [6,10]. The generated waste can be exploited to produce fermentable sugars to obtain commodities, such as organic acids or ethanol [11]. Indeed, glucose, galactose, mannitol and fucose, derived from green (*Ulva pertusa*), brown (*Laminaria* sp.) and red seaweed (*Gelidium amansii*), have been used as fermentable sugars for lactic acid bacteria. Moreover, it has been reported [12] that lactic acid yields from seaweeds are comparable to that obtained from lignocellulosic wastes. However, brown seaweed also contains non-fermentable sugars (mannuronic acid and guluronic acid) but requires recombinant microorganisms [6].

The release of monomeric sugars from seaweed requires the hydrolysis of polymeric carbohydrates [13]. Thus, to improve the enzyme's accessibility to these polymers, a previous pretreatment stage is usually used. In this sense, hydrothermal acid pretreatment, which is one of the most commonly applied to lignocellulosic biomass [14], has also been used for macroalgae [15,16]. However, in the case of brown seaweed, high yields could be reached even without pretreatment [17].

The utilization of *R. okamurae* to obtain high value-added products has been reported scarcely in the literature. There are some very recent studies in which *R. okamurae* has been used to produce reducing sugars [18], volatile fatty acids [19] as well as to obtain methane by anaerobic co-digestion [20]. Moreover, it has also been employed for other purposes, such as the obtention of precursors for biofertilizers or bioplastics production [21].

The main objective of this work was to evaluate the invasive macroalga *Rugulopteryx okamurae* as an alternative raw material for fermentable sugars production in a biorefinery approach. The dietary fibre composition of *R. okamurae* was determined by adaptation of the fibre detergent method used for lignocellulosic biomass. Indeed, this study includes an in-depth description of this methodology, correlating the main polysaccharides present in brown seaweed with the different fractions of the fibre determination method. Besides, the main enzymatic hydrolysis variables (i.e., biomass loading, enzyme dose and stirring rates) and two types of operation modes (fed-batch versus batch) have been evaluated to maximize the sugar concentration. In addition, an enzyme kinetic model has been developed, which considers the effect of enzyme diffusion through the inert solid biomass. This model, unlike the typical first-order kinetics, is capable to fit the slight increase in reducing sugars occurring for prolonged hydrolysis times.

2. Material and methods

2.1. Sampling and conditioning of seaweed biomass

The brown macroalga *Rugulopteryx okamurae* was collected at the coastal waters of Punta Camorro (Tarifa, Spain) during low tides in the spring. The seaweed was deposited in 25 L polyethylene drums and washed with tap water to remove salts and debris until the final conductivity was below 600 μS/cm. Then, washed seaweed was dried in a greenhouse for 24 h. Finally, the dried biomass was milled using a cutting mill to obtain a 1 mm particle size and stored in hermetically closed drums at room temperature until use.

2.2. Enzymatic saccharification

The commercial enzyme preparation Cellic Ctec2 (Novozymes, Denmark), containing cellulase (EC 3.2.1.4) and endo-β-1,4-xylanase (EC 3.2.1.8) was used for enzyme hydrolysis of macroalgal biomass.

In order to evaluate the best conditions for reducing sugars (RS) production, the enzymatic hydrolysis was carried out in batch mode at different solids loading (6, 8 and 10 % (w/v)) and enzyme doses (18, 30 and 50 FPU/g of dried biomass) working at 150 rpm. Subsequently, the assays producing the highest RS concentration were tested at 250 rpm to analyze the effect of the stirring rate on the saccharification.

Enzymatic hydrolysis was performed in 250 mL Erlenmeyer flasks containing 45 mL of 50 mM sodium phosphate buffer (pH 5.0). For batch hydrolysis assays, before adding the commercial enzyme preparation Cellic Ctec2, the biomass was added to the buffer and sterilised by autoclaving for 20 min at 121 °C.

On the other hand, to study the fed-batch hydrolysis, the best conditions of the batch tests were selected, and the biomass was added 4 times until reaching a final solid loading of 10 % (w/v) as follows: 0 h, 2 g; 3 h, 1.5 g; 6 h, 0.5 g; 9 h, 0.5 g. The first load was sterilised co-jointly with the buffer, but in subsequent additions, the biomass was autoclaved separately. At the same time, the appropriate enzyme dose was added in each case.

In both modes of operation, after the enzyme addition, the flasks were sealed with silicone stoppers and incubated in a rotary shaker at 50 °C. These conditions were selected based on other related studies [22]. Samples were withdrawn during the process and stored at −20 °C until use for RS analysis. The hydrolysis process was maintained for 165 h, and each assay was performed in duplicate.

