Optimisation of the production of fermentable monosaccharides from algal biomass grown in photobioreactors treating wastewater

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ABSTRACT

Biomass grown in wastewater treatment photobioreactors is a cheap raw material with high contents of carbohydrates, proteins and lipids. This work studies the production of fermentable monosaccharides from three biomasses grown in piggery wastewater (P), domestic wastewater (W) and synthetic medium (S) by applying chemical pretreatment and enzymatic hydrolysis, using a Taguchi design.

ANOVA identified temperature, chemical reagent type and chemical reagent concentration as significant operational parameters. However, the biomass concentration, pretreatment time, enzyme dosage and enzymatic hydrolysis time had no remarkable effect. The bacterial content of the biomass had no relevant impact on carbohydrate and protein solubilisation but had a remarkable effect on the degradation of the released carbohydrates (57, 60 and 37% for P, W and S), while also affecting lipid solubilisation. Pretreatment with HCl 2M at 120ºC resulted the optimal conditions, achieving a monosaccharide recovery of 53, 59 and 80% for P, W and S biomasses, respectively.

Highlights

- Temperature was the most influential factor on sugar production from algal biomass.
- HCl resulted in higher monosaccharide recovery than NaOH.
- No effect of enzymatic hydrolysis operational factors on sugar production was found
- High carbohydrate solubilisations were achieved from biomasses grown in wastewater.
- Biomass grown in synthetic medium achieved the highest monosaccharide recovery.

Keywords: Enzymatic hydrolysis; Lipids; Pig manure; Pretreatment; Proteins; Taguchi method

1. Introduction

Microalgae are considered a promising bio-based feedstock and a great source of carbohydrates, proteins and lipids, which has increased their use in the recent years. Microalgae photosynthetically consume CO_2 as a carbon source, use sunlight as an energy source, can treat different types of wastewaters and exhibit high areal productivities in non-arable land (Jankowska et al., 2017; Su et al., 2017). Nowadays, the cultivation of axenic microalgae is costly (Zhuang et al., 2018), but the integration of microalgae cultivation and wastewater treatment significantly reduces the production costs of microalgae biomass. By contrast, complex mixtures of different microalgae species and bacteria grow symbiotically in these treatment photobioreactors hinder the valorisation of the biomass (Kadir et al., 2018; Chen et al., 2013).

At an industrial scale, microalgae are currently used to produce extracts of specific high added value products, such as astaxanthin or pigments, but the rest of components are typically not valorised, which jeopardises the economic sustainability of these processes (Koutra et al., 2018). Thereby, one of the main challenges of microalgae cultivation is the valorisation of every fraction of the microalgae biomass. Among the different components, the carbohydrate fraction could be used as a carbon source for fermentation processes for the production of biofuels like bioethanol, biohydrogen, biobutanol (Sankaran et al., 2018) and even for the production of polyhydroxyalkanoates (Rahman and Miller, 2017).

Cell wall disruption is typically the main bottleneck to valorise the components of algal biomass. This step becomes even more critical for algal-bacterial biomass grown in wastewater treatment photobioreactors, due to the resistant and recalcitrant cell wall of microalgae species able to growth in these media (Onumaegbu et al., 2018). Among the possible alternatives, chemical pretreatments have been successfully tested to support microalgae cell wall disruption, resulting in a fast and relatively inexpensive cell breakdown

while providing high carbohydrate solubilisation. As examples of effective chemical pretreatments, Shokrkar et al., (2017) achieved a monosaccharide recovery of 94% from a mixture of pure microalgae species using 2M HCl at 120ºC for 30 min. Markou et al., (2013) obtained a carbohydrate solubilisation of 90% from *Spirulina platensis* using 0.5N HNO_3 at 100ºC for 3h. Likewise, Harun et al., (2011) pretreated *Chlorococcum infusionum* biomass with alkali, achieving a maximum yield of 0.350 $g_{glucose}\ g_{dw}^{-1}$ at 0.75% (w/v) NaOH, 120ºC for 30 min. In addition, the potential sterilisation effect of chemical pretreatment is of great interest when pretreating microalgae-bacteria consortia, due to the prevention of the microbial degradation of the released components by microorganisms present in the cultivation broth (Fuentes et al., 2016).

The high variability and the bacterial content of the biomass grown in wastewater treatment photobioreactors are also major challenges to be considered (Oh et al., 2018). Biomass grown in open photobioreactors is strongly dependent on uncontrollable factors, such as climatic and environmental conditions (Kumar et al., 2019), as well as on the characteristics of the wastewater (García et al., 2017; Iasimone et al., 2018; Lv et al., 2018; Ganeshkumar et al., 2018). A robust optimisation of the process that would be able to provide high extraction yields independently of the intrinsic variability of biomass grown in wastewater treatment photobioreactors is a requirement to successfully implement the process at both pilot and industrial scales (El-Dalatony et al., 2019).

This work aims at optimising the production of fermentable monosaccharides from the carbohydrate fraction of algal-bacterial biomass grown in photobioreactors. Based on previous results (Martín Juárez et al., 2018), a two-step process with a chemical pretreatment followed by an enzymatic hydrolysis was selected. A Taguchi $L_{27}(3^{13})$ design was used to evaluate the influence of the main experimental parameters and their interaction effects on carbohydrate solubilisation and monosaccharide recovery, and to analyse the loss

of released sugars via chemical or metabolic degradation. The effect of the pretreatment and the enzymatic hydrolysis on proteins and lipids was also evaluated by applying the concept of bio-refinery. In order to achieve a robust optimisation, independent of the substrate characteristics, the complete experimental design was applied to three types of biomass grown in piggery wastewater, domestic wastewater and a synthetic medium. These particular wastewater streams were selected in order to obtain a wide variation of bacterial content in the microalgae biomass, which is a main objective of this study. The microalgae grown in synthetic medium, without bacteria, is an extreme condition and is comparable to most of the previously published research in this field which worked with pure microalgae.

2. Materials and methods

2.1. Raw materials

The biomass used in this work was cultivated in a 1.2 m^3 outdoor thin-layer photobioreactor operating under steady-state at the facilities of the Cajamar Foundation (Almería, Spain) (Morales-Amaral et al., 2015). Three experiments were performed feeding the photobioreactor with different media: piggery wastewater (P), domestic wastewater (W) and synthetic culture medium (S). The different types of biomass cultivated were concentrated through centrifugation up to a concentration of 20% (P), 24% (W) and 18% (S). The biomass was refrigerated at 4 °C prior to use for a maximum of 48 h. The chemical composition of these fresh biomasses was as follows: 22.3% of carbohydrates (including 1.7% of starch), 51.7% of proteins and 13.4% of lipids for P grown biomass; 24.2% of carbohydrates (including 1.4% of starch), 45.4% of proteins and 14.0% of lipids for W grown biomass; and 21.9% (including 1.9% of starch) of carbohydrates, 58.0% of proteins and 13.7% of lipids for S grown biomass (percentages refer to dry mass).

The main microalgae species present in the three biomasses were as follows: *Scenedesmus acutus* (32%), *Chlorella kessieri* (23%), *Scenedesmus obliquus* (17%), *Scenedesmus* sp. (12%) and *Aphanothece saxicola* (12%) in biomass P; *Scenedesmus acutus* (65%), *Scenedesmus acuminatus* (27%) and *Chlorella kessieri* (7%) in biomass W; and *Scenedesmus acutus* (98%) in biomass S.

The identification and quantification measurements of the microalgae species were performed by microscopic examination (OLYMPUS IX70) using at least three different samples using a counting chamber according to Sournia, (1978). Biomass samples were fixed with lugol acid at 5% and stored at 4 °C prior to analysis.

2.2. Pretreatments

Weighted amounts of biomass and the corresponding volumes of 5 M HCl or NaOH and distilled water – to achieve a total volume of 300 mL of suspension – were introduced in 1 L borosilicate bottles. The bottles were introduced in a thermostatic bath or in an autoclave at the pre-established temperature during the time selected for each experiment. The pretreated suspensions were stored at 4 ºC for a maximum period of 24 h for further enzymatic hydrolysis experiments. Additional aliquots were centrifuged at 10,000 rpm for 6 min to separate the solid and liquid fractions, which were then weighted. The content of carbohydrates, proteins and lipids was analysed in the solid fractions and the monosaccharide concentration was measured in the liquid fractions. In order to check the mass balances, total and volatile solids were determined in the solid and liquid fractions, as well as in the whole suspensions.

2.3. Enzymatic hydrolysis

Assays to study the enzymatic hydrolysis conditions in the pretreated biomass were carried out at a biomass concentration of 5 % w/w and adjusting the final concentration with distilled water when necessary. The pH was adjusted to 4.9 ± 0.1. The tests were performed in 100 mL Erlenmeyer flasks with a working volume of 25 mL by adding the required enzyme dosage (Celluclast 1.5L - Cellulase from *Trichoderma reesei*) and a 1 M citrate buffer (Travaini et al., 2016). The assays were carried out in a rotatory shaker at 50 ºC and 300 rpm at the tested incubation times. The experiments were performed in duplicate.

The solid and liquid fractions were separated by centrifugation (10 min, 10,000 rpm) and weighted after the enzymatic hydrolysis. The carbohydrate, protein and lipid concentrations were determined in the solid fractions and the monosaccharide concentration was determined in the liquid fractions (Martín Juárez et al., 2016). Total and volatile solids were determined in the solid and liquid fractions as well as in the whole suspensions to check the mass balances. All analyses were carried out in duplicate.

2.4. Calculation of yields

The following parameters were defined to understand the process and to determine the solubilisation of carbohydrates, proteins and lipids, the loss of carbohydrates via degradation and the recovery of monosaccharides in the liquid fractions during the pretreatment step and the global process (pretreatment + enzymatic hydrolysis):

$$\text{Component solubilisation yield} = \left(1 - \frac{\text{g component in solid fraction}}{\text{g component in PR}}\right) \cdot 100 \quad \text{Eq. (1)}$$

$$\text{Monosaccharide recovery yield} = \frac{\text{g monosaccharides in liquid fraction}}{\text{g carbohydrates in PR}} \cdot 100 \quad \text{Eq. (2)}$$

$$\text{Carbohydrate degradation factor} = \left(1 - \frac{\text{g monosaccharides in liquid fraction}}{\text{g carbohydrates in PR} - \text{g carbohydrates in solid fraction}}\right) \cdot 100 \quad \text{Eq. (3)}$$

where “components” are carbohydrates, proteins and lipids and “PR” is the initial biomass. The solid and liquid fractions were from the pretreatment for the pretreatment step yields and from the enzymatic hydrolysis for the global yields.

