Rugulopteryx okamurae: effect of hydrothermal acid pretreatment on the saccharification process

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ABSTRACT

The biological invasion caused by the invasive macroalga *Rugulopteryx okamurae* is causing increasing concern in southern Europe. To reduce its impact, this brown alga can be treated from a biorefinery approach. In this study, the macroalga is used as raw material to obtain fermentable sugars, which can be converted into high value-added products. The alga was exposed to hydrothermal and hydrothermal acid pretreatment and the pretreated biomass was used for enzymatic hydrolysis, achieving a hydrolysate with a reducing sugar concentration of almost 25 g/L (49.2 % more than with non-pretreated alga). In addition, the combined severity factor was calculated to identify the best pretreatment conditions, finding the optimum in those pretreatments performed

with 0.2 N HCl concentration and 15 min reaction time. Based on the results, it would be interesting to carry out new studies using the saccharified medium obtained under optimal conditions to obtain value-added compounds by fermentation.

Keywords: macroalga; dietary fibre; enzyme hydrolysis; combined severity factor; reducing sugar

1. Introduction

Biological invasions are considered one of the most important threats to biodiversity loss and have both environmental and economic repercussions. In this regard, more than 40 % of the invasive alien species in the European Union are invasive macroalgae and their ecological effects have been comprehensively studied in the last decades. Invasive macroalgae are capable of displacing native species by competing for light and nutrients, monopolising space. One of these harmful algae is *Rugulopteryx okamurae*, known for its rapid spread and capacity to alter marine habitats (Faria et al., 2022; Roca et al., 2022).

The first occurrence of *R. okamurae* in Spain was reported in 2015 in the Strait of Gibraltar (Tarifa and Ceuta) during the autumn of 2015. By 2016, it had already established itself in most of the rocky bottoms along the Strait's coastline (García-Gómez et al., 2020). This invasive alga is not only having an impact on the environment but also on the economy of the affected regions. An example of the economic repercussion given by this seaweed is that, only in the first summer after its occurrence, 5000 tonnes of macroalgae deposits had to be removed from 15.5 km of the coastline of Ceuta. It implies that beaches must be restored, sometimes causing the closure of very

popular beaches during the tourist beach season. To this must be added the economic losses for the fishing sector, harmed by the accumulation of algae in fishing nets (Carrillo et al., 2016; García-Gómez et al., 2020). The most disturbing is that the brown alga *R. okamurae* is spreading along the Mediterranean coast, both eastward and westward, according to the most recent report published by the Spanish Ministry for Ecological Transition and the Demographic Challenge (MITECO). These worrying data have led to the macroalgae being included in the EU list of alien species of concern.

The use of invasive macroalgae to produce high value-added products could be a promising strategy to reduce their impact. Given the environmental problems and the depletion of non-renewable resources, the use of this biomass as an alternative raw material to obtain valuable bio-products from biorefinery methods is increasingly investigated. Furthermore, macroalgae biomass offers significant economic benefits compared to other types of biomass. Seaweeds show a low recalcitrant material content and are rich in carbohydrates, making them an interesting material for the production of high-value compounds like bioplastic precursors (e.g. polyhydroxyalkanoates), succinic acid, biogas and liquids biofuels. In addition, its cultivation does not depend on freshwater or arable land, thus avoiding competition with conventional agriculture that utilizes terrestrial plants for food production (Fernand et al., 2017; Greiserman et al., 2019; Jiang et al., 2016; Yun et al., 2016). However, for a biorefinery process to be cost-effective, it is highly recommended that the raw material be entirely utilized. In this sense, the valorization of macroalgae has advanced considerably in recent years.

The issue of *R. okamurae* is recent and research to obtain value-added products from this biomass is very scarce. Even so, there are very recent publications that demonstrate the potential of this macroalgae, given its nitrogen-rich composition, to balance the carbon/nitrogen ratio in anaerobic co-digestion processes to produce methane (Barcellos

et al., 2023; de la Lama-Calvente et al., 2021). It has also been demonstrated that this brown alga is useful for obtaining biofertilizers and animal proteins by vermicomposting (Patón et al., 2023). Due to its high sesquiterpene content, *R. okamurae* also shows pharmacological applications, given that these compounds have anti-inflammatory and antibacterial activity (Barcellos et al., 2023). One of the potential applications suggested for this alga is its use for the production of fermentable compounds (e.g. reducing sugars, volatile fatty acids), which can be used to obtain value-added products. For this purpose, *R. okamurae* has been subjected to biological pretreatment, resulting in a sugar hydrolysate with almost 96 % glucose (Agabo-García et al., 2023). Microwave pretreatment has also been tested on this alga, which resulted in an improvement in the production of reducing sugars (RS) and volatile fatty acids (VFA) of 35 % and 45 % respectively, compared to alga without pretreatment (Fernández-Medina et al., 2022). In another study, this macroalga was subjected to enzymatic hydrolysis without pretreatment. Here, the conditions of biomass loading, enzyme dose, stirring rate and mode of operation (batch and fed-batch) were studied in the hydrolysis process, and a concentration of RS of almost 14 g/L was obtained (Romero-Vargas et al., 2023a). This concentration is much higher than those obtained in the aforementioned studies, in which pretreatment was performed.

The primary aim of this study was to assess the potential of the invasive macroalga *Rugulopteryx okamurae* as an alternative feedstock for the production of fermentable sugars in the biorefinery framework. In this work, hydrothermal acid pretreatment is used for the first time on *R. okamurae* and no scientific literature has been found where this type of pretreatment is applied to this alga. Both acid concentration and reaction time have been studied. In addition, enzymatic hydrolysis of each pretreated biomass was performed and the RS values were fitted to a recently developed kinetic model. The

dietary fibre composition of pretreated *R. okamurae* was compared with the non-pretreated one and different sugars contained in the filtrate and hydrolysate were also analyzed. On the other hand, the combined severity factor (CSF), recently implemented in macroalgae, is used here for the first time to calculate the optimal pretreatment conditions. Indeed, this study includes an in-depth description of the applicability of CSF in macroalgae.

2. Material and methods

2.1 Sampling and conditioning of seaweed biomass

Rugulopteryx okamurae was collected from the coastal waters of Punta Camorro (Tarifa, Spain) during low tide periods in the spring season. The macroalga was placed in 25 L polyethylene drums and thoroughly washed with tap water to eliminate salts and debris until the final conductivity reached a value below 600 µS/cm. Subsequently, the washed macroalga was dried in a greenhouse for 24 hours. Finally, the dried seaweed was milled using a cutting mill to achieve a particle size of 1 mm. Then, the ground biomass was stored in hermetically closed drums at room temperature until use.