2.3. Analytical techniques

Before fibre composition analysis, solid biomass was dried in an oven for 2 h at 105 °C and, after that, it was kept in a desiccator at room temperature for 30 min. Analysis of fibre of algae biomass was carried out following the Detergent Fibre Analysis methodology described in EN ISO 13906:2008 and AOAC 973.18 to determine acid detergent fibre (ADF) and acid detergent lignin (ADL). The methodology described in EN ISO 16472:2006 and AOAC 2002:04 was used to determine neutral detergent fibre (NDF). These methods were carried out in Fibertec™ 8000 (FOSS IBERIA, Barcelona, Spain) and FT 121 Fibertec (FOSS IBERIA, Barcelona, Spain). This analysis was carried out in triplicate.

The proposed methodology allowed the quantification of different fractions in the seaweed: lipid compounds (fats, oils and waxes), which are the result of acetone extraction; Soluble dietary fibre (SDF), which contains non-cellulosic polysaccharides (acid-labile carbohydrates), obtained by treatment with ADF (0.5 M H_2SO_4); Insoluble dietary fibre (IDF), which is the result of ADL treatment and it consists of a liquid fraction solubilized with 12 M H_2SO_4, composed mainly of cellulose, and a solid fraction consisting of non-eliminable compounds (acid-insoluble lignin and ashes). Non-fibrous matter (NFM) is composed mainly of non-fibrous carbohydrates (starch), soluble salts and proteins, obtained from the amylase-treatment NDF. By comparing the various fibre fractions obtained with this gravimetric method, the total amounts of lipids, NFM, SDF and IDF (cellulose and lignin) could be determined (Fig. 1).

RS were measured in the hydrolysates from the enzymatic saccharification by the DNS method adapted to microplates [23,24]. Samples were previously centrifuged, and the supernatant was used for analysis. The measurements were performed in triplicate.

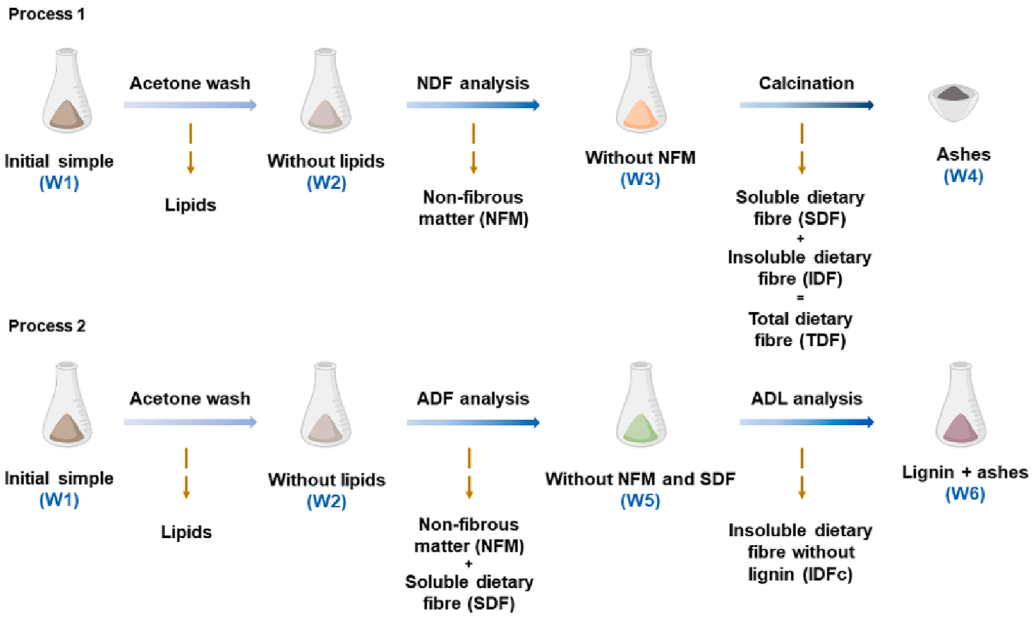

Fig. 1. General outline of the fibre analysis process. Methodology described in EN ISO 13906:2008 and AOAC 973.18 to determine acid detergent fibre (ADF) and acid detergent lignin (ADL). Methodology described in EN ISO 16472:2006 and AOAC 2002:04 to determine neutral detergent fibre (NDF). W_n: dry weight of the sample "n". $W_1 - W_2$ = Lipids; $W_2 - W_3$ = NFM; $W_3 - W_5$ = SDF; $W_5 - W_6$ = IDF_C; $W_6 - W_4$ = Lignin; $W_3 - W_4$ = TDF. The result of the analysis is given in % (w/w).