165

166 **2.5. Optimisation of operational conditions by Taguchi's robust parameter design**

167 Seven operational parameters (control factors) were selected in this study based on
168 previous works on monosaccharide production from solid wastes by applying chemical
169 pretreatments and enzymatic hydrolysis: biomass concentration (C_A), chemical reagent (H),
170 chemical reagent concentration (C_Q), temperature (T) and pretreatment time (t) on the
171 pretreatment step and enzyme dosage (E) and time (t_H) for the enzymatic hydrolysis.
172 Interaction effect of some control factors ($C_Q \times T$, $C_Q \times t$ and $T \times t$) were also considered. The
173 optimisation was carried out using the Taguchi's orthogonal arrays (OA) $L_{27}(3^{13})$ design.
174 This experimental design, with 27 freedom degrees, permits three levels for each control
175 factor in order to detect quadratic or non-linear effects of the parameters and to obtain
176 information over a wide range of the factors. Additionally, this design provides information
177 about the interaction effect of 3 combinations of control factors (Taguchi et al., 2007).

178 The range as well as the specific values of each operational parameter were selected
179 based on previous results and unpublished research (Table 1). Individual control factors and
180 interactions of control factors were assigned to the columns of the OA according to the
181 adequate triangular table and linear graph (Taguchi and Konishi, 1987). The chemical
182 reagent type (H) was tested at only two levels, using HCl and NaOH solutions. The dummy
183 treatment allowed for the accommodation of the factor H at only two levels into a column
184 with three levels while orthogonality was maintained by repeating one of the two levels
185 (Ross, 1995). The experimental design matrix is shown in Table 2. The execution order of
186 each set of 27 experiments was randomised.

187 The variability of the microalgae biomass, inherent and uncontrollable in a real
188 wastewater treatment process, was introduced in the experimental design as a noise factor by
189 using three microalgae biomass grown in rather different media to achieve a robust

response. Each of the 27 combinations of factor levels defined by the OA were run at the three levels of the noise factor.

The effect of the individual control factors and the interactions of control factors on the different target responses was studied by analysis of variance (ANOVA). No replicate of experiments was performed, and hence residual error was estimated from the results of the unassigned degree of freedom of the design (dummy error in factor H, e_H). Sums of squares and degrees of freedom of dummy error and of its interaction with the noise factor, $e_H \times N$, were pooled for a first estimation of the residual variance. Non-significant factors/interactions were then iteratively pooled into the residual error until only significant effects arose. To estimate the experimental conditions less affected by the variability of microalgal biomass, the ANOVA of the signal-to-noise ratio (S/N) of the 27 combinations was analysed (Taguchi et al., 2007).

For those factors that contributed considerably to the target responses, the Duncan multiple range test was used. This test allowed for the evaluation of the statistically significant differences between the tested factor values for the identification of the factor level that yielded the optimum response (Ross, 1988). A significance level $p=0.05$ was used in all statistical calculations.

2.6. Analytical methods

The total and volatile solid contents were measured according to the NREL protocols in the raw material, solid and liquid fractions, and whole suspensions to check the mass balance in all the experiments (Van Wychen and Laurens, 2015a). The lipid content was determined using a modified protocol based on a chloroform-methanol 2:1 extraction by applying the Kochert method (Kochert, 1978) and the protein content was calculated by multiplying the Kjeldahl Total Nitrogen by a factor of 5.95 (González Lopez et al., 2010).

215 The carbohydrate content was determined as total monosaccharides in the raw
216 materials and solid fractions by using an NREL procedure (Van Wychen and Laurens,
217 2015b). The biomass samples (300 mg dry biomass) were subjected to a concentrated acid
218 hydrolysis for 1 h by adding 3 mL of 72% w/w H_2SO_4 at 30 ºC. Then, 84 mL of deionised
219 water was added to dilute the acid concentration to 4% w/w and the samples were
220 autoclaved at 121 ºC for 1 h. Then, solid and liquid fractions were separated by filtration and
221 the resulting liquid fraction was stored at 4 ºC for in order to determine the total
222 carbohydrate content by HPLC-RI.
223 A Bio-Rad HPX-87H ion-exclusion column installed in a Waters e2695 separation
224 module was used for the quantification of the monosaccharide content. A refractive index
225 detector (Waters 2414) was used to quantify the monosaccharide concentration obtained in
226 the liquid fractions. An aqueous solution of 0.025 M H_2SO_4 was eluted at a flow rate of 0.6
227 mL/min and 50ºC (Martín-Juárez et al., 2016). The external calibration method was used for
228 quantification. Multi-standard calibration solutions were prepared by adequate dilution of
229 individual standards commercially available with a purity >95% (Sigma Aldrich, Spain).
230 The starch content was determined using the polarimetric methodology using an internal
231 procedure of the Laboratory of Animal Nutrition (Serida, Spain).
232

233 **3. Results and discussion**

234 *3.1. Effect of the experimental parameters on the performance of the pretreatment step*

235 High solubilisation yields of the different macromolecular components of biomass
236 were achieved in the pretreatment step for some of the combinations of the operational
237 parameters (Table 2). Specifically, an average carbohydrate solubilisation yield of 64% was
238 obtained, with similar values ranging from 25% to 94% for biomasses grown in piggery and
239 domestic wastewaters and slightly lower (from 13% to 85%) for microalgae grown in

synthetic medium. A high protein solubilisation yield was also achieved, with average yields of 53% (identical for the three biomass) and experimental values ranging from 13% to 96%. These similar carbohydrate and protein solubilisation yields concurred with the analogous composition and predominant microalgae species determined in the three biomasses used in this study. Therefore, these results could indicate the insignificant effect of the bacteria present in the biomass in the release of these components during acid or basic diluted pretreatment. Lipid solubilisation resulted in the largest differences with average yields of only 18% for biomass grown in piggery wastewater, while 48% and 52% of the lipid fraction was solubilised from biomass W and S, respectively.

The experimental design applied allowed for the elucidation of the individual effects that each operational parameter, interaction of selected factors and noise factor had on carbohydrate, protein and lipid solubilisation, as well as on the monosaccharide recovery.

3.1.1. Carbohydrate solubilisation and monosaccharide recovery

The effect of each factor level on the mean values of carbohydrate solubilisation yields during the pretreatment step is shown in Figure 1a. The mean results at the different noise factor levels have been represented separately to highlight the variability of the type of biomass.

The ANOVA analysis revealed that temperature, chemical reagent concentration and chemical reagent type were the most influential parameters with the respective percentages of contributions of 38, 13 and 12%, being higher than the residual error (8%). Similarly, the ANOVA S/N disclosed the most influential factors in the robustness of the carbohydrate solubilisation during the pretreatment step against the variability of microalgae biomass used as a substrate. The main parameters identified by ANOVA were confirmed by the ANOVA S/N, with a contribution of 48% for temperature and 15% for the chemical reagent

265 concentration and a residual contribution of 9%. It was also determined that the effect of the
266 reagent type depended on the biomass.

267 The effect of temperature was very similar for the three types of biomass, with a
268 rapid increase in the yields between 80 and 100°C and slight differences between 100 and
269 120 ºC. For instance, the carbohydrate solubilisation yield in experiments with microalgae
270 grown in synthetic medium pretreated with HCl 0.5 M increased from 13% at 80ºC to 69%
271 at 100ºC and to 75% at 120ºC. HCl provided higher carbohydrate solubilisation yields than
272 NaOH, increasing the significance of the type of chemical reagent with the concentration of
273 chemical reagent (Figure 1a). The biomass type exhibited a significant influence on the
274 effect of the chemical reagent factor, with significant differences for algal-bacteria biomass
275 grown in wastewater, but minor variances for microalgae grown in synthetic medium.

276 Despite the insignificant effect of the pretreatment time in the mean responses of the
277 three biomasses, this control factor had a significant impact on the results from microalgae
278 grown in synthetic medium. Indeed, carbohydrate solubilisation yields increased remarkably
279 from Level 1(10 minutes) to Level 2 (20 minutes) in the S biomass. The bacteria present in
280 the biomasses grown in wastewater jeopardised the effect of pretreatment time.

281 Monosaccharide recovery yields varied from 3% to 76% for biomass grown in
282 piggery wastewater, from 8% to 62% for biomass grown in domestic wastewater and from
283 4% to 80% for microalgae grown in synthetic medium (Table 2). These values were low
284 compared with the high monosaccharide recovery yields reported by Shokrkar et al., (2017),
285 who achieved a maximum yield of 94% from mixed microalgae grown in synthetic medium
286 by applying acid pretreatment with 2M HCl at 121ºC for 30 min. This difference could be
287 attributed to the previous drying and grinding applied to the biomass or to the microalgae
288 species composition (data not provided).

289 Despite the fact that comparable average carbohydrate solubilisation yields were
290 obtained for the three types of biomass, the average monosaccharide recovery yields were
291 significantly higher for the S microalgae (41%) than for biomasses grown in wastewaters
292 (31% for P and 28% for W). These differences revealed average carbohydrate degradation
293 factors of 37% for the S microalgae and ~ 60% for the P and W biomasses. The presence of
294 bacteria in the biomass exerted a relevant and negative influence on monosaccharide
295 recovery by increasing the microbial degradation of the monosaccharides released (Fuentes
296 et al., 2016).
297 The impact of the control factor levels on the mean monosaccharide recovery yields
298 during the pretreatment step is shown in Figure 2a. According to the ANOVA analysis, the
299 effects of temperature (33% of the share) and the reagent concentration (9% of the share) in
300 the monosaccharide recovery were very similar to those obtained for carbohydrate
301 solubilisation. However, a higher contribution of the chemical reagent type was calculated
302 for monosaccharide recovery (20% of the share) than for carbohydrate solubilisation.
303 Chemical degradation of the solubilised carbohydrates could also increase with the severity
304 of the pretreatment conditions, resulting in lower recovery yields (Anburajan et al., 2018).
305 No significant contributions were found for the rest of individual and combined operational
306 parameters in the pretreatment step. Some authors have reported the significant influence of
307 the microalgae concentration (Shokrkar et al., 2017) and the pretreatment time
308 (Sivaramakrishnan and Incharoensakdi, 2018) on monosaccharide recovery, but these
309 studies only used microalgae species grown in synthetic media and conducted non-statistical
310 analysis.
311 The ANOVA S/N confirmed that temperature was the most influential factor (with a
312 share of 42%). The effect of the other factors was rather variable dependent on the different
313 biomass and, hence, common conclusions cannot be drawn (23% of residual). Higher impact

of temperature on monosaccharide recovery was recorded from Level 1 (80ºC) to 2 (100ºC) than from Level 2 to 3 (120ºC). Sivaramakrishnan and Incharoensakdi, (2018) observed a similar effect of temperature during the chemical pretreatment of *Scenedesmus* sp. with 0.3M NaOH, with an increase in the monosaccharide recovery yield, from 45% at 60ºC to 78% at 100ºC, but with no further improvement at 120ºC.

Despite the differences among biomasses, the mean values of monosaccharide recovery were higher using HCl instead of NaOH (Figure 2a). Therefore, a monosaccharide recovery of 80% was achieved with HCl, while the maximum monosaccharide recovery using NaOH was only 40%. The superior performance of acid reagents was also reported by Shokrkar et al., (2017) when comparing the hydrolysis of microalgae mixtures with different acid reagents (H_2SO_4, HCl, H_3PO_3) and NaOH. However, Sivaramakrishnan and Incharoensakdi, (2018) achieved higher monosaccharide recovery yields with NaOH (45%) instead of HCl (28%) under mild pretreatment conditions (0.2M, 80ºC).