2.2 Pretreatment process

Hydrothermal acid pretreatment was adopted to disrupt complex molecules in *R. okamurae* cells to facilitate sugar production during the subsequent enzymatic hydrolysis. In this regard, the most commonly used acids for this type of pretreatment are hydrochloric acid (HCl) and sulfuric acid (H_2SO_4). However, HCl is easier to recover and has shown better pretreatment efficiency than those using H_2SO_4 (Kassim et al., 2022). In addition, HCl is cheaper than H_2SO_4 (around 40 %). Different reaction times (15, 30 and 60 min) and several HCl concentrations (0.05, 0.1 and 0.2 N) were studied to optimise the pretreatment. Both the reaction time and acid concentration

range were selected according to other similar studies (Abd-Rahim et al., 2014; Azizi et al., 2017). In those studies, higher reaction times and acid concentrations did not show improvements in RS release. Furthermore, as far as acid is concerned, a higher concentration of acid will result in more water needed to wash the biomass in the next step and would have a more corrosive effect on the equipment and higher operating costs. 125 mL of acid suspensions with 10 % (w/v) dried biomass were included in 250 mL Pyrex® bottles and introduced in the autoclave at different reaction times. The pretreatment temperature was set at 121 ºC, following other related studies (Romero-Vargas et al., 2023b). Subsequently, the solid biomass was separated by vacuum filtration with a Whatman No.1 filter paper and washed with tap water until it showed a pH close to 6.0. This solid biomass had a high moisture content, with the subsequent risk of contamination and degradation of the sugars. Therefore, the biomass was dried in a forced convection oven at 40 ºC for 24 h and stored at room temperature until its use in the enzymatic hydrolysis stage. NaOH micropearls were added to the filtrate to adjust the pH to 5.0 for sugars analysis. As a control assay, the same pretreatment was performed with distilled water instead of the acid solution (hydrothermal pretreatment). All pretreatments were performed in triplicate.

2.3 Enzymatic saccharification

The commercial enzyme preparation Cellic Ctec2 (Novozymes, Denmark) was used for the enzymatic hydrolysis of macroalgae biomass. This enzyme preparation was composed of cellulase (EC 3.2.1.4) and endo-β-1,4-xylanase (EC 3.2.1.8) and has been considered the most optimal for carrying out the saccharification process using macroalgae biomass (*Gracilaria verrucosa)*. This is because it shows better yields compared to other commonly used enzymes (Celluclast 1.5 L and Viscozyme L) and because it has an advanced cellulase for industrial use (Sukwong et al., 2019).

Enzymatic hydrolysis was carried out in 250 mL Erlenmeyer flasks, containing 45 mL of 50 mM sodium phosphate buffer (pH 5.0). The biomass was added to the buffer and autoclaved for 20 minutes at 121 °C before the addition of the commercial enzyme preparation Cellic Ctec2. Following enzyme addition, the flasks were tightly sealed with silicone stoppers and incubated on a rotary shaker at 50 °C. The hydrolysis conditions were 10 % (w/v) loading biomass, 50 FPU/g biomass of enzyme dose at 250 rpm of agitation rate. These specific conditions were chosen in accordance with previous related studies (Díaz et al., 2017; Romero-Vargas et al., 2023a). Samples were collected periodically throughout the process and stored at -20 °C for subsequent sugars analysis. The hydrolysis process was performed for 72 hours, and each assay was carried out by duplicate.

2.4 Analytical techniques

The analysis of the fibre composition of the pretreated macroalgae was performed by the AOAC method following the methodology described in a previous study for the analysis of algal biomass fibre (Romero-Vargas et al., 2023a). This methodology was carried out in Fibertec™ 8000 (FOSS IBERIA, Barcelona, Spain) and FT 121 Fibertec (FOSS IBERIA, Barcelona, Spain), and the analysis was performed in triplicate. The different fractions quantified were: lipid compounds (fats, oils and waxes); Soluble dietary fibre (SDF), containing non-cellulosic polysaccharides (acid labile carbohydrates); Insoluble dietary fibre (IDF), mainly composed of cellulose (IDF_C fraction); acid-insoluble lignin (Klason lignin) and ash and Non-fibrous matter (NFM), mainly composed of non-fibrous carbohydrates (starch), soluble salts and proteins. Through the comparison of the different fibre fractions obtained by the gravimetric method, it was possible to determine the total amounts of lipids, NFM, SDF, IDF_C, Klason lignin and ashes.

The RS concentrations of the filtrates from the pretreatment and the saccharified media from the enzymatic hydrolysis were quantified using the DNS method adapted for microplates (Gonalves et al., 2010; Miller, 1959). Before analysis, samples were centrifuged at 10,000 rpm for 10 min in a high-speed mini centrifuge (Gusto®, Vernon Hills, USA), and the resulting supernatant was used for measurements. The glucose, xylose and galactose content was measured by an enzymatic biochemical analyser YSI 2900 (Vertex Technics S.L., Barcelona, Spain). Moreover, arabinose, cellobiose, fructose, lactose, maltose, sucrose, glycerol, mannitol and sorbitol were analysed by ion chromatography (Metrohm 930 Compact IC Flex, Herisau, Switzerland), using a pulse amperometric detector and a working gold electrode. For the separation, it was used the column Metrosep Carb 2-150/4.0 (Metrohm, Herisau, Switzerland) and the elution was performed in isocratic, at a 0.35 mL/min flow rate with 300 mM sodium hydroxide (NaOH) and 1 mM sodium acetate (NaOAc). The supernatant was filtered through a 0.22 µm filter before analysis by the enzymatic biochemical analyser and ion chromatography. The measurements were performed in triplicate for each sample to ensure accuracy.

The pH was measured in a pH-meter Basic20 (Crison®, Barcelona, Spain).

2.5 Study of enzymatic hydrolysis kinetics

For the analysis of enzymatic hydrolysis, RS concentrations were plotted as a function of time and the data were fitted to a kinetic model recently developed in a previous study (Romero-Vargas et al., 2023a). The model is based on a first-order kinetic model, typically used in this type of hydrolysis, to which the authors added a new term that significantly improved the fit of the model to the experimental RS data. Thus, Eq. 1 distinguishes two stages in hydrolysis, both occurring simultaneously: the term on the left, which considers that the substrate of the enzymatic hydrolysis is present in the

liquid medium (stage 1), and the term on the right, which considers that the substrate is located inside the algal biomass particles (stage 2).

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

where P is the RS concentration (g/L) at time t, Po is the initial RS concentration (g/L), β is the hydrolysis yield coefficient for the stage 1 and k_1 is the rate constant (h^{-1}) also for this stage, α is the hydrolysis yield coefficient for stage 2 and k_2 is the rate constant ($g \cdot L^{-1} \cdot h^{-1}$) also for this stage, 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.

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

In this equation, 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 and ash (w/w), which was considered to contain the total hydrolysable polysaccharides (Romero-Vargas et al., 2023a), and it was calculated as the sum of the SDF and IDF_C fractions.