2.4. Kinetics analysis of the enzymatic hydrolysis

For the study of enzymatic hydrolysis, the RS concentrations were plotted as a function of time and the data were fitted to a first-order kinetic model Eq. (1), characteristic of enzymatic hydrolysis [22].

$$P = P_o + \beta \cdot S_o \left(1 - exp^{-k_1 \cdot t}\right) \quad (1)$$

where P is the RS concentration (g/L) at time t, P_o is the initial RS concentration (g/L), β is the hydrolysis yield coefficient, k_1 is the rate constant (h^{-1}), t is the hydrolysis time (h) and S_o is the initial substrate concentration that can be converted to product (g/L) which was calculated from Eq. (2).

$$S_0 = \frac{B \cdot TDF_C}{V_H} \quad (2)$$

where B is the biomass loading (g) used in the hydrolysis, V_H is the hydrolysis volume (L), and TDF_C is the total fibre dietary without lignin (w/w), which was considered to contain the total hydrolysable polysaccharides, and it was calculated as the sum of the SDF and IDF_C fractions. It is known that the main carbohydrates of brown algae are alginates, fucoidan, laminarin and cellulose [25], and according to the literature [26], the SDF fraction contains alginates, fucoidan and laminarin, and IDF fraction contains mainly cellulose, but also includes Klason lignin (KL), which is subtracted to calculate IDF_C. In addition to this, the NFM fraction is a heterogeneous mixture that may contain proteins and non-fibrous carbohydrates, mainly starch, but since the starch is not present in brown algae [27], this fraction represents mainly nitrogenous compounds and, therefore, it was not considered for the estimation of S_o.

The fit of the experimental data to the first-order model shows that it does not predict the increase in RS concentration for long hydrolysis times. Therefore, a new kinetic term has been added to the previous first-order equation. This term takes into account that the product is generated as a consequence of enzymes reaction on the substrate inside the solid particle not dissolved in the liquid medium. In short, the new proposed model considers that the substrate for enzymatic hydrolysis could be present both in the liquid medium and inside the algal biomass particles. It is assumed that the hydrolysis of the dissolved substrate fraction in the liquid medium will follow conventional first-order kinetics. However, the new term considers the hydrolysis of the substrate contained in the biomass less accessible to the enzymes. The proposed new model relies on the well-known unreacted core model for particles of constant size [28]. This model is used for heterogeneous solid–liquid reactions and considers that an inert-material layer remains consolidated inside the particles when the reaction occurs and, therefore, the particle size is constant. In this model, the reaction takes place in 3 successive stages: (1) diffusion of the enzyme through the liquid to the particle surface (2) penetration and diffusion of the enzyme through the inert-material layer to the unreacted core surface (3) biological reaction on this reaction surface. The transport rate for stage 2 is given by Fick's law. Considering the rate-limiting step approximation and assuming that stage 2 is the slowest stage (the rate-limiting step) in the overall process, the other stages should reach equilibrium. In this way, a simple expression can be obtained, in which the flux of the enzyme, and hence the rate of substrate consumption, are constant. Thus, the reaction rate of the enzyme at any instant is given by its rate of diffusion towards the surface where the substrate is located, with the rate being constant [28]. The integration of the corresponding expression to obtain Eq. (3) for the product generation includes an additional linear-type term over time.

$$P = P_o + \beta \cdot S_o \left(1 - exp^{-k_1 \cdot t}\right) + \alpha \cdot k_2 \cdot t \quad (3)$$

where α is the hydrolysis yield coefficient for the enzyme diffusion stage and k_2 is the rate constant ($g \cdot L^{-1} \cdot h^{-1}$), also for this stage.

2.5. Enzymatic hydrolysis yield

Hydrolysis yield (Y_{RS}) was calculated considering the RS (in grams) produced in the hydrolysis with respect to the theoretical monosaccharides derived from polysaccharides in the biomass (in grams) as it is described below in Eq. (4).

$$Y_{RS} = \frac{P \cdot V_H}{B \cdot TDF_C \cdot 1.1} \quad (4)$$

where theoretical monosaccharides were estimated based on TDF_C and they were calculated using the correction factor (1.1) according to [29]. This factor takes into account the presence of water in the

polysaccharides' conversion to monosaccharides during the hydrolysis process.

2.6. Statistical analysis

A one-factor analysis of variance (ANOVA) using the software Statgraphics© Centurion 19 (StatPoint Technologies, Inc, Princeton, NJ, USA) was performed to evaluate the existence of significant differences between the means in the analysis of dietary fibre. A difference between means with a *p-value* less than 0.05 was considered significant.

The values of the kinetics parameters were calculated with the *Solver* tool of Microsoft® Excel® 2016 MSO (16.0.4266.1001) 64 bits.

3. Results and discussion

3.1. Fibre composition of Rugulopteryx okamurae

The results of the fibre analysis of *Rugulopteryx okamurae* are shown in Table 1.