Monosaccharide recovery increased with the chemical reagent concentration in the three types of biomass tested in this study. Only a slight difference was observed in monosaccharide recovery from the W biomass, where the recovery yield increased slightly when the reagent concentration increased from 1M to 2M. In this context, the carbohydrate solubilisation from the W biomass using acid pretreatment at 80°C increased from 28% at HCl 0.5M to 84% at HCl 2M. Similarly, Sivaramakrishnan and Incharoensakdi, (2018) also reported an increment on the monosaccharide recovery yields with a chemical reagent concentration from 35% at 0.1M NaOH to 60% at 0.3M NaOH.

According with the carbohydrate solubilisation results, the contribution of pretreatment time on monosaccharide recovery was particularly relevant in microalgae grown in synthetic medium, but it was not significant for the mean values of the three biomasses.

339
340 *3.1.2. Protein and lipid solubilisation*

341 The application of chemical pretreatments resulted in the solubilisation of other
342 macromolecular components of the biomass (proteins and lipids) (Lorenzo Hernando et al.,
343 2018). Thus, similar protein solubilisation yields were obtained for the three types of
344 biomass, ranging from 13% to 96% (Table 2). Figure 3a displays the effect of the control
345 factors on the mean protein solubilisation yields for the three noise levels. No divergence on
346 protein solubilisation for the three microalgae was detected and, hence, a great robustness of
347 this result against the variations of microalgae biomass in the process was determined.

348 The ANOVA analysis provided the contributions of the most influential parameters
349 to protein solubilisation: temperature (39%), chemical reagent type (21%), and the chemical
350 reagent concentration (11%), with residual of 8%. These results, analogous to those obtained
351 for the carbohydrate solubilisation yields, were confirmed by ANOVA of S/N.

352 Protein solubilisation increased with temperature and chemical reagent
353 concentration, reaching the maximum at 2M and 120ºC, which confirmed the simultaneous
354 solubilisation of carbohydrates and proteins. However, the best chemical reagent for protein
355 solubilisation was NaOH. It is well known that alkaline pHs promote protein solubilisation,
356 whereas carbohydrates are better solubilised under acidic conditions (Phong et al., 2018).
357 The highest protein solubilisation yield was obtained for the S microalgae with NaOH 2M
358 and 120ºC (96%), while only a maximum yield of 75 % was achieved for this biomass with
359 HCl 2M at 120ºC.

360 The noise effect exerted a significant impact on lipid solubilisation yields along with
361 chemical reagent type used according to the ANOVA. The impact of the type of biomass is
362 shown in Figure 4a. The lipid solubilisation yields from the P biomass were remarkably
363 lower than those obtained from the W and S biomasses. HCl solubilised lower amounts of
364 lipids than NaOH under all experimental conditions tested. This effect was especially

365 notable for the S microalgae. The chemical reagent was also the only significant factor in
366 ANOVA signal to noise, with 55% of the share (residual: 45%). Therefore, the use of acid
367 reagents was selected as the best option to minimise lipid release.
368
369 *3.2. Effect of the operational parameters on the global process yields*
370 The application of enzymatic hydrolysis after chemical pretreatment was also
371 evaluated using the same experimental design. Two additional factors of the enzymatic
372 process were also included (enzyme dosage, E, and time, t_H). Considering the low
373 concentration of starch in the microalgae biomasses used in this work, a commercial cocktail
374 containing cellulases and β-glucosidases was selected for the enzymatic hydrolysis in order
375 to obtain fermentable monosaccharides, as previously reported by other authors (González-
376 Fernández et al., 2012; Hernández et al., 2015; Passos et al., 2014; Yin et al., 2010). The
377 assessment of global yields (pretreatment followed by enzymatic hydrolysis) was
378 investigated in this section in order to determine the feasibility of an additional enzymatic
379 hydrolysis step compared to a single chemical pretreatment stage. Despite the use of specific
380 enzymes for carbohydrates, enzymatic hydrolysis increased the average global solubilisation
381 values of all the macromolecular components to 83% for carbohydrates, 77% for proteins
382 and 59% for lipids. This simultaneous solubilisation of intracellular content (carbohydrates,
383 proteins and lipids) could be attributed to the cell wall breakthrough by the enzymatic
384 hydrolysis. The multilayer cell wall of microalgae present in these biomasses contain
385 structural polysaccharides (cellulose and hemicellulose) which were degraded by the
386 enzymes actions (Cordova et al., 2018). Proteins are also an integral cell wall constituent,
387 covalently linked to algaenan or carbohydrates (Zhang et al., 2018). Thus, it could be
388 expected that these proteins release in the media after polysaccharides hydrolysis.

389 The effect of enzymatic hydrolysis was different depending on the type of biomass.
390 Therefore, enzymatic hydrolysis resulted in a lower impact on the global carbohydrate
391 solubilisation of the P biomass (average of 78%) than in the W biomass (average of 89%)
392 and the S microalgae (average of 81%). The opposite effect was found in the global protein
393 solubilisation, with the highest yields recorded in the P biomass (average of 83%) compared
394 to the W and S biomass (76% and 70%, respectively).
395 The enzymatic hydrolysis also boosted the global monosaccharide recovery yields,
396 but to a lower extent than the global carbohydrate solubilisation yields, with average yields
397 of 39% in the P biomass, 44% in the W biomass and 53% in the S microalgae. The
398 maximum global monosaccharide recovery yields were 86% for the P biomass, 72% for the
399 W biomass and 91% for the S biomass. The biomass cultivated in the synthetic medium also
400 provided the highest global monosaccharide recoveries. Differences between the global
401 carbohydrate solubilisation yields and the global monosaccharide recovery yields allowed
402 for an estimation of the global carbohydrate degradation factors – 57% for the P biomass,
403 60% for the W biomass and 37% for the S microalgae. These factors, very similar to those
404 previously estimated for the chemical pretreatment step highlighted the metabolic
405 degradation of solubilised carbohydrates by the bacteria present in biomasses grown in
406 wastewater.
407
408 *3.2.1. Global carbohydrate solubilisation and monosaccharide recovery*
409 The effect of the operational parameters on the global carbohydrate solubilisation
410 yields is shown in Figure 1b. The ANOVA showed that temperature was the only factor
411 with an important contribution on the global yields (37%). The enzymatic hydrolysis stage
412 counteracted the differences found in the pretreatment step for the rest of the operational
413 parameters. No influence of the analysed operational factors of the enzymatic hydrolysis

414 was identified. Rehman and Anal, (2019) also detected no impact of enzyme concentration
415 on sugar yields from *Chlorococcum* sp. using cellulase enzyme at 45ºC, 72h.
416 Regarding the noise effect, the W biomass provided higher global carbohydrate
417 solubilisation yields than the P and S biomass. The ANOVA S/N confirmed that temperature
418 was the most influential factor with a 58% of the share, where an increase in the
419 carbohydrate solubilisations yields was observed at increasing temperatures.
420 Temperature was also the most influential parameter on the mean values of global
421 monosaccharide recovery (Figure 2b), with a 41% of the share. The ANOVA S/N of the
422 global monosaccharide recovery yields confirmed this major contribution of temperature
423 (51%, with a residual of 30%).
424 Regarding the results for each biomass, temperature, chemical reagent type and
425 chemical reagent concentration exhibited a noteworthy impact on the global monosaccharide
426 recovery yields in the P biomass. Average global monosaccharide recoveries of 45% were
427 obtained using HCl, whereas a recovery of 26% was reached with NaOH. Moreover, an
428 increase in chemical reagent concentrations greatly improved the yields (24% at 0.5M and
429 58% at 2M). However, only temperature and chemical reagent type exerted a significant
430 effect on global monosaccharide recovery yields in the W biomass. In this case, the average
431 values were 49% using HCl and 34% using NaOH. Finally, only temperature exhibited a
432 relevant impact on the global monosaccharide recovery yields in the S biomass. Therefore,
433 the effect of the chemical reagent type and concentration on monosaccharide recovery yields
434 seems to be related to the sterilising effect of the pretreatment, and with the metabolic
435 degradation of solubilised carbohydrates by the viable bacteria remaining after pretreatment.
436
437 *3.2.2. Global protein and lipid solubilisation*

Figure 3b shows the effect of the control factors on the mean values of global protein solubilisation yields. The trend was similar to the results obtained in the protein solubilisation tests conducted with a single pretreatment step. However, the significant operational parameters had a lower influence on these yields. Temperature and chemical reagent type were the most influential factors with 29% and 18% of the share, respectively (residual 13%). Unlike of the results obtained in the chemical pretreatment step, the noise factor exerted a significant impact on this global yield, with remarkably different results among the three types of biomass tested. The enzymatic hydrolysis step increased the average protein solubilisation yield by 30% in the P biomass, 31% in the W biomass and 17% in the S biomass. The bacteria present in the biomass could contribute to the proteins release during the enzymatic hydrolysis step. It could be corroborated with the fact that Maffei et al., (2018) obtained constant protein content after the application of cellulase on pure *Nannochloropsis* at 53ªC and pH 4.4.

The ANOVA S/N confirmed the key role of temperature (39% of the share) and the chemical reagent type (23% of the share) on the global protein solubilisation, but to a lesser extent than the ANOVA analysis, because of the differences between the biomasses (38% of residual). The global protein solubilisation yields increased with temperature and NaOH as the chemical reagent. These results were consistent with those previously recorded for the pretreatment step.

On the other hand, the effect of the individual parameters on the global lipid solubilisation yields was identical to that found in the chemical pretreatment tests (Figure 4b). The only difference was the increase in the yields after enzymatic hydrolysis in all the experiments. The chemical reagent and biomass type were identified as the only influential control factors on the global lipid solubilisation yields. The highest global lipid solubilisation yields were recorded in microalgae grown in the synthetic medium and the

lowest yields were recorded in microalgae grown in piggery wastewater. The ANOVA established the global lipid solubilisation dependence of only these two parameters, with contributions of 40% for the type of biomass and 13% for the chemical reagent type (residual of 24%). In this regard, Zhang et al., (2018) identified temperature, enzyme dosage and enzymatic hydrolysis time as the key variables in the optimisation of lipid solubilisation in *Scenedesmus* sp. using enzymatic hydrolysis, although these tests were conducted with an initial chemical pretreatment step.

Finally, the ANOVA S/N demonstrated that the chemical reagent type was significant in every biomass, with a 61% of the share. HCl was the chemical reagent that caused minimal global lipid solubilisation and was less sensitive to noise.

3.3. Process optimisation

In order to optimise a robust process capable of coping with a variable biomass composition, the typical effects of the main significant control factors should be used. A Duncan multiple range test of the most influential parameters was performed to elucidate the factor levels providing the highest improvement of the target variables. The analysis of the protein solubilisation yields showed an inevitable co-solubilisation of carbohydrates and proteins. Most of the operational conditions mediating a carbohydrate release also caused a solubilisation of proteins. Therefore, the protein solubilisation yields cannot be used as a target response and process optimisation should target maximising carbohydrate solubilisation and/or monosaccharide recovery and minimising lipid solubilisation. Thus, a fractional valorisation of macromolecular components of microalgae-based biomass using HCl or NaOH pretreatment would require a further step to separate monosaccharides and proteins (Suarez Garcia et al., 2018).