2.6 Yield of enzymatic hydrolysis

The hydrolysis yield (Y_{RS}) was determined by calculating the amount of reducing sugars (RS) produced during hydrolysis (in grams) in relation to the theoretical monosaccharides derived from the polysaccharides present in the biomass (in grams), according to Eq. 3.

$$Y_{RS} = \frac{P \cdot V_H}{B \cdot TDF_C \cdot 1.\bar{1}} \quad (3)$$

The theoretical monosaccharide content was estimated based on the TDF_C and calculated using a correction factor (1.1), which considers the presence of water during

the conversion of polysaccharides to monosaccharides in the hydrolysis process (Sluiter et al., 2004).

2.7 Analysis of severity parameters

The combined severity factor (CSF) was defined originally for lignocellulosic biomass (Ruiz et al., 2017). However, this parameter has recently been applied to macroalgae (Park et al., 2018; Sukwong et al., 2019), despite their very low lignin content. To the best of our knowledge, there is no study that provides a sufficient explanation of the applicability of this parameter to this type of biomass. According to the literature (Chen et al., 2007; Ziegler-Devin et al., 2021), the severity factor is a concept derived from the H-factor determined by (Vroom, K. E., 1957), which combines reaction time and temperature into a single kinetic parameter Eq. 4. For the calculation of the H-factor it is assumed that the rate constant obeys the Arrhenius law Eq. 5.

$$H_{(t)} = \int_0^t \frac{k_{T_p}}{k_{T_r}}\, dt \quad (4) \qquad ; \qquad k = \frac{E_a}{R \cdot T} \quad (5)$$

where $H_{(t)}$ is the H-factor value at time t, k_{T_p} is the rate constant for the pretreatment temperature T_p, k_{T_r} is the rate constant for the reference temperature T_r (373 K), t is the pretreatment time, k is the rate constant for a temperature T, E_a is the activation energy (in this study 134 kJ·mol^{-1}) and R is the gas constant (8.314 J·mol^{-1}·K^{-1}). The H-factor is used to measure the delignification rate in the Kraft cooking process and it has been widely used in the paper industry to control the degree and cooking time required (process severity) to dissolve a certain amount of lignin. Later on, in a study dedicated to the optimisation of the pretreatment of *Pinus radiata* (Brasch and Free, 1965), an equivalent concept was introduced, the P-factor (Eq. 6), which involves the rate of solubilisation of hemicellulose in the cellulose separation process. An approximation of

the Arrhenius relation is applied for its calculation, where the reaction rate approximately doubles with every 10 °C increase in temperature (Eq. 7).

$$P_{(t)} = \int_0^t \frac{k_{T_p}}{k_{T_r}}\, dt \quad (6) \qquad ; \qquad \frac{k_{T_p}}{k_{T_r}} = 2^{\frac{T_p - T_r}{10}} \quad (7)$$

where $P_{(t)}$ is the P-factor value at time t.

To compensate for the duration and temperature of the pretreatment so that the final effects were equivalent, the severity function arises (Eq. 8) (Overend and Chornet, 1987).

$$R_o = t \cdot exp^{\left(\frac{T_p - T_r}{\omega}\right)} \quad (8) \qquad ; \qquad \omega = \frac{T_f^2 \cdot R}{E_a} \quad (9)$$

where R_o is the severity coefficient, T_f is the room temperature, and ω is the typical activation energy for glycosidic bond cleavage of carbohydrates. Most studies assume a value for ω of 14.75, supposing an hydrothermal process and an overall first-order conversion (Ruiz et al., 2021). With this value, the reaction rate has an increase of 1.971 (almost double) for every 10 °C increase in temperature (Chen et al., 2007), similar to what occurs in Eq. 7. This severity function (R_o) was initially proposed for aqueous pretreatments, and the effect of the acid concentration was incorporated later for hydrothermal acid pretreatments (Chum et al., 1990). For this purpose, it is assumed that hydrogen ion activity is involved in the polymer dissolution, which follows first-order kinetics. Thus, the CSF can be calculated with Eq. 10.

$$CSF = Log\,(Ro) - pH \quad (10)$$

where *CSF* is the combined severity factor value, *Log(Ro)* is the logarithm of *Ro* (above defined, T_r = 100 ºC and ω = 14.75) and *pH* is the concentration of hydrogen ions in the solution before pretreatment, measured in pH units.

CSF is also used to evaluate the release of inhibitory compounds during hydrothermal acid pretreatment, assuming that the reaction follows first-order kinetics (Chen et al., 2007) and that the release of these products occurs at a reaction rate that approximately doubles with every 10 ºC increase in temperature. In these cases, it can be accepted ω = 14.75 as a generic value for the definition of R_o.

2.8 Statistical analysis

The kinetics parameters were calculated using the Solver tool in Microsoft® Excel® 2016 MSO (16.0.4266.1001) 64 bits.

To assess the presence of significant differences between means in the analysis of kinetic parameters and sugars, a one-factor and two-factor analysis of variance (ANOVA) was performed. Effects were considered significant for *p-value* < 0.05. To determine the best pretreatment conditions, the interaction between reaction time and acid concentration and between CSF and RS production after hydrolysis was analysed. For this purpose, a two-factor multilevel factorial design experiment was performed. Also, the desirability function provided by this same tool was used. The goal was to maximize the RS concentration and reduce the CSF value. The optimal conditions were those producing extracts with a maximum RS concentration and a minimum CSF value. The analyses were run using Statgraphics© Centurion 19 (StatPoint Technologies, Inc, Princeton, NJ, USA).

3. Results and discussion

3.1 Pretreatment effect on the fibre composition

Twelve conditions were tested in the hydrothermal acid pretreatment of *R. okamurae,* and the pretreated solids were then subjected to fibre analysis. The acid concentrations studied were 0, 0.05, 0.1, and 0.2 N HCl (Zero-Z, Low-L, Medium-M and High-H,

294 respectively), and the reaction times were 15, 30 and 60 min (T15, T30 and T60,
295 respectively) for each acid concentration. Figure 1 shows the comparison between SDF
296 (soluble dietary fibre), IDF_C (insoluble dietary fibre without lignin) and TDF_C (total
297 dietary fibre without lignin) fractions in dry basis (% w/w) present in non-pretreated and
298 pretreated *R. okamurae* biomass in each case. Data on fibre composition in the non-
299 pretreatment alga were obtained from a previous study (Romero-Vargas et al., 2023a).
300 TDF_C was higher in all assays with pretreated macroalga. However, only the change in
301 acid concentration affected the TDF_C fraction significantly (*p-value* = 0.02). The effect
302 of using acid in the pretreatment is noteworthy, as for example can be seen by
303 comparing the results obtained for the pretreatments ZT15 and HT15, without acid and
304 with maximum acid concentration (0.2 N) respectively, at the same reaction time. Both
305 showed practically the same percentage of TDF_C (40.06 ± 1.65 % and 40.12 ± 3.52 %,
306 respectively) but with differences in the IDF_C and SDF fractions. When acid was used,
307 IDF_C increased by 7.3 % (w/w) and SDF decreased by 7.2 % (w/w). SDF is very
308 sensitive to the pretreatment since it is composed of acid-labile polysaccharides, as
309 detailed in the AOAC analysis method. The highest TDF_C and IDF_C were achieved in
310 the pretreatment with 0.2 N HCl and 60 min reaction time (40.9 % and 28.4 %,
311 respectively), with an increase of 13.6 % and 14.8 % respectively over the non-
312 pretreated alga. The high increase in TDF_C observed in the biomass after pretreatment is
313 a positive aspect for the next step, as this fraction has the polysaccharides that can be
314 hydrolysed by the enzyme in the enzymatic hydrolysis step.