A relevant aspect of the study of TDF is the IDF ratio. According to the Standardization of Analytical Methodology for Feed, IDF represents the insoluble fraction in an acid concentration equal to or less than 0.5 M (SDF being the soluble fraction) and is mainly composed of cellulose and lignin [30]. Although different methods exist in the literature to separate lignin from cellulose, the procedure carried out in this study using Acid Detergent Lignin (ADL) is the most commonly used for dietary fibre analysis [31]. In the protocol, IDF is obtained after treatment with ADL, which consists of a 12 M acid solution (72% mass fraction), and the solubilized ADL residue contains mainly cellulose, while the lignin remains in the solid. This acid-insoluble lignin was defined as Klason lignin (KL) [30,32]. The industrial extraction of lignin for different applications, such as the production of phenol–formaldehyde resins, is also carried out with the ADL method [33,34]. Since lignin is not a substrate for the enzymes used in this study, the cellulosic portion of IDF (IDF_c) is the most relevant fraction. For this reason, IDF_c and acid-insoluble lignin (KL) from *R. okamurae* are shown separately in Table 1, where they are compared with other brown and red macroalgae species. No studies of dietary fibre analysis with green algae applying a comparable protocol have been found in the literature.

The results showed that, unlike other macroalgae, *R. okamurae* contained similar IDF_c and SDF fractions (13.59 % and 13.70 %, respectively). Also, this macroalga and *Himanthalia elongate* showed the highest proportion of TDF_C of the brown algae (27.29 % and 27.60 %, respectively), but *R. okamurae* had the highest cellulosic fraction, which was similar to the one of *Laminaria digitate*. This aspect is advantageous for enzymatic saccharification with the typical commercial enzyme cocktails containing cellulases. Nevertheless, no significant differences were observed in IDF_C, SDF and KL fractions between the brown and red algae compared.

On the other hand, the non-fibrous fractions present in *R. okamurae* biomass are shown in Table 2. Although the lipid content of *R. okamurae* was similar to that of other macroalgae (*Bifurcaria bifurcata*), this did not occur for protein and ash content. Thus, the protein fraction (NFM) of *R. okamurae* showed the highest value of all the macroalgae (49.05 %), followed by *Porphyra tenera* (29.80 %), although the ashes content was much lower (15.15 %) than for the rest.

This difference in protein fraction may be due to the season in which the macroalgae were collected, as has already been reported in the literature [35]. Also, other studies have highlighted the high capacity of *R. okamurae* for nitrogen accumulation compared to other species [36], which would explain the high percentage observed in this fraction. In the cases compared in this study, macroalgae were collected during spring-summer, a broad period from March to September. There are studies in which it is reported that from May and throughout the summer, there is a decrease in nitrogen in brown algae such as *Laminaria longicrusis*, which is associated with its low growth rate in this period [35]. Therefore, macroalgae collected in late summer will have less protein content than ones collected in spring. In this study, *R. okamurae* was collected in early spring (April), which may explain the higher proportion of protein observed regarding the other macroalgae.

Another aspect reported for brown algae, such as *Laminaria hyperborea*, is that protein synthesis is influenced by the laminarin content. This is the reserve polysaccharide in brown algae, which increases from

Table 1
Composition of SDF (soluble dietary fibre), IDF_C (insoluble dietary fibre without lignin), KL (Klason lignin) and TDF_C (total dietary fibre without lignin) in dry basis (% w/w) of *R. okamurae* and other brown and red algae.

Brown algae	TDF_C	IDF_C	SDF	KL	Reference
Himanthalia elongata	27.60 ± 0.86	3.97 ± 0.45	23.63 ± 0.48	9.54 ± 0.40	[18]
Bifurcaria bifurcata	26.59 ± 0.78	11.96 ± 0.97	14.64 ± 0.68	10.83 ± 0.84	[18]
Laminaria saccharina	26.34 ± 0.85	9.22 ± 0.56	17.12 ± 0.84	3.89 ± 0.12	[18]
Laminaria digitata	23.03 ± 2.46	13.89 ± 1.97	9.15 ± 0.48	13.09 ± 0.84	[19]
Undaria pinnatifida	24.26 ± 1.31	6.94 ± 0.79	17.31 ± 0.51	9.32 ± 0.29	[19]
Fucus vesiculosus	19.01 ± 1.77	9.21 ± 0.98	9.80 ± 0.78	31.08 ± 0.52	[19]
Rugulopteryx okamurae	27.29 ± 1.49	13.59 ± 0.37	13.70 ± 1.34	4.49 ± 0.60	This study
Red algae					
Mastocarpus stellatus	28.25 ± 0.23	5.40 ± 0.67	22.85 ± 0.19	3.45 ± 0.90	[18]
Gigartina pistillata	26.07 ± 0.34	4.17 ± 0.12	21.90 ± 0.22	3.24 ± 1.13	[18]
Chondrus crispus	28.36 ± 3.89	6.11 ± 2.89	22.25 ± 0.99	5.93 ± 1.95	[19]
Porphyra tenera	23.39 ± 3.38	8.83 ± 2.05	14.56 ± 1.33	10.39 ± 0.52	[19]

Table 2
Composition of lipids, proteins and ashes in dry basis (% w/w) of *R. okamurae* and other brown and red algae.