The temperature of the pretreatment was identified as the most important factor, with higher temperature increasing carbohydrate and protein solubilisation and monosaccharide recoveries in both the chemical pretreatment tests and the global process. Interestingly, no significant influence of temperature on lipid solubilisation yields was recorded. Differences between temperature levels were all significant for carbohydrate solubilisation and monosaccharide recovery, with Level T3 (120°C) being selected as the optimal temperature. The reagent type exerted a higher influence on the pretreatment step than on the global process. The use of HCl favored carbohydrate solubilisation and monosaccharide recovery, mainly in the pretreatment step, while the NaOH pretreatment favored protein and lipid solubilisation. Therefore, HCl was selected as the optimal chemical reagent. The increase in the chemical reagent concentration induced higher carbohydrate and protein solubilisation and monosaccharide recovery in both the chemical pretreatment tests and the global process but exhibited no impact on lipid solubilisation. The Duncan Test conducted revealed that the only significant difference was between Level 1 (0.5M) and Level 3 (2M), and between Level 2 (1M) and Level 3 (2M) during carbohydrate solubilisation and monosaccharide recovery. Therefore, Level 3 was selected as the optimal concentration.

Carbohydrate solubilisation increased with the pretreatment time from Level 1 (10 minutes) to Level 2 (30 minutes), but no significant differences were found from Level 2 to Level 3 (60 minutes). Nevertheless, the effect of the pretreatment time on the monosaccharide recovery was highly dependent on the type of biomass, with the degradation factor increasing remarkably in biomass grown in wastewater. An optimal pretreatment time of 10 minutes was selected based on economic considerations. Finally, economic or technical criteria should be applied for the values selection of the rest of the operational parameters since no significant impact was recorded (Lam et al., 2017).

The results obtained in experiment number 25, which involved all the selected levels of the influential parameters, provided carbohydrate solubilisations of 85%, 85% and 84% in the pretreatment step, and monosaccharide recoveries of 53%, 59% and 80% in the P, W and S biomasses, respectively. Likewise, protein solubilisation yields of 85%, 85% and 84% and lipid solubilisation yield of 16%, 1% and 59% were obtained in the chemical pretreatment tests in the P, W and S biomasses, respectively, under optimal operational conditions.

In the particular case of the P biomass, experimental conditions numbers 8 and 15 provided high monosaccharide recovery yields (76 and 73%, respectively). Carbohydrate solubilisation was similar or lower in these experiments than in experiment number 25. The high monosaccharide recovery recorded in experiments 8 and 15 was likely due to the low degradation of the solubilised carbohydrates under these particular combinations of operational parameters.

The enzymatic hydrolysis of pretreated samples obtained under the selected optimal conditions supported global carbohydrate solubilisation values of 97%, 98% and 95% and, therefore, global monosaccharide yields of 64%, 68% and 91% in the P, W and S biomasses, respectively. This slight improvement in the yield was not likely sufficient to counterbalance the additional cost of the enzymatic step. The economic viability of applying an enzymatic hydrolysis step could be considered only in the case that a relevant enhancement of the monosaccharide recovery is achieved. Interestingly, enzymatic hydrolysis did not solubilise additional proteins under these conditions, but lipid solubilisation yields increased up to 49%, 46% and 66% in the P, W and S biomass, respectively.

4. Conclusions

This study optimised the operational conditions of the chemical pretreatment and the enzymatic hydrolysis for the fermentable monosaccharide production from microalgae

biomass. The experimental design provided the optimal conditions for the significant control factors (120ºC, 2M HCl) independently of the kind of microalgae biomass. The other parameters (10 min, 75g/L) were selected applying economic considerations. At these conditions, the carbohydrate solubilisations were 84% for all biomasses with a degradation of 37, 31 and 5% for biomass grown in piggery wastewater, domestic wastewater and synthetic medium, respectively. The global process improved the solubilisation up to 97% while the degradation remained constant.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version.

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Figure Captions

Figure 1. Main effect plots on the carbohydrate solubilisation yields (in %) for **(a)** the chemical pretreatment step, **(b)** the global process (pretreatment followed by an enzymatic hydrolysis). Plotted values represent the mean yields for each factor level considering individual noise levels P (◇), W (○) and S (□) and the mean response of the three noise levels (✳).

Figure 2. Main effect plots on the monosaccharide recovery yields (in %) for **(a)** the pretreatment step, **(b)** the global process (pretreatment followed by an enzymatic hydrolysis). Plotted values represent the mean yields for each factor level considering individual noise levels P (◇), W (○) and S (□) and the mean response of the three noise levels (✳).

Figure 3. Main effect plots on the protein solubilisation yields (in %) for **(a)** the pretreatment step, **(b)** the global process (pretreatment followed by an enzymatic hydrolysis). Plotted values represent the mean yields for each factor level considering individual noise levels P (◇), W (○) and S (□) and the mean response of the three noise levels (✳).

Figure 4. Main effect plots on the lipid solubilisation yields (in %) for **(a)** the pretreatment step, **(b)** the global process (pretreatment followed by an enzymatic hydrolysis). Plotted values represent the mean yields for each factor level considering individual noise levels P (◇), W (○) and S (□) and the mean response of the three noise levels (✳).

721 **Tables**

Table 1: Levels of the studied control factors[a] for the optimisation by Taguchi design compared with other published works.

References	Statistical design	Microalgae biomass	C_Q	T	T min	C_A g/L	H	E FPU/g	t_H hours	Remarks
This study	Taguchi design, three levels (1, 2, 3)	P, W: Mixed microalgae-bacteria S: *Scenedesmus Almeriense*	1: 0.5M 2: 1M 3: 2M	1: 80 ºC 2: 100 ºC 3: 120 ºC	1: 10 2: 30 3: 60	1: 50 g/L 2: 75 g/L 3: 100 g/L	1: HCl 2: NaOH 3[b]: HCl	1: 10 2: 30 3: 60	1: 3 2: 6 3: 12	
Asyraf Kassim and Bhattacharya, (2016)	Response surface method	*Chlorella sp.*	0.1 to 0.5 M	60 to 120ºC	30 to 120		NaOH	-	-	Sugar yield: 88mg/g at 120ºC, 2% NaOH, 30 min
Harun et al., (2011)	Central composite design	*Chlorococcum humicola*	0.2 to 0.75 M	60 to 140ºC	15 to 60		NaOH			Glucose yield: 350 mg/g at 0.75%, 120ºC, 30 min
Hernández et al., (2015)		*C.sorokiniana* *N.gaditana* *S. almeriensis*	0 to 2.5M	121ºC	30		H_2SO_4	15 Celluclast 1.5L		Maximum sugar release *C.sorokiniana*: 100mg/g *N.gaditana:* 125mg/g *S. almeriensis:* 50mg/g
Pancha et al., (2016)		*Scenedesmus sp.* CCNM 1077	0.1 to 3M	121ºC	15 to 60	20 to 100	HCl, H_2SO_4, NaOH, KOH	Cellulase	6, 24, 48, 72	HCl, 60 min, 0.5M, 6% of biomass, 72h.
Shokrkar et al., (2017)		Mixed microalgae-bacteria biomass	0.5, 1 and 2M	121ºC	10 to 40		HCl, H_2SO_4, NaOH			Sugar yield: 95% at HCl, 2M, 30 min
Sivaramakrishnan and Incharoensakdi, (2018)		*Scenedesmus sp.*	0.1, 0.2 and 0.3N	60 to 120ºC	10 to 40		HCl, H_2SO_4, NaOH, KOH			Sugar yield: 80% at 0.3N, 120ºC, 20min, NaOH

[a]C_Q: Concentration of chemical reagent; T: Temperature; t: time; C_A: concentration of microalgae biomass; H: reagent; E: dosage of enzyme; t_H: time during the enzymatic hydrolysis.
[b]Level 3 for chemical reagent corresponds again to HCl (dummy effect).

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Table 2: Taguchi's $L_{27}(3)^{13}$ orthogonal array and experimental results of carbohydrate, protein and lipid solubilisation yields, and monosaccharide recovery yields during the pretreatment step.