315 To visualise the effect of pretreatment more clearly, each fibre fraction was plotted as a
316 function of CSF and the results are shown in Figure 2. When the relationship between
317 fibre fractions and CSF was compared, an increase in the IDF_C was observed as the
318 pretreatment severity level increased. This can be seen by the positive trend observed in

319 the data. In contrast, no clear trend of increase or decrease in SDF was observed,
320 remaining stable for all pretreatments tested. Thus, the biomass enrichment in the total
321 hydrolysable polysaccharide (TDF_C) fraction observed was due to the enrichment of the
322 cellulosic fraction. However, the range of conditions under which the pretreatment has
323 been carried out has not allowed us to determine the maximum value of IDFc that could
324 be achieved with higher CSFs. Further enrichment of IDF_C can still be achieved, as is
325 the case in other studies that associate CSF with the generation or degradation of other
326 pretreatment-related compounds such as inhibitors (levulinic acid) or fermentable (sugar
327 monomers) (Banerji et al., 2013; Park et al., 2018). In the case of the relationship
328 between SDF and CSF, this fraction is very sensitive to acid pretreatment as commented
329 above. Therefore, the monomers released during hydrothermal acid pretreatment
330 (alginate, laminarin and fucoidan in the case of brown algae) are mostly derived from
331 SDF, and the biomass would be easily depleted of this fraction. It has been
332 demonstrated in other studies that increasing the CSF to values close to 1.76 is very
333 favourable for monosaccharide release during pretreatment, however, above this value,
334 the yield decreases considerably (Banerji et al., 2013; Sukwong et al., 2019). Here, the
335 CSF was increased to a value of 1.44, very close to the value indicated above, so the
336 SDF was close to its minimum values at these conditions, which would explain why the
337 increase in CSF did not affect this fraction. The susceptibility of SDF to low CSF values
338 is also reflected in the decrease in TDF_C observed in Figure 1, in those pretreatments
339 that were carried out without acid (from ZT15 to ZT60). This demonstrates that the
340 increase in reaction time also favoured the release of SDF-derived monomers.

341 The effect of pretreatment on lipid, protein (NFM), Klason lignin and ash fractions were
342 also plotted as a function of CSF and the results are shown in Figure 3. It can be seen
343 that the percentage of protein decreases drastically as harsher conditions are used, with a

reduction of up to 32.6 % between the HT60 pretreatment and the non-pretreated alga (Figure 3 a). The same trend is observed for the ash content (Figure 3 b). The removal of nitrogen compounds from the biomass indicates that these were transferred to the aqueous solution used for pretreatment, where soluble sugars were also present. In this respect, when a solution with a mixture of amino acids and sugars is exposed to elevated or even moderate temperatures (Romero-Vargas et al., 2023b), it can give rise to Maillard reaction and caramelized compounds such as 5-hydroxymethylfurfural (5-HMF), fermentation inhibitors, can be obtained. Also, as a consequence of the reaction, fermentable monosaccharides are degraded (Ajandouz et al., 2008; Chen et al., 2007; Şen and Gökmen, 2022). Considering these results and given that the purpose of the medium obtained after enzymatic hydrolysis is to be used for fermentations, it should be considered not to use the liquid resulting from the pretreatment. Finally, the lipid fraction did not show any dependence on the CSF values (Figure 3 c). The very low percentages observed (between 0.36 – 0.98 %) could indicate that the lipid content of the biomass was practically removed. Klason lignin, however, is recalcitrant and not soluble in acid, so, as expected, this fraction was not reduced in the biomass with increasing pretreatment severity, in fact, it even increased due to those non-recalcitrant fractions that were impoverished in the biomass. (Figure 3 d).

3.1 Pretreatment effect on the hydrolysis process

The pretreated macroalgae were subjected to enzymatic hydrolysis. The parameter values obtained after fitting the kinetic model to the experimental RS concentration data are shown in Table 1. These values are compared with those obtained in hydrolysis with non-pretreated alga (Romero-Vargas et al., 2023a). The same table also shows the CSF values calculated for each pretreatment. Although CSF is specific for hydrothermal acid

368 pretreatments, it was also calculated for the hydrothermal pretreatments assays (without
369 acid). For this reason, negative values were obtained.

370 Firstly, it is important to highlight that the kinetic model fitted perfectly to the
371 experimental data, showing R^2 values between 98.8 and 99.7. Secondly, in the previous
372 section, the acid's effect on the TDF's composition was discussed. A comparison of the
373 same cases (ZT15 and HT15) shows that the yield of the hydrolysis with the alga
374 enriched in the cellulosic fraction (46.4 %) was much better than that of the enriched in
375 acid labile polysaccharides (28.0 %). Therefore, the cellulosic fraction is the most
376 important for fermentable monosaccharides production when the commercial enzyme
377 preparation Cellic Ctec2 is used. Another important observation is that the yields
378 obtained for hydrolysis performed with pretreated alga at conditions below LT60 (from
379 ZT15 to LT30) were lower than those for non-pretreated alga (41.3 %). The higher yield
380 for the non-pretreated alga is due to the fact that although it had the lowest TDF_C
381 content (27.3 %), that is, the lowest theoretical hydrolysable monosaccharide content,
382 the concentration of RS in the hydrolysates derived from it (12.52 g/L) and from ZT15
383 and ZT30 (both with 12.47 g/L) were quite similar. However, for those last ones, the
384 TDF_C content was 40.1 % and 38.9 % respectively, higher than those of the non-
385 pretreated seaweed, and as a result, the yield of RS generated by theoretical
386 hydrolysable monosaccharides was lower. This effect could be explained if it is taken
387 into account that biological inhibitors could have been generated during pretreatment, as
388 mentioned above. Hexoses such as D-glucose and pentoses such as D-xylose generated
389 from biomass can be thermally degraded during pretreatment, giving rise to phenolic
390 compounds that inhibit the cellulase activity of the enzyme (Rasmussen et al., 2014).
391 Only when the hydrolysis was carried out with pretreated alga under HT60 and MT15
392 conditions, TDF_C enrichment (36.8 % and 34.7 % respectively) resulted in better yields

(41.8 % and 41.4 % respectively), with RS production of 17.11 g/L and 15.94 g/L respectively. Thus, the pretreatment conditions that allowed the highest yields to be achieved were HT30 and HT60 (both with 54.3 %), with the highest RS production being achieved with the hydrolysis carried out with the alga pretreated under HT60 conditions (24.67 g/L).