Brown algae	Ashes	Lipids	Protein	Reference
Himanthalia elongata	36.41 ± 0.15	0.94 ± 0.07	14.08 ± 0.21 [a]	[18]
Bifurcaria bifurcata	34.34 ± 0.21	5.67 ± 0.32	10.92 ± 0.10 [a]	[18]
Laminaria saccharina	34.78 ± 0.08	0.79 ± 0.07	25.70 ± 0.11 [a]	[18]
Laminaria digitata	37.60 ± 0.32	n/d	9.99 ± 0.02 [a]	[19]
Undaria pinnatifida	39.82 ± 0.05	n/d	15.97 ± 0.04 [a]	[19]
Fucus vesiculosus	30.12 ± 0.48	n/d	6.86 ± 0.01 [a]	[19]
Rugulopteryx okamurae	15.15 ± 0.37	4.02 ± 0.29	49.05 ± 1.36[b]	This study
Red algae				
Mastocarpus stellatus	24.99 ± 0.12	0.39 ± 0.02	21.30 ± 0.18 [a]	[18]
Gigartina pistillata	34.56 ± 0.47	0.57 ± 0.06	15.59 ± 0.28 [a]	[18]
Chondrus crispus	21.44 ± 0.14	n/d	20.90 ± 0.14 [a]	[19]
Porphyra tenera	21.00 ± 0.42	n/d	29.80 ± 0.04 [a]	[19]

n/d: not detailed by the author.
a: Protein content was determined with a Leco FP-2000.
b: Protein content was determined with a gravimetric methodology described in EN ISO 16472:2006 and AOAC 2002:04 to determine neutral detergent fibre (NDF).

2 % in May to 30 % in October-November [35]. Thus, the SDF fraction of brown algae collected in spring will be poorer in laminarin than the ones collected at the end of summer.

3.2. Effect of solid loading, stirring and enzyme dose on the enzymatic hydrolysis

3.2.1. Comparison of kinetic models

The experimental data and the predicted trends by both the simple first-order kinetic model and the proposed one are shown in Fig. 2. In this case, the experiment with a 10 % biomass loading, 30 FPU/g biomass and a stirring rate of 150 rpm has been selected.

The classical model to describe enzymatic hydrolysis corresponds to a simple first-order kinetic model. However, as can be seen in Fig. 2, the experimental data obtained in this work show that, for high hydrolysis times, the first-order kinetics does not appropriately represent their evolution. Thus, a slight increase in the reducing-sugars production can be observed from 9 h on, which cannot be explained by first-order kinetics. Consequently, the proposed new model includes an additional term to consider the diffusion of enzymes into the solid matrix of biomass. This term would be responsible for the slight increase in RS observed experimentally.

Regarding the simple model, the predicted trend showed that the saccharification process was practically completed after approximately 30 h of hydrolysis and did not predict the production of subsequently generated RS.

In this way, the proposed kinetic model showed a much better fitting to experimental data (r^2: 88.9 % and 97.6 %, respectively). On average, the regression coefficient values (r^2) improved by 5.8 %, with a maximum improvement of 8.7 %.

According to the proposed model, two differentiated stages in the hydrolysis process can be seen. During approximately the first 9 h, the hydrolysis followed a first-order kinetics. In this case, the kinetics is dominated by the hydrolysis of the fraction of substrate solubilised in the medium liquid. In fact, during the sterilisation stage part of the solid substrate was dissolved and, hence, was easily accessible to the enzymes. Nevertheless, from these first hours onwards, the hydrolysis process followed a linear behaviour. This effect is related to the hydrolysis of the substrate contained inside the solid biomass particles, which is less easily accessible to the enzymes. To consider this fact, the proposed model assumes that the behaviour is similar to that of the unreacted core model for particles of constant size, when the diffusion of the enzyme through the inert-material layer is the rate-limiting step. Obviously, the hydrolysis of both soluble and particulate substrates occurs simultaneously. However, since the hydrolysis of the readily accessible (soluble) substrate is much faster than that of the less accessible substrate (inside the particles) the effect of the latter on the overall kinetics is only appreciated when all the soluble substrate has been consumed.

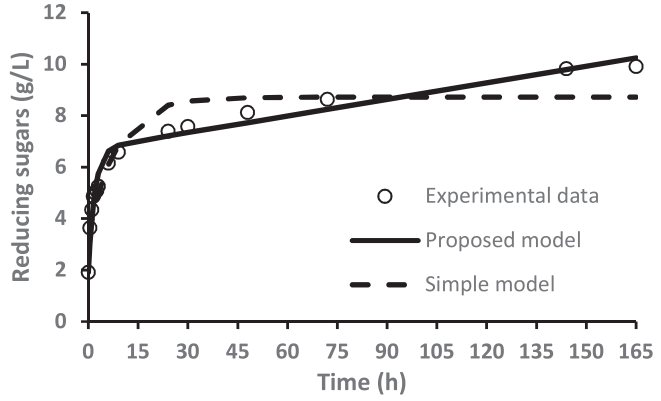

Fig. 2. Comparison between the fitting of the simple first-order (R^2: 88.9 %) and proposed (R^2: 97.6 %) kinetic model to the experimental data for hydrolysis carried out at 10 % of biomass loading, 30 FPU/g biomass and agitation rate of 150 rpm.