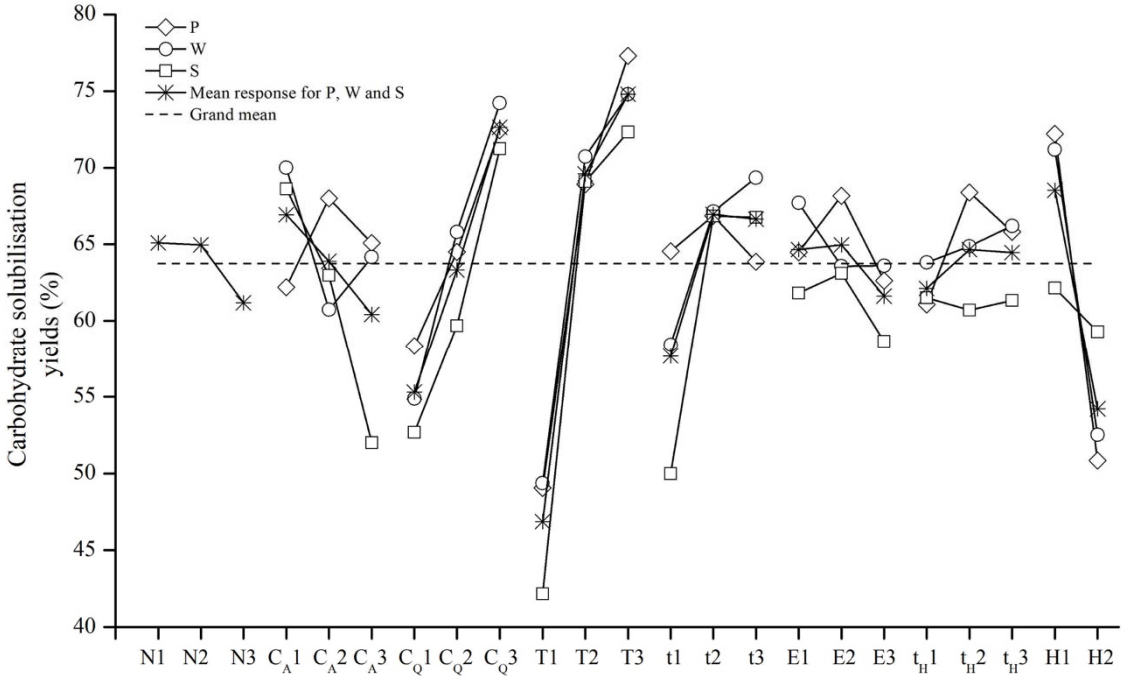
Orthogonal array matrix														Experimental results, in %										
	1	2	3	4	5	6	7	8	9	10	11	12	13	Carbohydrates			Monosaccharides			Proteins			Lipids	
Exp. No.	C_Q[a]	T[b]	C_Q×T	C_Q×T	t[c]	C_Q×t	C_Q×t	T×t	C_A[d]	E[e]	Txt	t_H[f]	H[g]	P[h]	W[i]	S[j]	P[h]	W[i]	S[j]	P[h]	W[i]	S[j]	P[h]	W[i]	S[j]
1	1	1	1	1	1	1	1	1	1	1	1	1	1	28	37	13	4	10	4	18	13	18	1	62	44
2	1	1	1	1	2	2	2	2	2	2	2	2	2	45	33	48	9	8	17	37	34	48	2	40	78
3	1	1	1	1	3	3	3	3	3	3	3	3	1'	40	54	20	5	9	7	26	23	33	11	67	26
4	1	2	2	2	1	1	1	2	2	2	3	3	1'	75	44	57	10	15	31	34	46	29	30	69	45
5	1	2	2	2	2	2	2	3	3	3	1	1	1	73	67	69	16	15	30	45	26	38	20	29	14
6	1	2	2	2	3	3	3	1	1	1	2	2	2	40	45	76	12	17	32	67	73	88	63	71	88
7	1	3	3	3	1	1	1	3	3	3	2	2	2	55	54	40	4	12	28	62	56	51	59	65	77
8	1	3	3	3	2	2	2	1	1	1	3	3	1'	85	85	78	76	56	70	67	57	68	12	32	16
9	1	3	3	3	3	3	3	2	2	2	1	1	1	85	75	75	56	52	51	58	49	35	7	19	23
10	2	1	2	3	1	2	3	1	2	3	1	2	1'	52	34	22	3	9	8	13	17	20	7	66	46
11	2	1	2	3	2	3	1	2	3	1	2	3	1	57	64	51	4	8	16	13	21	22	13	44	44
12	2	1	2	3	3	1	2	3	1	2	3	1	2	25	53	67	14	10	27	67	53	81	5	50	89
13	2	2	3	1	1	2	3	2	3	1	3	1	2	61	61	45	19	9	30	64	54	64	9	7	77
14	2	2	3	1	2	3	1	3	1	2	1	2	1'	82	84	67	44	47	57	56	58	58	16	44	49
15	2	2	3	1	3	1	2	1	2	3	2	3	1	74	80	74	73	51	64	54	61	55	2	39	28
16	2	3	1	2	1	2	3	3	1	2	2	3	1	87	88	78	54	62	72	52	63	62	12	34	41
17	2	3	1	2	2	3	1	1	2	3	3	1	2	58	45	65	22	15	37	86	75	86	14	64	78
18	2	3	1	2	3	1	2	2	3	1	1	2	1'	85	82	67	55	58	52	71	63	43	22	30	22
19	3	1	3	2	1	3	2	1	3	2	1	3	2	52	28	28	8	8	21	56	61	34	3	53	92
20	3	1	3	2	2	1	3	2	1	3	2	1	1'	60	67	67	24	15	31	28	50	35	10	20	51
21	3	1	3	2	3	2	1	3	2	1	3	2	1	84	74	64	60	30	55	54	24	41	17	41	43
22	3	2	1	3	1	3	2	2	1	3	3	2	1	86	94	85	49	44	77	51	92	75	10	78	59
23	3	2	1	3	2	1	3	3	2	1	1	3	2	55	76	79	13	14	32	82	67	89	37	93	96
24	3	2	1	3	3	2	1	1	3	2	2	1	1'	75	84	71	68	52	59	42	71	51	5	48	18
25	3	3	2	1	1	3	2	3	2	1	2	1	1'	85	85	84	53	59	80	60	67	75	16	1	59
26	3	3	2	1	2	1	3	1	3	2	3	2	1	88	83	78	48	50	67	67	72	51	26	40	33
27	3	3	2	1	3	2	1	2	1	3	1	3	2	67	77	87	33	21	40	86	78	96	44	93	96

[a]Concentration of chemical reagent (mol/L). 1=0.5, 2=1, 3=2.
[b]Temperature (°C). 1=80, 2=100, 3=120
[c]time (min). 1=10, 2=30, 3=60.
[d]Concentration of microalgae biomass (g/L). 1=50, 2=75, 3=100.
[e]Dosage of enzyme (FPU/g). 1=10, 2=30, 3=60.
[f]Time during the enzymatic hydrolysis (h). 1=3, 2=6, 3=12.
[g]Chemical reagent. 1=HCl, 2=NaOH, 1'=HCl.
[h]P: microalgae biomass grown in pig manure wastewater.
[i]W: microalgae biomass grown in domestic wastewater.
[j]S: microalgae biomass grown in synthetic media.

724

Figure 1

(a)


P
W
S
Mean response for P, W and S
Grand mean
Carbohydrate solubilisation yields (%)
80
75
70
65
60
55
50
45
40
N1 N2 N3 C_A1 C_A2 C_A3 C_Q1 C_Q2 C_Q3 T1 T2 T3 t1 t2 t3 E1 E2 E3 t_H1 t_H2 t_H3 H1 H2

(b)

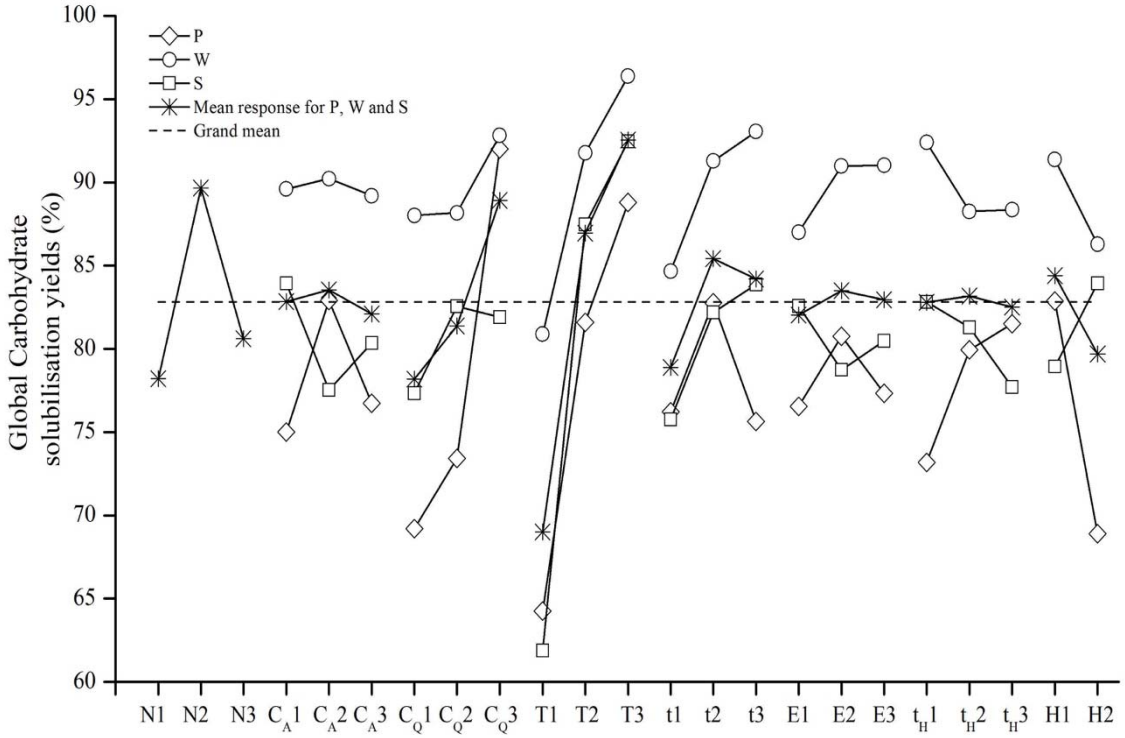
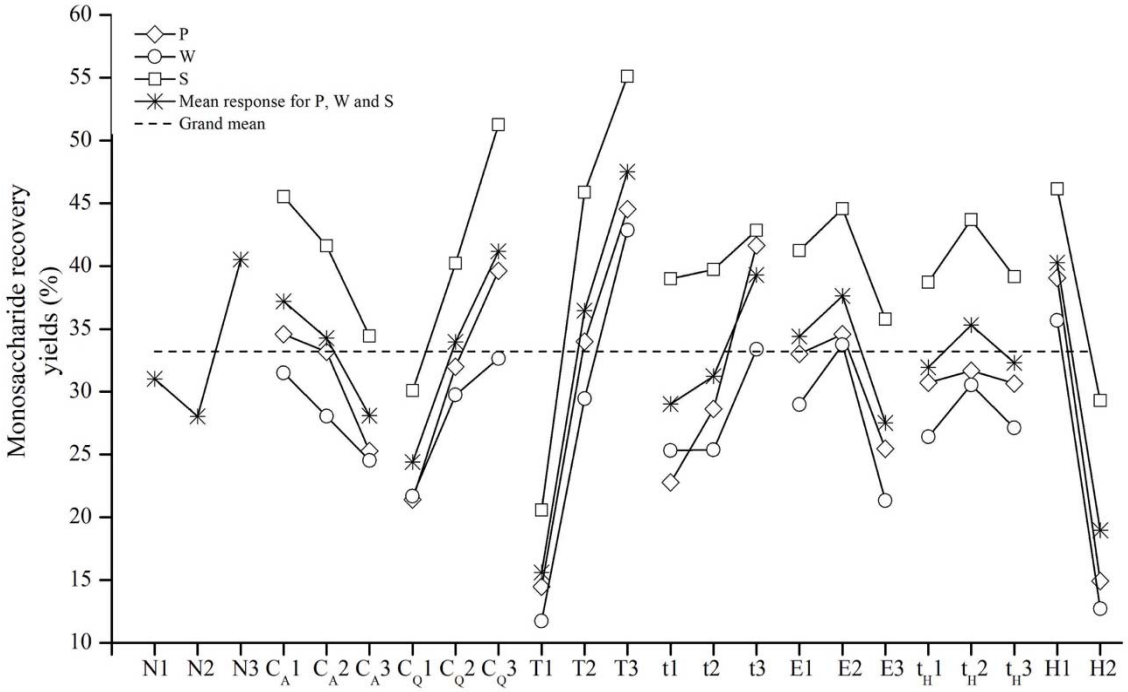

P
W
S
Mean response for P, W and S
Grand mean
Global Carbohydrate solubilisation yields (%)
100
95
90
85
80
75
70
65
60
N1 N2 N3 C_A1 C_A2 C_A3 C_Q1 C_Q2 C_Q3 T1 T2 T3 t1 t2 t3 E1 E2 E3 t_H1 t_H2 t_H3 H1 H2

Figure 2

(a)


P
W
S
Mean response for P, W and S
Grand mean
Monosaccharide recovery yields (%)
60
55
50
45
40
35
30
25
20
15
10
N1 N2 N3 C_A1 C_A2 C_A3 C_Q1 C_Q2 C_Q3 T1 T2 T3 t1 t2 t3 E1 E2 E3 t_H1 t_H2 t_H3 H1 H2

(b)