Finally, for a better comparison of the pretreatment effect, each parameter has been plotted as a function of CSF and the results are shown in Figure 4. It can be seen in Figure 4 (a) that increasing pretreatment severity (between LT15 and HT60) caused no effect on the rate of enzymatic hydrolysis at either stage. Note the absence of positive or negative trends in the data. In order to assess the influence of acid and reaction time on hydrolysis rates, the means of k_1 and k_2 were compared by groups: hydrolysis carried out with non-pretreated alga (NP) (k_1 = 0.382 h^{-1} and k_2 = 0.184 $g \cdot L^{-1} \cdot h^{-1}$); with hydrothermal pretreatment (ZT15, ZT30 and ZT60) (k_1 = 0.330 h^{-1} and k_2 = 0.280 $g \cdot L^{-1} \cdot h^{-1}$); with hydrothermal acid pretreatment (from LT15 to HT60) (k_1 = 0.221 h^{-1} and k_2 = 0.224 $g \cdot L^{-1} \cdot h^{-1}$). The results showed that k_1 was affected by pretreatment (*p-value* = 0.02) but not k_2 (*p-value* = 0.31). Then, both the acid concentration and the reaction time applied to the macroalga pretreatment affected the amount of polysaccharides available for enzymatic hydrolysis in the liquid phase with respect to the non-pretreated alga. This effect can be seen in Figure 5, which shows the increase in the duration of stage 1 from 9 hours in the hydrolysis with non-pretreated alga (NP) to 24 hours in the hydrolysis with pretreatment alga (Z, L, M, and HT60). When there was a higher concentration of free polysaccharides in the liquid phase (higher CFS value), it took more time to convert them into monomers in stage 1, reflected in a lower k_1 value. If the available substrate for the enzyme in stage 1 increases, there would be less substrate to hydrolyse in the solid in stage 2. It would imply that the hydrolysis in the solid phase

418 would occur faster. However, the constant values observed here for k_2 could indicate
419 that the hydrolysis was carried out with an excess of solid biomass.

420 The effect of pretreatment on the yields at each hydrolysis stage can be seen in Figure 4
421 (b). It was observed that the increase in pretreatment severity did not affect stage 2
422 yields, with a mean α value of 0.124. However, stage 1 did show a positive trend in
423 yields as pretreatment severity increased, with β yields improving by 53.4 % from the
424 lowest CSF value to the highest. Both acid concentration and reaction time showed a
425 significant effect (with a *p-value* of 4.2×10^{-5} and 0.02 respectively). As a result, the
426 overall yield (Y_{RS}) improved by 16.5 % (Figure 4 c) over the lowest CSF pretreatment
427 (LT15), and 13.0 % over hydrolysis with non-pretreated alga. The observed increase in
428 stage 1 yields can only be due to a higher amount of hydrolysable polysaccharides
429 available in the liquid phase of the hydrolysis medium, which is consistent with the
430 justifications made above for the observed effects on k_1 and k_2 rates. In the same way,
431 RS production increased with a similar trend (Figure 4 d), resulting in an increase in the
432 amount of RS produced at the end of hydrolysis of up to 44.3 % compared to hydrolysis
433 carried out with pretreated alga in the lower CSF condition and 49.2 % compared to
434 hydrolysis carried out with non-pretreated alga.

435 To calculate the optimal pretreatment conditions, multilevel factorial analysis of two
436 factors (HCl and reaction time) and 3 levels (0.05, 0.1, 0.2 N and 15, 30, 60 min,
437 respectively) with two response variables (RS concentration and CSF) was carried out
438 by Statgraphics®. The desirability function provided by this same tool was also used and
439 the result of the calculation is shown in Figure 6. The desirability (with values from 0 to
440 1, with 1 being the most desirability) was assigned to the hydrolysis that produced the
441 highest RS concentration using the pretreated alga under the pretreatment condition
442 with the lowest possible CSF value. From the industry's point of view, besides

producing the highest RS concentration with the mildest pretreatment, it would also be desirable that the enzymatic hydrolysis process takes the shortest possible time. However, as seen above, the hydrolysis rates did not show significant differences with the variation of acid concentrations and reaction time used during the pretreatment of the macroalgae. Therefore, the optimisation could be simplified to the variables of RS concentration produced and pretreatment severity. Thus, the highest desirability range (0.6 - 0.7) was observed with pretreatment conditions of 0.17 - 0.2 N HCl and 15 - 24 min reaction time. The optimal value calculated for the acid concentration was 0.2 N and 15 min for the reaction time. One effect worth mentioning is that at acid concentrations above 0.1 N, the increased reaction time in the pretreatment of the alga negatively affects the desirability. Based on the results obtained, increasing the acid concentration rather than the reaction time is preferable to achieving higher desirability data.

The results of the sugar analysis performed on the filtrates obtained after the pretreatment and enzymatic hydrolysis are shown in Table 2. As can be seen, the RS concentrations present in the liquids resulting from pretreatments (filtrate) are not too high, reaching the highest concentration in the HT60 condition (5.54 g/L). Comparing these liquids, in these conditions, the highest concentrations of glucose (101.5 mg/L), galactose (276.9 mg/L) and xylose (450.8 mg/L) were observed. In the case of liquids resulting from enzymatic hydrolysis (hydrolysate), the highest concentration of glucose (22.74 g/L) was also obtained in the HT60 conditions. The highest concentrations of galactose and xylose (175 mg/L and 790 mg/L, respectively) were observed in the HT30 and HT15 conditions, respectively. In the hydrolysates, glucose was the most representative sugar monomer, constituting between 79.8 – 98.1 % of the RS concentration. On the contrary, the pretreated liquids were very poor in glucose, with a

468 percentage of 1.8 - 22.5 % with respect to the RS concentration. These pretreated
469 liquids could be used for enzymatic hydrolysis, but their RS content was very low,
470 possibly because they reacted to give degradation compounds during pretreatment
471 (Rasmussen et al., 2014). Moreover, as mentioned above, the presence of biological
472 inhibitors could lead to undesired effects in subsequent fermentations. For these
473 reasons, it was decided not to use these liquids in the hydrolysis stage. As for the other
474 sugars measured after enzymatic hydrolysis, fructose, sorbitol, cellobiose, maltose, and
475 lactose appeared in very low concentrations and these results have not been shown.
476 Among these, fructose was the only one that barely exceeded 100 mg/L. The presence
477 of arabinose, sucrose, glycerol and mannitol was not detected.