3.2.2. Comparison between hydrolysis conditions

Table 3 shows the kinetics parameters, conversion, production and yield values obtained in the fitting of the proposed kinetic model to experimental data for each hydrolysis tested. The r^2 values are indicative of a good fitting for all cases, reaching up to 99.4 %.

The highest RS concentration was obtained for 10 % of biomass loading, 50 FPU/g biomass and 250 rpm in batch mode (13.65 g/L), reaching a yield (Y_{RS}) of 45 %. A significant improvement in the RS concentration has been observed operating under these conditions compared to those reported in the literature. Thus, data obtained by other authors [18] for the hydrolysis of non-pretreated *R. okamurae* showed that the maximum RS concentration obtained was 1.5 g/L after 72 h. However, in this study, 12.22 g/L was obtained at that time (Fig. 3 a). Taking into account that the biomass was not previously pretreated, it is interesting to point out that the quantities of RS generated are even higher than those of other studies carried out with pretreated brown algae. For example, under the conditions detailed above, after 48 h of hydrolysis, 11.85 g/L of RS was measured, which is significatively higher than the concentration of 8.07 g/L obtained with *Sargassum* sp. subjected to a thermo-acid pretreatment (0.15 N H_2SO_4, at 121 °C for 30 min) [37]. There are also studies in which enzymatic hydrolysis was specifically carried out with pretreated *R. okamurae*. In one of the cases, the algal biomass was pretreated biologically, but the RS concentration obtained did not exceed 3 g/L [18]. In addition, *R. okamurae* has also been subjected to hydrothermal-microwave pretreatment, and the RS concentration reached was lower than 12 g/L after 50 h of hydrolysis [19]. In both cases, the enzyme dose used was 112 FPU/g dry biomass, much higher than the maximum amount of enzyme used in this study. The relevance of these results lies in the use of non-pretreated biomass is advantageous as the total cost of the process is reduced, and it is more environmentally friendly since do not use aggressive chemical reagents. Another important aspect is that the formation of fermentation inhibitor products, which can be generated in severe pretreatments, is minimised [38].

Regarding the hydrolysis rates, it can be observed that increasing the solid loading significantly improved it in both reaction stages. The k_1 value increased progressively until 43 % (from 0.237 h^{-1} to 0.416 h^{-1}) and, equally, the k_2 value by 40 % (from 0.099 $g{\cdot}L^{-1}{\cdot}h^{-1}$ to 0.165 $g{\cdot}L^{-1}{\cdot}h^{-1}$). In contrast, the same trend was not observed when more enzyme was added, obtaining values between 0.410 h^{-1} and 0.522 h^{-1} for k_1, while for k_2, the values remained without appreciable changes in 0.165 $g{\cdot}L^{-1}{\cdot}h^{-1}$. According to these results, the ratio substrate-to-enzyme used in the hydrolysis has been too low; i.e., the enzyme was in excess. Indeed, increasing the biomass loading significantly improved the hydrolysis rate.

On the other hand, increasing the amount of biomass was not favourable for improving the yields in any of the reaction stages, obtaining an average value of 0.177 ± 0.011 for β and 0.127 ± 0.007 for α. As for the effect of enzyme dose on yields, only the β values increased from 0.151 to 0.181 (average value of 0.169 ± 0.013) when more enzyme was used. However, this slight increase in the stage 1 yield may be due to the RS contained in the enzyme cocktail itself, which was experimentally determined, resulting in 0.2 g RS/g enzyme ± 0.03. The α value showed no dependence on the enzyme doses tested (average value of 0.123 ± 0.006). The results show that both variables, biomass loading and enzyme dose, caused similar variations in the range of values of the parameters β and α. Thus, neither of these two variables had an appreciable effect on the yields of either hydrolysis step. Based on the results obtained, it can be said that the hydrolysis was probably carried out close to the maximum attainable yields for the conditions tested.

An effective strategy to increase the amounts of sugars obtained in the hydrolysis is to use higher solids loading; however, the viscosity of

Table 3
Values of the parameters obtained after fitting the enzymatic kinetic model proposed to the experimental data. The values of maximum RS concentration and the yields were calculated from the model (t = 165 h).