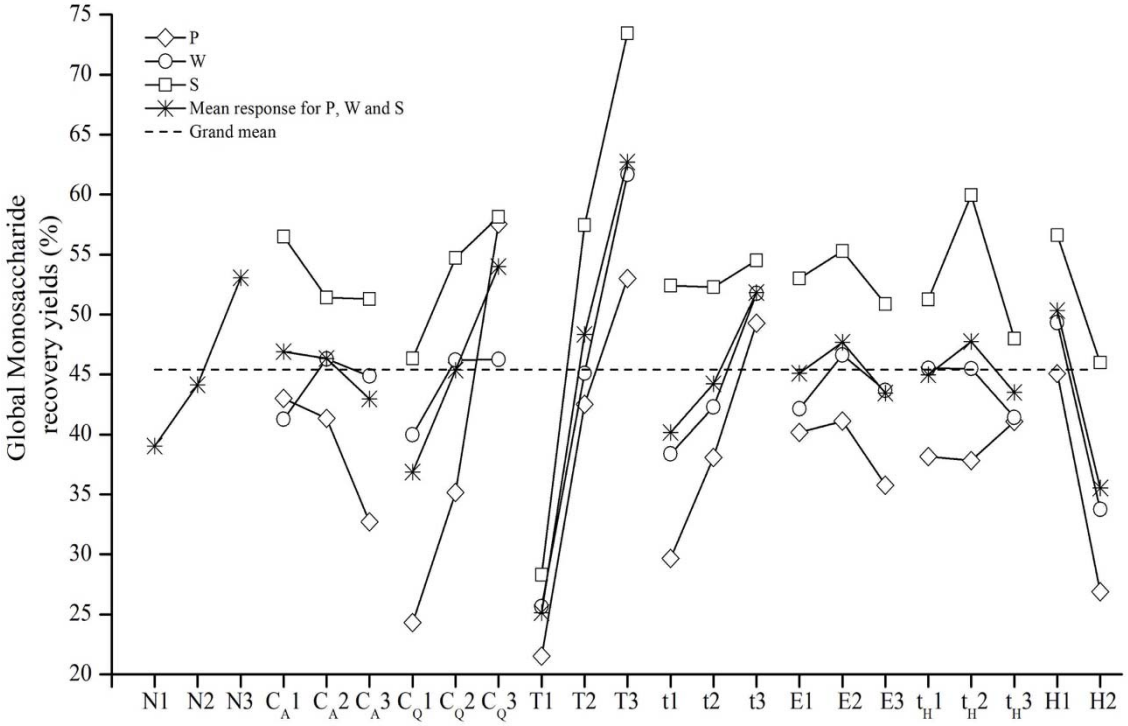
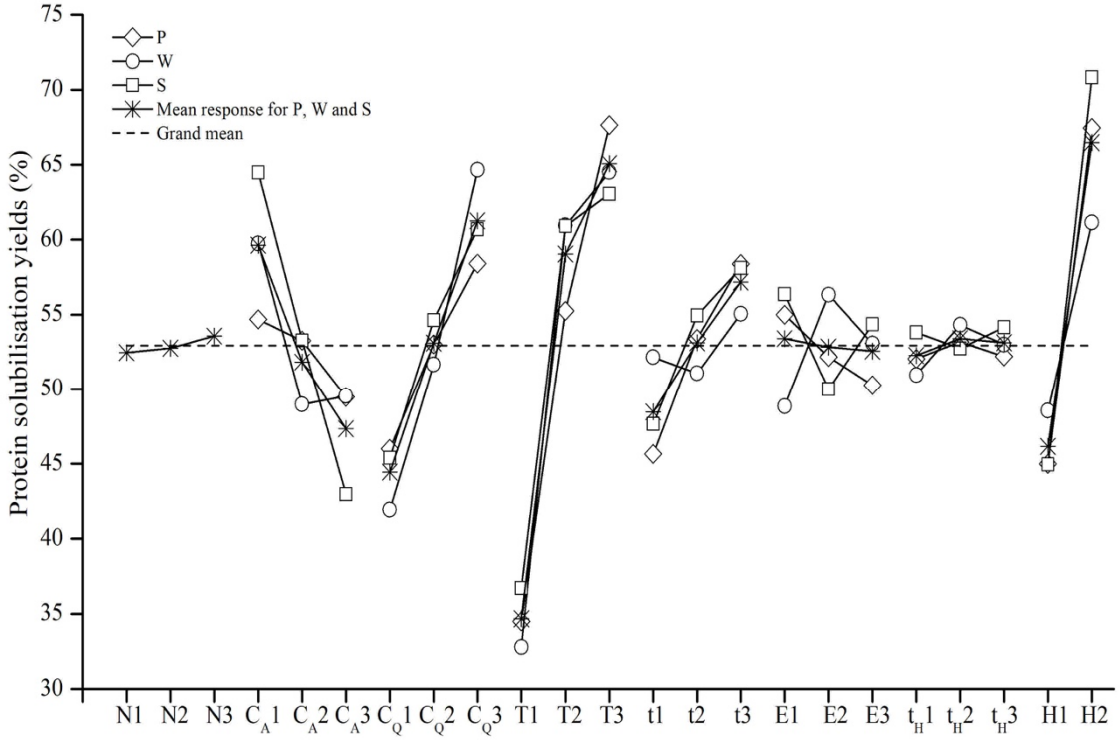

P
W
S
Mean response for P, W and S
Grand mean
Global Monosaccharide recovery yields (%)
75
70
65
60
55
50
45
40
35
30
25
20
N1 N2 N3 C_A1 C_A2 C_A3 C_Q1 C_Q2 C_Q3 T1 T2 T3 t1 t2 t3 E1 E2 E3 t_H1 t_H2 t_H3 H1 H2

Figure 3

(a)


P
W
S
Mean response for P, W and S
Grand mean
Protein solubilisation yields (%)
75
70
65
60
55
50
45
40
35
30
N1 N2 N3 C_A1 C_A2 C_A3 C_Q1 C_Q2 C_Q3 T1 T2 T3 t1 t2 t3 E1 E2 E3 t_H1 t_H2 t_H3 H1 H2

(b)

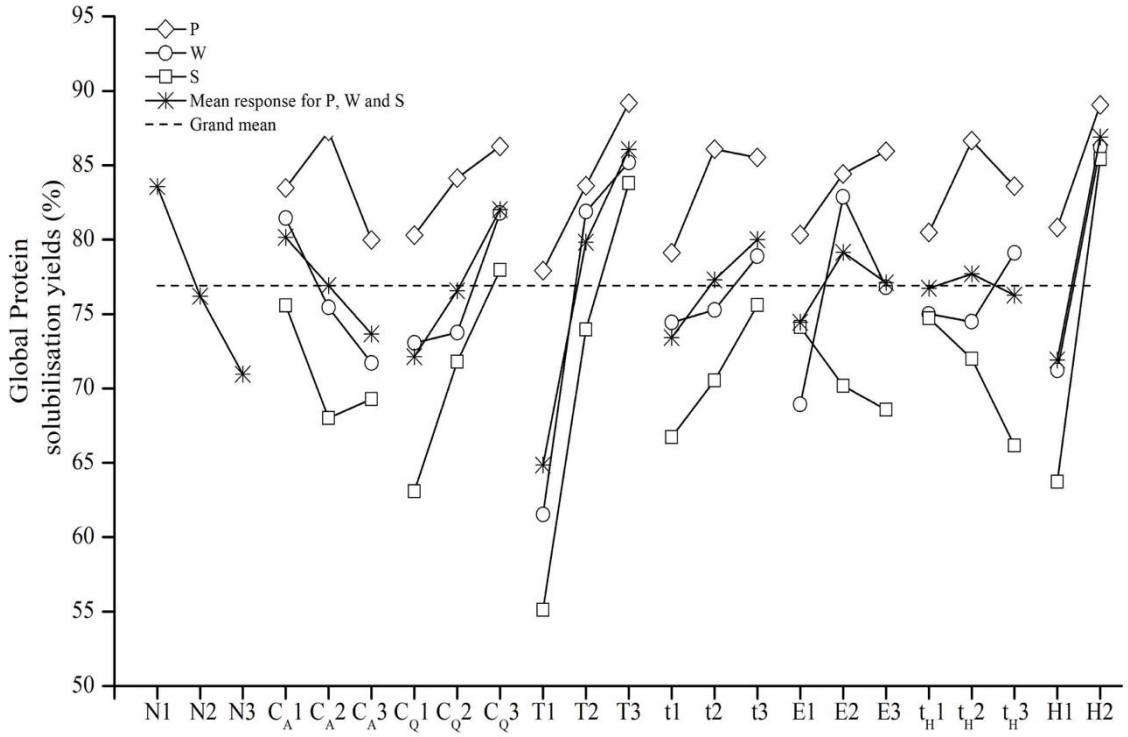
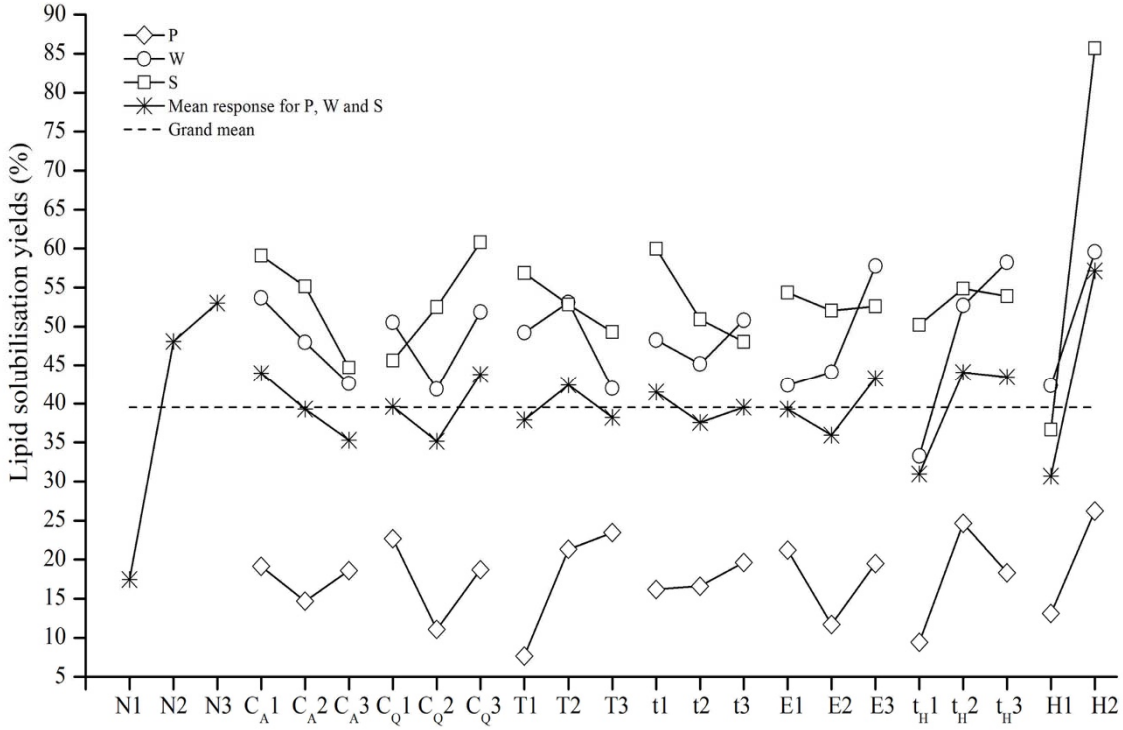
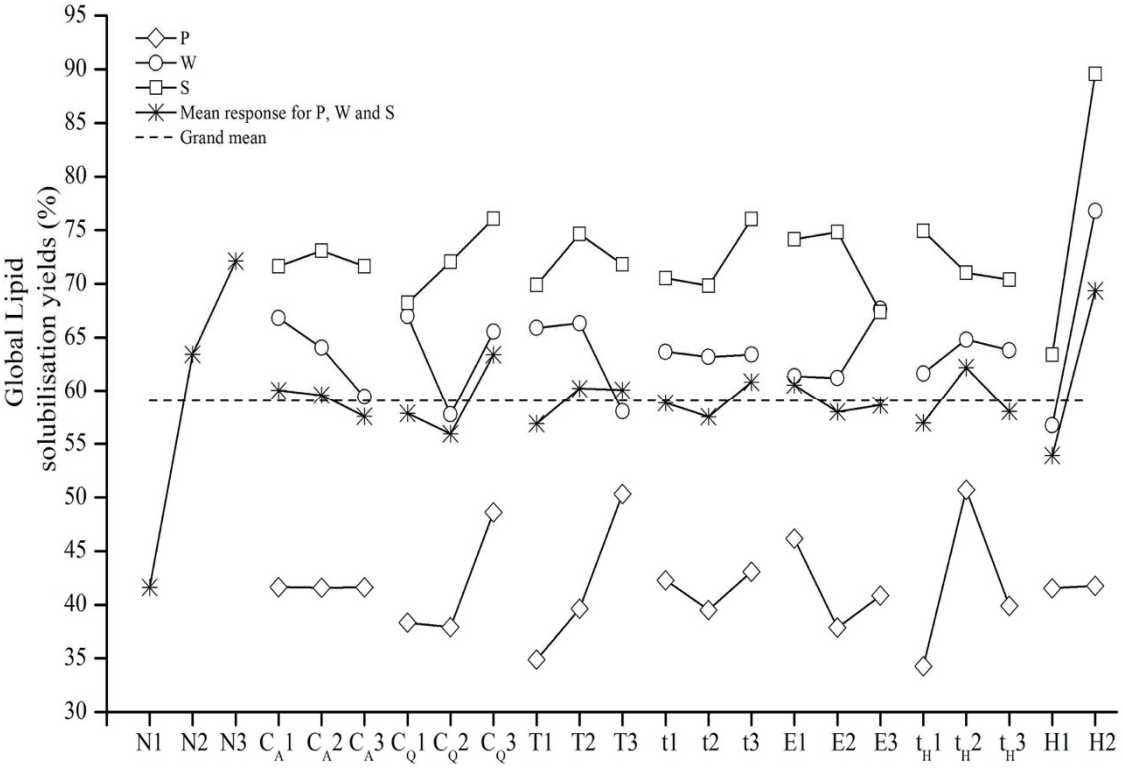