478 **4. Conclusions**

479 The hydrothermoacid pretreatment allowed the enrichment of macroalga in
480 hydrolysable polysaccharides. Macroalga pretreated with HCl 0.2 N for 60 minutes
481 showed the highest proportion of TDF_C and IDF_C (40.9 % and 28.4 %, respectively).
482 Hydrolysates from pretreated seaweed at these conditions had the highest RS
483 concentration (24.67 g/L), with IDF_C being the major responsible for RS release. A two-
484 factor multilevel factorial analysis determined that optimum pretreatment conditions
485 (highest RS concentration and lowest CSF) were HCl 0.2 N for 15 min. Conducting
486 new studies using the saccharified medium obtained under optimal conditions to obtain
487 value-added compounds by fermentation would be interesting.

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FIGURE CAPTIONS

Figure 1: Comparison of the fibre composition of *R. okamurae* biomass in dry basis (% w/w) before and after each pretreatment: NP: non-pretreated; Z, L, M, H: 0, 0.05 N, 0.1 N, 0.2 N of HCl concentration; T15, T30, T60: 15 min, 30 min, 60 min of reaction time. Composition of SDF (soluble dietary fibre), IDF_C (insoluble dietary fibre without lignin) and TDF_C (total dietary fibre without lignin).

Figure 2: Biomass fraction (SDF, IDF_C and TDF_C) in dry basis (% w/w) as a function of CSF.

Figure 3: Percentage of (a) protein, (b) ash, (c) lipids and (d) Klason lignin fractions in dry basis (% w/w) as a function of CSF.

Figure 4: Kinetic parameters (a) conversion (b), yields (c) and production (d) as a function of CSF.

Figure 5: Comparison between enzymatic hydrolysis carried out with non-pretreated (NP) and pretreatment algae for 60 minutes (T60) without acid (Z) and with acid at 0.05 N (L), 0.1 N (M) and 0.2 N (H) concentration.

Figure 6: Optimisation of the pretreatment process for the variables HCl concentration (0.05 - 0.2 N) and reaction time (15 - 60 min). Optimal condition: maximum RS production for the lowest possible CSF value; 1, most desirable condition; 0, least desirable condition.

Table 1: Parameter values obtained after fitting the enzyme kinetic model to the experimental data. RS concentration values and yields were calculated from the model (t = 72 h). NP: non-pretreated; Z, L, M, H: 0, 0.05 N, 0.1 N, 0.2 N of HCl concentration; T15, T30, T60: 15 min, 30 min, 60 min of reaction time.

Condition		R^2	Kinetics parameters		Conversion		Production	Yield
	CSF	%	k_1 (h^{-1})	k_2 ($g \cdot L^{-1} \cdot h^{-1}$)	β	α	RS (g/L)	Y_{RS} (%)
NP	-	98.1	0.382	0.184	0.164	0.240	12.52	41.3
ZT15	-5.53	98.9	0.366	0.415	0.102	0.109	12.47	28.0
ZT30	-5.23	99.0	0.407	0.175	0.115	0.251	12.47	28.8
ZT60	-4.93	99.3	0.218	0.249	0.156	0.159	14.07	34.5
LT15	0.43	99.4	0.226	0.193	0.197	0.167	13.75	37.7
LT30	0.73	99.4	0.282	0.242	0.194	0.156	14.49	38.5
LT60	1.03	99.6	0.170	0.222	0.244	0.154	17.11	41.8
MT15	0.62	99.5	0.179	0.289	0.248	0.106	15.94	41.4
MT30	0.93	99.3	0.160	0.226	0.333	0.133	19.33	51.1
MT60	1.23	99.7	0.197	0.201	0.315	0.156	19.45	47.2
HT15	0.83	99.5	0.247	0.207	0.336	0.118	20.68	46.4
HT30	1.14	99.5	0.261	0.221	0.413	0.144	23.93	54.3
HT60	1.44	99.7	0.267	0.213	0.416	0.131	24.67	54.3

669 **Table 2:** Carbohydrate content analysed in filtrates after the pretreatment and hydrolysates from enzymatic hydrolysis for each condition

670 assayed.

Condition	Filtrate				Hydrolysate		
	***RS* (g/L)**	***Glucose* (mg/L)**	***Galactose* (mg/L)**	***Xylose* (mg/L)**	***Glucose* (g/L)**	***Galactose* (mg/L)**	***Xylose* (mg/L)**
NP	n/a	n/a	n/a	n/a	10.06 ± 0.03	160.0 ± 60.0	250.0 ± 25.0
ZT15	0.37 ± 0.02	56.0 ± 4.3	96.5 ± 2.5	0.0 ± 0.0	9.89 ± 0.26	175.0 ± 5.0	315.0 ± 75.0
ZT30	0.37 ± 0.01	77.6 ± 12.2	158.0 ± 23.4	0.0 ± 0.0	12.19 ± 0.32	160.0 ± 25.0	120.0 ± 45.0
ZT60	0.36 ± 0.01	80.2 ± 6.4	177.4 ± 53.0	44.0 ± 14.6	12.38 ± 0.14	170.0 ± 15.0	0.0 ± 0.0
LT15	0.60 ± 0.03	80.4 ± 9.0	191.3 ± 31.7	224.9 ± 35.3	11.42 ± 0.00	140.0 ± 20.0	355.0 ± 25.0
LT30	0.64 ± 0.02	35.0 ± 2.4	84.6 ± 1.3	0.0 ± 0.0	14.28 ± 0.20	150.0 ± 0.0	70.0 ± 65.0
LT60	0.93 ± 0.12	63.3 ± 6.7	124.0 ± 23.7	0.0 ± 0.0	14.52 ± 0.15	110.0 ± 25.0	0.0 ± 0.0
MT15	1.40 ± 0.11	80.8 ± 15.6	158.4 ± 32.5	70.1 ± 22.8	12.92 ± 0.59	150.0 ± 5.0	440.0 ± 205.0
MT30	1.80 ± 0.14	91.9 ± 26.8	234.8 ± 31.9	363.5 ± 46.4	17.21 ± 0.30	130.0 ± 0.0	0.0 ± 0.0
MT60	2.43 ± 0.02	32.8 ± 7.9	70.6 ± 29.0	0.0 ± 0.0	17.93 ± 0.33	170.0 ± 0.0	0.0 ± 0.0
HT15	3.00 ± 0.08	56.0 ± 13.2	137.3 ± 16.8	2.7 ± 1.2	19.14 ± 0.20	180.0 ± 0.0	790.0 ± 30.0
HT30	3.98 ± 0.20	100.4 ± 49.1	209.2 ± 5.8	117.2 ± 21.8	21.91 ± 0.62	190.0 ± 5.0	0.0 ± 0.0
HT60	5.54 ± 0.09	101.5 ± 22.4	276.9 ± 15.6	450.8 ± 21.9	22.74 ± 0.37	150.0 ± 10.0	0.0 ± 0.0