Effect	Condition	r^2	Kinetics parameters		Conversion		P	Yield
Biomass loading	**50 FPU/g – 150 rpm**	**%**	**k_1 (h^{-1})**	**k_2 ($g{\cdot}L^{-1}{\cdot}h^{-1}$)**	**β**	**α**	**RS (g/L)**	**Y_{RS}**
	6 % (w/v)	99.2	0.237	0.099	0.188	0.121	8.12	44.6 %
	8 % (w/v)	99.4	0.287	0.137	0.162	0.136	10.22	42.1 %
	10 % (w/v)	98.2	0.416	0.165	0.181	0.123	12.51	41.3 %
Enzyme dose	**10 % (w/v) – 150 rpm**	**%**	**k_1 (h^{-1})**	**k_2 ($g{\cdot}L^{-1}{\cdot}h^{-1}$)**	**β**	**α**	**RS (g/L)**	**Y_{RS}**
	18 FPU/g	98.0	0.410	0.165	0.151	0.114	8.58	28.3 %
	30 FPU/g	97.6	0.522	0.165	0.175	0.130	10.25	33.8 %
	50 FPU/g	98.2	0.416	0.165	0.181	0.123	12.51	41.3 %
Stirring rate	**10 % (w/v) – 50 FPU/g**	**%**	**k_1 (h^{-1})**	**k_2 ($g{\cdot}L^{-1}{\cdot}h^{-1}$)**	**β**	**α**	**RS (g/L)**	**Y_{RS}**
	150 rpm	98.2	0.416	0.165	0.181	0.123	12.51	41.3 %
	250 rpm	98.1	0.319	0.165	0.204	0.128	13.65	45.0 %
Batch vs Fed-Batch	**50 FPU/g – 250 rpm**	**%**	**k_1 (h^{-1})**	**k_2 ($g{\cdot}L^{-1}{\cdot}h^{-1}$)**	**β**	**α**	**RS (g/L)**	**Y_{RS}**
	10 % (w/v) Batch	98.1	0.319	0.165	0.204	0.128	13.65	45.0 %
	10 % (w/v) Fed-Batch	98.6	0.139	0.165	0.268	0.051	10.39	34.3 %

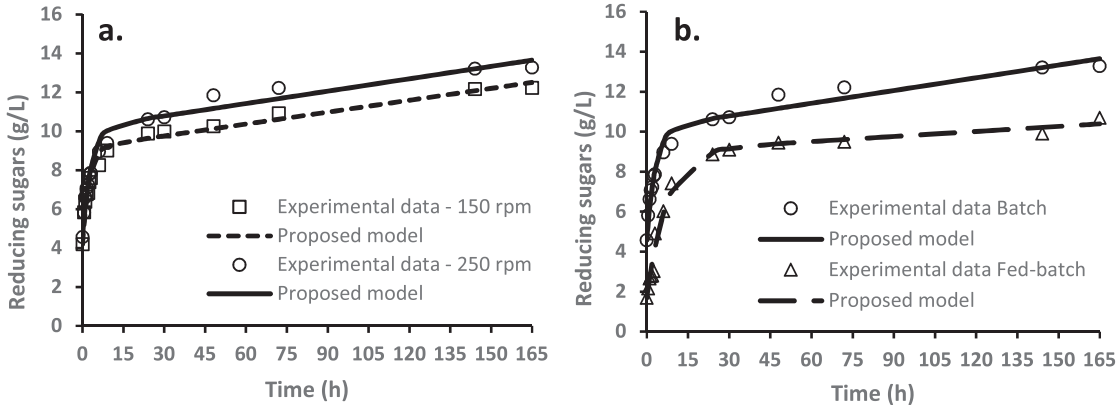

Fig. 3. Comparison of RS produced at different agitation rates (150 and 250 rpm) operating at 10 % of biomass loading and 50 FPU/g biomass (a). Comparison between batch and fed-batch mode hydrolysis, operating at 10 % of biomass loading, 50 FPU/g biomass and 250 rpm (b).

the reaction medium would increase, reducing mass transfer and mixing intensity [39]. In this sense, increasing the stirring rate is one of the ways that can help to overcome this problem, although high stirring rates could cause problems such as the mechanical deactivation of the enzyme by shearing [40].

To analyze the effect of the stirring rate on the process, the same conditions of the hydrolysis leading to the highest production of RS at 150 rpm (10 % biomass loading and 50 FPU/g dry biomass) were used, and the additional stirring rate of 250 rpm was tested. Fig. 3 (a) shows the experimental data together with the predictions of the proposed kinetic model, for the two stirring rates assayed. It was observed that increasing the stirring rate from 150 rpm to 250 rpm mainly affected stage 1, improving its hydrolysis yield. Thus, operating at 250 rpm, a higher RS concentration was obtained in stage 1. In contrast, the rate constant at this stage (k_1) decreased slightly, which could be related to a moderate delay in the inflexion point (the time at which the change in the controlling stage of the reaction occurs). A cause that may explain this is that less substrate dissolved in the liquid medium is available for the enzyme when a lower stirring rate (150 rpm) is used. Thus, the substrate in stage 1 would be depleted faster than at higher stirring rate (250 rpm), where there is more dissolved substrate. Based on this, it can be said that higher stirring rates would have a positive effect on the amount of substrate available to be converted by the enzyme in the liquid medium.