P
W
S
Mean response for P, W and S
Grand mean
Global Protein solubilisation yields (%)
95
90
85
80
75
70
65
60
55
50
N1 N2 N3 C_A1 C_A2 C_A3 C_Q1 C_Q2 C_Q3 T1 T2 T3 t1 t2 t3 E1 E2 E3 t_H1 t_H2 t_H3 H1 H2

Figure 4

(a)


P
W
S
Mean response for P, W and S
Grand mean
Lipid solubilisation yields (%)
N1 N2 N3 C_A1 C_A2 C_A3 C_Q1 C_Q2 C_Q3 T1 T2 T3 t1 t2 t3 E1 E2 E3 t_H1 t_H2 t_H3 H1 H2

(b)


P
W
S
Mean response for P, W and S
Grand mean
Global Lipid solubilisation yields (%)
N1 N2 N3 C_A1 C_A2 C_A3 C_Q1 C_Q2 C_Q3 T1 T2 T3 t1 t2 t3 E1 E2 E3 t_H1 t_H2 t_H3 H1 H2

SUPPLEMENTARY MATERIALS

Optimisation of the production of fermentable monosaccharides from algal biomass grown in photobioreactors treating wastewater

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CONTENT

Table S1: Volatile solids solubilisation yields of the pretreatment and the global process (pretreatment and enzymatic hydrolysis)

Orthogonal array matrix														Experimental results, in %					
	1	2	3	4	5	6	7	8	9	10	11	12	13	P[h]		W[i]		S[j]	
Exp. No.	C_Q[a]	T[b]	C_Q×T	C_Q×T	t[c]	C_Q×t	C_Q×t	T×t	C_A[d]	E[e]	Txt	t_H[f]	H[g]	PR	Global	PR	Global	PR	Global
1	1	1	1	1	1	1	1	1	1	1	1	1	1	22	47	7	51	8	28
2	1	1	1	1	2	2	2	2	2	2	2	2	2	24	67	10	80	39	64
3	1	1	1	1	3	3	3	3	3	3	3	3	1’	16	30	17	29	18	44
4	1	2	2	2	1	1	1	2	2	2	3	3	1’	26	51	26	69	32	54
5	1	2	2	2	2	2	2	3	3	3	1	1	1	30	55	24	49	26	55
6	1	2	2	2	3	3	3	1	1	1	2	2	2	62	85	32	69	77	94
7	1	3	3	3	1	1	1	3	3	3	2	2	2	46	71	34	62	41	79
8	1	3	3	3	2	2	2	1	1	1	3	3	1’	51	83	50	80	48	86
9	1	3	3	3	3	3	3	2	2	2	1	1	1	53	86	45	69	28	85
10	2	1	2	3	1	2	3	1	2	3	1	2	1’	15	38	18	38	12	22
11	2	1	2	3	2	3	1	2	3	1	2	3	1	15	43	12	38	19	51
12	2	1	2	3	3	1	2	3	1	2	3	1	2	54	65	33	87	68	87
13	2	2	3	1	1	2	3	2	3	1	3	1	2	25	52	40	65	44	82
14	2	2	3	1	2	3	1	3	1	2	1	2	1’	49	76	52	79	38	75
15	2	2	3	1	3	1	2	1	2	3	2	3	1	44	73	50	74	43	73
16	2	3	1	2	1	2	3	3	1	2	2	3	1	43	74	53	79	58	83
17	2	3	1	2	2	3	1	1	2	3	3	1	2	51	77	38	78	66	92
18	2	3	1	2	3	1	2	2	3	1	1	2	1’	53	77	47	75	28	85
19	3	1	3	2	1	3	2	1	3	2	1	3	2	16	35	41	61	13	35
20	3	1	3	2	2	1	3	2	1	3	2	1	1’	27	64	19	58	26	62
21	3	1	3	2	3	2	1	3	2	1	3	2	1	51	82	26	53	35	73
22	3	2	1	3	1	3	2	2	1	3	3	2	1	38	77	54	78	70	85
23	3	2	1	3	2	1	3	3	2	1	1	3	2	55	79	75	92	72	92
24	3	2	1	3	3	2	1	1	3	2	2	1	1’	39	79	51	81	38	84
25	3	3	2	1	1	3	2	3	2	1	2	1	1’	54	87	52	83	66	86
26	3	3	2	1	2	1	3	1	3	2	3	2	1	66	94	56	85	53	79
27	3	3	2	1	3	2	1	2	1	3	1	3	2	58	83	80	94	91	97

[a]Concentration of chemical reagent (mol/L). 1=0.5, 2=1, 3=2.
[b]Temperature (°C). 1=80, 2=100, 3=120
[c]time (min). 1=10, 2=30, 3=60.
[d]Concentration of microalgae biomass (g/L). 1=50, 2=75, 3=100.
[e]Dosage of enzyme (FPU/g). 1=10, 2=30, 3=60.
[f]Time during the enzymatic hydrolysis (h). 1=3, 2=6, 3=12.
[g]Chemical reagent. 1=HCl, 2=NaOH, 1’=HCl.
[h]P: microalgae biomass grown in pig manure wastewater.
[i]W: microalgae biomass grown in domestic wastewater.
[j]S: microalgae biomass grown in synthetic media.

Table S2: ANOVA tables of the results from the pretreatment step showing degrees of freedom (DF), sum of squares (SS), p-value (p) and percentages of contributions (C) of factors and interactions for the experimental design at three noise levels. In italics, non-significant factors/interactions pooled to estimate the residual variance.

Source of variation[a]	Carbohydrates				Monosaccharides				Proteins				Lipids			
	DF	SS	p	C	DF	SS	p	C	DF	SS	p	C	DF	SS	p	C
C_Q	2	4063	0.000	13	2	3829	0.000	9	2	3819	0.000	11	*2*	*1009*		
T	2	11913	0.000	38	2	141809	0.000	34	2	13985	0.000	39	*2*	*342*		
C_Q x T	*4*	*349*			4	2022	0.000	5	*4*	*92*			*4*	*1826*		
t	2	1508	0.000	5	2	1579	0.000	4	2	1023	0.001	3	*2*	*206*		
C_Q x t	4	831	0.010	3	4	2787	0.000	7	4	1034	0.004	3	*4*	*2796*		
T x t	4	1523	0.000	5	4	1306	0.004	3	4	886	0.008	2	*4*	*301*		
C_A	2	576	0.009	2	2	1173	0.001	3	2	2096	0.000	6	*2*	*1015*		
H	1	3685	0.000	12	1	8179	0.000	20	1	7430	0.000	21	1	12607	0.000	21
N	*2*	*265*			2	2303	0.000	6	*2*	*18*			2	19993	0.000	33
C_QxN	*4*	*109*			*4*	*277*			*4*	*289*			*4*	*1183*		
TxN	*4*	*164*			*4*	*156*			*4*	*343*			*4*	*1820*		
(C_QxT)xN	8	1265	0.011	4	*8*	*430*			*8*	*681*			*8*	*2365*		
txN	4	834	0.010	3	*4*	*568*			*4*	*313*			*4*	*696*		
($C_Q$xt)xN	*8*	*270*			*8*	*355*			*8*	*390*			*8*	*919*		
(Txt)xN	*8*	*418*			*8*	*474*			8	1370	0.008	4	*8*	*1537*		
C_AxN	4	1254	0.001	4	*4*	*70*			4	781	0.016	2	*4*	*636*		
HxN	2	1197	0.000	4	*2*	*183*			2	571	0.011	2	2	4641	0.001	8
Residual	45	2477		8	57	4365		10	49	2818		8	75	22698		38
Total	80	31126			80	41722			80	35814			80	59939		

[a]C_Q: Concentration of chemical reagent, T: Temperature, t: time, C_A: Concentration of microalgae biomass, H: Chemical reagent, and N:microalgae biomass harvested from different wastewater treatments (noise).

Table S3: ANOVA tables for the signal to noise values of the 27 experiments for pretreatment results, showing degrees of freedom (DF), sum of squares (SS), p-value (p) and percentages of contributions (C) of factors and factor interactions for the experimental design at three noise levels.