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672 **Figure 1**

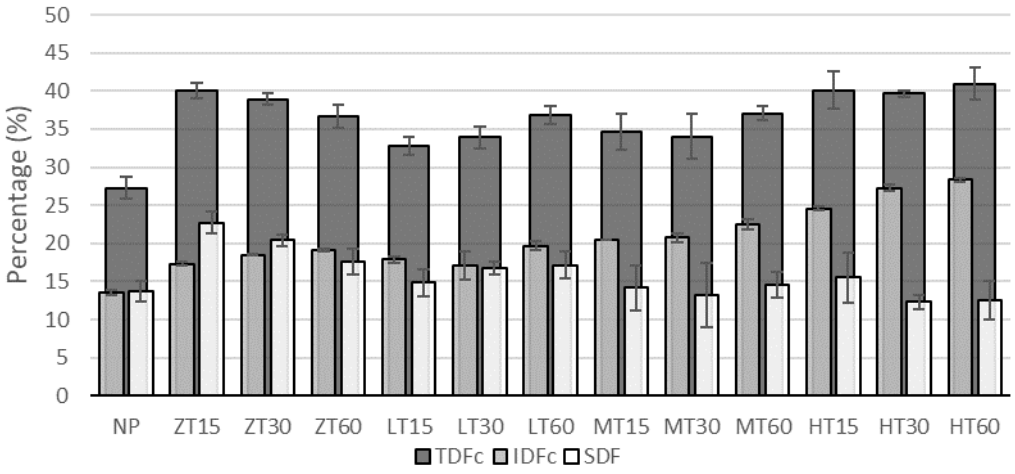

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Figure 2

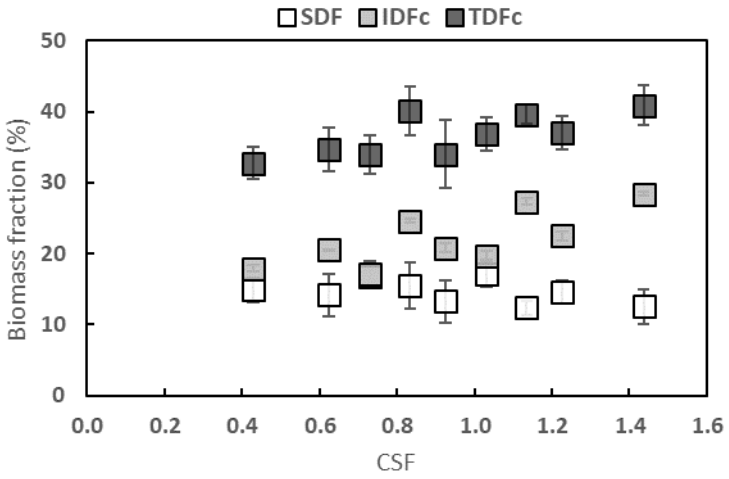

SDF
IDFc
TDFc
Biomass fraction (%)
50
40
30
20
10
0
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
CSF

Figure 3

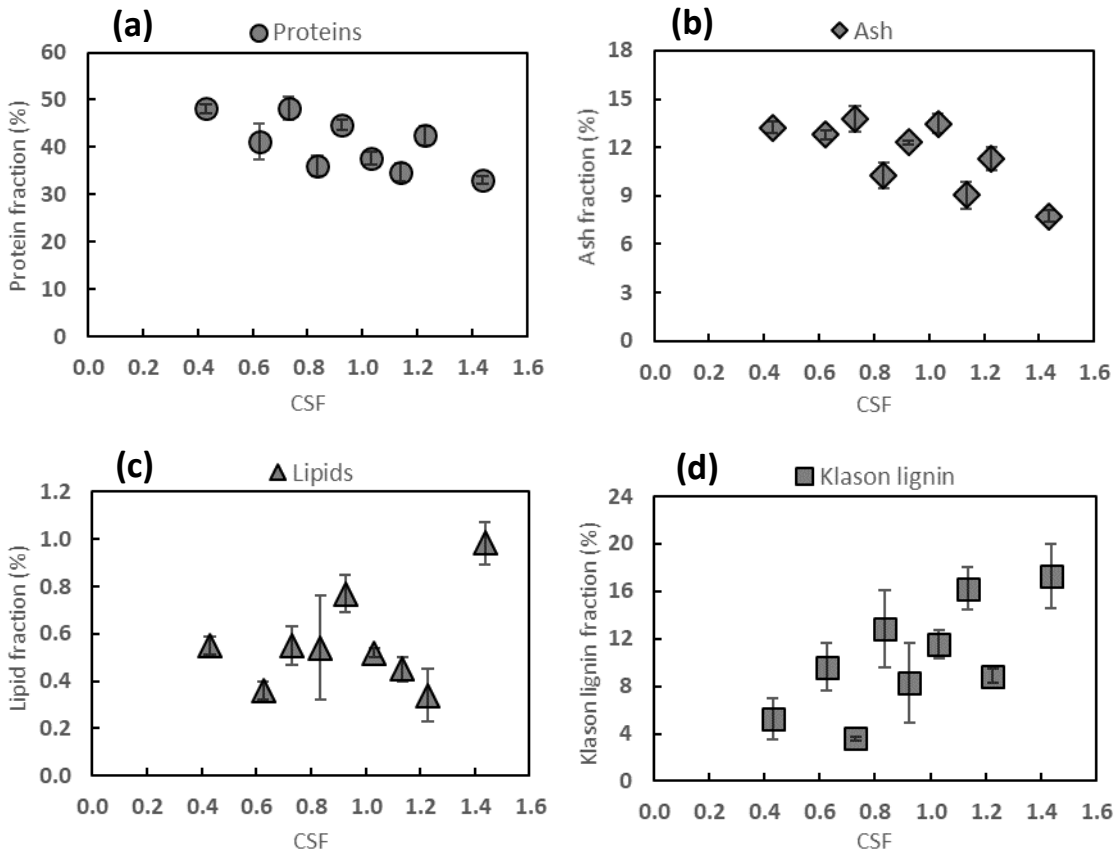

(a)
Proteins
Protein fraction (%)
CSF
(b)
Ash
Ash fraction (%)
CSF
(c)
Lipids
Lipid fraction (%)
CSF
(d)
Klason lignin
Klason lignin fraction (%)
CSF