On the other hand, the parameters for stage 2 were not affected by the stirring rate. Thus, the hydrolysis rate constant (k_2 was 0.165 $g{\cdot}L^{-1}{\cdot}h^{-1}$ in both cases) and the hydrolysis yield (α ranges from 0.123 at 150 rpm to 0.128 at 250 rpm) were maintained practically constant. This stage is related to the enzyme diffusion inside the solid and was therefore expected to be unaffected, as stirring can mainly influence the hydrolysis kinetics of the substrate in the liquid phase.

Another promising way to overcome mass transfer and mixing problems due to using high solids loadings is to apply the fed-batch mode [39,41]. In this work, fed-batch hydrolysis was carried out using the conditions that lead to the best utilisation of the substrate (10 % biomass loading, 50 FPU/g dry biomass and 250 rpm) in batch assays. Fig. 3 (b) compares the hydrolysis evolutions for the saccharification process in batch and fed-batch modes. In stage 1, the hydrolysis yield increased from 0.204 in batch mode to 0.268 in fed-batch mode (Table 3). It is worth noting that the hydrolysis rate of stage 2 was not affected regardless of the hydrolysis mode, but its yield decreased in the fed-batch operation mode. In addition, the final RS concentration in the fed-batch mode was reduced by 23.88 % with respect to batch mode. In order to improve the RS production in the fed-batch regime, parameters such as feeding intervals, enzyme dose or solids loading at each addition should be studied further. This operational mode has been applied to lignocellulosic biomass [39,41]. However, to the best of our knowledge, no previous studies have reported its use for macroalgae biomass.

It is important to emphasize that the proposed kinetic model allows calculating the amount of RS generated in both stages of the hydrolysis.

For example, in the hydrolysis conducted with 10 % biomass loading, 50 FPU/g dry biomass and 250 rpm in batch mode, stage 1 produced up to 10.15 g/L RS in 24 h, compared to 0.51 g/L generated up to that time from stage 2. Thereafter, only stage 2 contributed to the generation of RS, producing 2.99 g/L after a further 141 h of hydrolysis. According to these data, a saccharification process over 24 h does not seem to be cost-effective since a high hydrolysis time increase leads only to a moderate rise in RS production, about 29.5 %. However, it should be considered that if hydrolysis is stopped at 24 h, part of the substrate would not be utilized.

4. Conclusions

The present study has demonstrated that the brown macroalga *Rugulopteryx okamurae,* even without any pretreatment, represents a promising feedstock for RS production, which can be converted into value-added products by fermentation. It has been found that detergent fibre analysis, which is commonly used for lignocellulosic biomass, is appropriate for the characterization of the polysaccharide content in macroalgae biomass. Thus, this analysis showed that *R. okamurae* contains fractions rich in hydrolysable polysaccharides, in a higher proportion than even many other algae used as a dietary supplement in food. To improve the production of RS in the enzyme hydrolysis of the macroalga, different biomass loadings, enzyme doses and stirring rates were tested in batch mode and compared to the fed-batch regime. The highest RS concentration (13.65 g/L) was achieved with 10 % (w/v) biomass loading, 50 FPU/g dry biomass and 250 rpm in batch mode. These conditions significantly improved sugar production from *R. okamurae* without pretreatment and produced higher sugar concentrations than those for the alga subjected to biological or hydrothermal-microwave pretreatments. Due to the relevant improvement in the production of fermentable sugars achieved in this work, it would be interesting to carry out new studies with the pretreated alga, but carrying out the enzymatic hydrolysis under the proposed conditions. Furthermore, it would be convenient to test other types of pretreatment on *R. okamurae*, such as thermo-acid pretreatment, which is widely used on lignocellulosic biomass due to its good yields.

The experimental data were fitted to a new kinetic model developed in this study. The proposed model was compared to the simple first-order kinetics typically used for enzymatic hydrolysis. The fitting showed that the proposed model was able to represent the observed hydrolysis trend better than the simple first-order approach, improving the value of the regression coefficient by up to 8.7 %. The proposed model considers that hydrolysis occurs through two stages. Stage 1 corresponds to the hydrolysis of the dissolved polysaccharides in the liquid medium and is responsible for the majority production of RS, mainly during the first 9 h. However, stage 2 is related to the hydrolysis of the non-dissolved polysaccharides available inside the algal biomass and, consequently, requires enzyme diffusion into the solid matrix. Stage 1 is much faster than stage 2 as the enzyme diffusion rate is slower than the enzymatic reaction. However, the analysis of data from stage 2 would allow a better evaluation of the pretreatments applied to the algal biomass, since they cause the modification of the internal structure of the biomass facilitating enzyme diffusion. In future work, it would be interesting to work on pretreatments that reduce the enzymatic diffusion effect observed here.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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