Figure 4

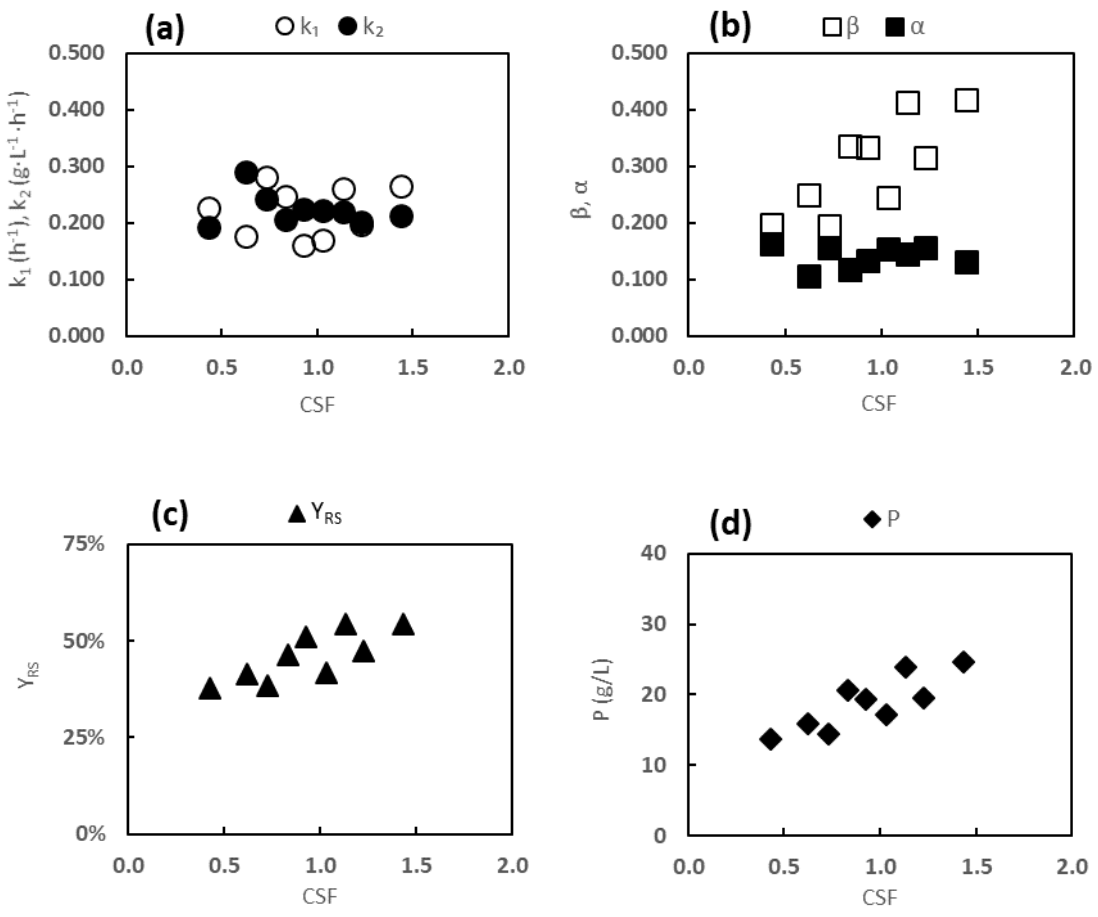
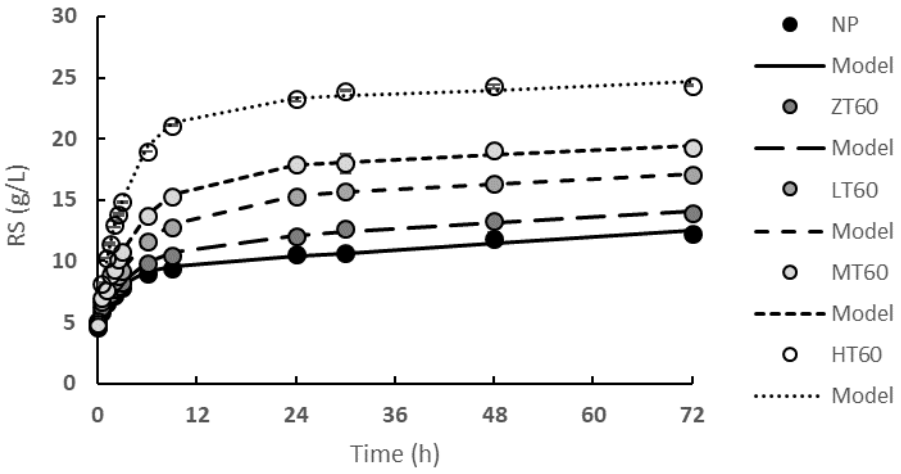
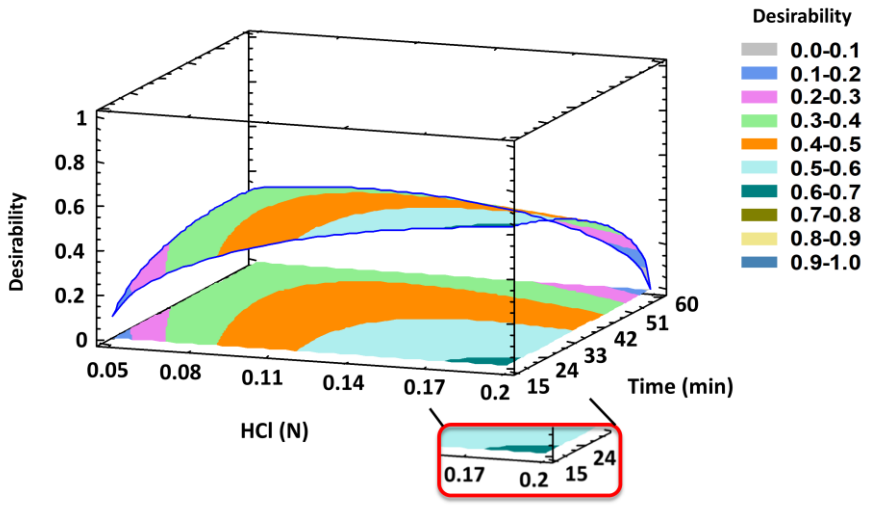

(a)
k1
k2
0.500
0.400
0.300
0.200
0.100
0.000
k1 (h-1), k2 (g·L-1·h-1)
0.0
0.5
1.0
1.5
2.0
CSF
(b)
β
α
0.500
0.400
0.300
0.200
0.100
0.000
β, α
0.0
0.5
1.0
1.5
2.0
CSF
(c)
YRS
75%
50%
25%
0%
YRS
0.0
0.5
1.0
1.5
2.0
CSF
(d)
P
40
30
20
10
0
P (g/L)
0.0
0.5
1.0
1.5
2.0
CSF

Figure 5


30
25
20
15
10
5
0
RS (g/L)
0
12
24
36
48
60
72
Time (h)
NP
Model
ZT60
Model
LT60
Model
MT60
Model
HT60
Model

740 **Figure 6 (color)**



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