Source of variation[a]	Carbohydrates				Monosaccharides				Proteins				Lipids			
	DF	SS	p	C	DF	SS	p	C	DF	SS	p	C	DF	SS	p	C
C_Q	2	47	0.001	15	2	208	0.014	13	2	41	0.023	9	*2*	*12*		
T	2	153	0.000	48	2	676	0.000	42	2	231	0.000	50	*2*	*16*		
C_Q x T	*4*	*13*			*4*	*77*			*4*	*7*			*4*	*25*		
t	2	28	0.007	9	2	142	0.045	9	*2*	*17*			*2*	*20*		
C_Q x t	*4*	*10*			*4*	*105*			*4*	*23*			*4*	*32*		
T x t	4	32	0.020	10	*4*	*31*			*4*	*21*			*4*	*3*		
C_A	*2*	*2*			*2*	*76*			*2*	*19*			*2*	*10*		
H	1	26	0.003	8	1	212	0.004	13	1	94	0.000	21	1	185	0.000	55
Residual	15	30		10	19	368		23	21	93		20	25	150		45
Total	26	315			26	1606			26	459			26	335		

[a]C_Q: Concentration of chemical reagent, T: Temperature, t: time, C_A: Concentration of microalgae biomass, H: Chemical reagent, and N: noise.

Table S4: Taguchi's $L_{27}(3)^{13}$ orthogonal array and experimental results for carbohydrates, proteins and lipids solubilisation, and monosaccharides recovery in the global process (pretreatment followed by enzymatic hydrolysis).

Orthogonal array matrix														Experimental results, in %									
	1	2	3	4	5	6	7	8	9	10	11	12	13	Carbohydrates			Monosaccharides			Proteins			Lipids		
Exp. No.	C_Q[a]	T[b]	C_QxT	C_QxT	t[c]	$C_Q$xt	$C_Q$xt	Txt	C_A[d]	E[e]	Txt	t_H[f]	H[g]	P[h]	W[i]	S[j]	P[h]	W[i]	S[j]	P[h]	W[i]	S[j]	P[h]	W[i]	S[j]
1	1	1	1	1	1	1	1	1	1	1	1	1	1	38	75	39	5	14	9	54	41	40	11	77	65
2	1	1	1	1	2	2	2	2	2	2	2	2	2	67	87	58	11	20	24	89	78	68	14	87	83
3	1	1	1	1	3	3	3	3	3	3	3	3	1'	47	99	54	6	53	15	67	66	50	26	68	46
4	1	2	2	2	1	1	1	2	2	2	3	3	1'	86	87	69	12	25	34	77	72	35	40	71	60
5	1	2	2	2	2	2	2	3	3	3	1	1	1	82	98	91	19	46	47	85	61	50	31	57	53
6	1	2	2	2	3	3	3	1	1	1	2	2	2	50	69	100	15	34	55	86	85	91	68	79	89
7	1	3	3	3	1	1	1	3	3	3	2	2	2	69	87	97	15	35	83	86	77	79	60	69	78
8	1	3	3	3	2	2	2	1	1	1	3	3	1'	91	92	97	78	61	87	88	91	81	37	53	58
9	1	3	3	3	3	3	3	2	2	2	1	1	1	94	97	92	57	72	64	91	87	74	57	41	82
10	2	1	2	3	1	2	3	1	2	3	1	2	1'	65	61	49	7	23	18	80	45	42	56	76	61
11	2	1	2	3	2	3	1	2	3	1	2	3	1	72	70	67	7	11	25	71	32	38	47	53	61
12	2	1	2	3	3	1	2	3	1	2	3	1	2	34	89	84	16	18	38	91	84	87	23	80	89
13	2	2	3	1	1	2	3	2	3	1	3	1	2	66	91	94	20	38	41	77	79	95	40	55	94
14	2	2	3	1	2	3	1	3	1	2	1	2	1'	89	98	86	48	59	76	83	82	72	36	48	71
15	2	2	3	1	3	1	2	1	2	3	2	3	1	81	91	86	76	57	75	91	84	64	16	41	65
16	2	3	1	2	1	2	3	3	1	2	2	3	1	93	100	93	56	74	84	81	88	70	48	42	51
17	2	3	1	2	2	3	1	1	2	3	3	1	2	71	98	90	30	67	61	97	95	93	27	76	82
18	2	3	1	2	3	1	2	2	3	1	1	2	1'	91	96	94	57	70	74	87	75	85	48	51	75
19	3	1	3	2	1	3	2	1	3	2	1	3	2	76	63	57	27	19	32	85	91	64	30	58	98
20	3	1	3	2	2	1	3	2	1	3	2	1	1'	86	85	77	46	21	38	78	68	58	44	50	64
21	3	1	3	2	3	2	1	3	2	1	3	2	1	95	98	71	69	53	56	86	50	49	62	44	62
22	3	2	1	3	1	3	2	2	1	3	3	2	1	98	99	91	61	49	80	91	99	82	46	79	62
23	3	2	1	3	2	1	3	3	2	1	1	3	2	91	94	87	46	31	39	92	91	94	53	94	98
24	3	2	1	3	3	2	1	1	3	2	2	1	1'	92	99	84	86	67	71	70	84	81	26	73	80
25	3	3	2	1	1	3	2	3	2	1	2	1	1'	97	98	95	64	68	91	82	77	93	49	46	66
26	3	3	2	1	2	1	3	1	3	2	3	2	1	97	99	86	57	66	74	92	80	80	65	51	59
27	3	3	2	1	3	2	1	2	1	3	1	3	2	97	99	89	62	42	42	99	96	99	61	94	96

[a]Concentration of chemical reagent (mol/L). 1=0.5, 2=1, 3=2.
[b]Temperature (°C). 1=80, 2=100, 3=120
[c]time (min). 1=10, 2=30, 3=60.
[d]Concentration of microalgae biomass (g/L). 1=50, 2=75, 3=100.
[e]Dosage of enzyme (FPU/g). 1=10, 2=30, 3=60.
[f]Time during the enzymatic hydrolysis (h). 1=3, 2=6, 3=12.
[g]Chemical reagent. 1=HCl, 2=NaOH, 1'=HCl.
[h]P: microalgae biomass grown in pig manure wastewater.
[i]W: microalgae biomass grown in domestic wastewater.
[j]S: microalgae biomass grown in synthetic media.

Table S5: ANOVA tables for the global process (pretreatment and enzymatic hydrolysis) responses showing degrees of freedom (DF), sum of squares (SS), p-value (p) and percentages of contributions (C) of factors and interactions for the experimental design at three levels of noise. In italics, non-significant factors/interactions pooled to estimate the residual variance.

Source of variation[a]	Carbohydrates				Monosaccharides				Proteins				Lipids			
	DF	SS	p	C	DF	SS	p	C	DF	SS	p	C	DF	SS	p	C
C_Q	2	1639	0.000	7	2	3953	0.000	8	2	1319	0.000	6	2	819	0.048	2
T	2	8158	0.000	37	2	19387	0.000	41	2	6409	0.000	29	*2*	190		
C_Q x T	*4*	359			4	2874	0.001	6	*4*	317			*4*	1098		
t	2	651	0.032	3	2	1903	0.001	4	2	591	0.009	3	*2*	151		
C_Q x t	*4*	349			4	1640	0.016	3	4	1442	0.000	6	*4*	549		
T x t	4	1274	0.011	6	4	1710	0.013	4	*4*	181			*4*	603		
C_A	*2*	29			*2*	249			2	571	0.011	3	*2*	94		
E	*2*	29			*2*	247			*2*	299			*2*	98		
t_H	*2*	6			*2*	251			*2*	29			*2*	412		
H	1	395	0.040	2	1	3940	0.000	8	1	4032	0.000	18	1	4311	0.000	13
e_H	*1*	16			*1*	1			*1*	66			*1*	47		
N	2	1973	0.000	9	2	2734	0.000	6	2	2171	0.000	10	2	13341	0.000	40
C_QxN	4	1292	0.011	6	4	2121	0.004	5	*4*	278			*4*	567		
TxN	*4*	683			*4*	522			4	938	0.006	4	4	1432	0.034	4
(C_QxT)xN	*8*	1045			*8*	1097			*8*	736			8	3322	0.004	10
txN	*4*	312			*4*	718			*4*	137			*4*	120		
($C_Q$xt)xN	*8*	358			*8*	1159			*8*	175			*8*	1267		
(Txt)xN	*8*	1017			*8*	1026			*8*	132			*8*	578		
C_AxN	*4*	472			*4*	581			*4*	399			*4*	170		
ExN	*4*	223			*4*	81			4	881	0.009	4	*4*	769		
t_HxN	*4*	572			*4*	592			4	603	0.046	3	*4*	1000		
HxN	2	1083	0.004	5	*2*	177			2	543	0.013	2	2	2203	0.001	7
e_HxN	*2*	2			*2*	5			*2*	198			*2*	129		
Residual	61	5472		25	55	6704		14	51	2946		13	61	7842		24
Total	80	21938			80	46966			80	22447			80	33269		

[a]C_Q: Concentration of chemical reagent, T: Temperature, t: time, C_A: Concentration of microalgae biomass, E: dosage of enzyme, t_H: time of enzymatic hydrolysis, H: Chemical reagent, e_H: dummy effect, and N: noise.

Table S6: ANOVA tables for the signal to noise values of the 27 experiments for global (pretreatment and enzymatic hydrolysis) results, showing degrees of freedom (DF), sum of squares (SS), p-value (p) and percentages of contribution (C) of factors and factor interactions for the experimental design at three noise levels.

Source of variation[a]	Carbohydrates				Monosaccharides				Proteins				Lipids			
	DF	SS	p	C	DF	SS	p	C	DF	SS	p	C	DF	SS	p	C
C_Q	2	12	0.014	13	2	203	0.004	20	*2*	*9*			*2*	*4*		
T	2	53	0.000	58	2	527	0.000	51	2	45	0.000	39	*2*	*0*		
C_Q x T	*4*	*5*			*4*	*97*			*4*	*5*			*4*	*6*		
t	*2*	*5*			*2*	*55*			*2*	*5*			*2*	*1*		
C_Q x t	*4*	*3*			*4*	*63*			*4*	*14*			*4*	*2*		
T x t	*4*	*7*			*4*	*11*			*4*	*1*			*4*	*5*		
C_A	*2*	*1*			*2*	*18*			*2*	*4*			*2*	*0*		
E	*2*	*0*			*2*	*22*			*2*	*3*			*2*	*0*		
t_H	*2*	*1*			*2*	*6*			*2*	*1*			*2*	*2*		
H	*1*	*4*			*1*	*35*			1	27	0.001	23	1	34	0.000	61
e_H	*1*	*0*			*1*	*1*			*1*	*1*			*1*	*0*		
Residual	22	26		29	22	310		30	23	43		38	25	21		39
Total	26	91			26	1040			26	114			26	55		

[a] C_Q: Concentration of chemical reagent, T: Temperature, t: time, C_A: Concentration of microalgae biomass, E: dosage of enzyme, t_H: time of enzymatic hydrolysis, H: Chemical reagent, and e_H: dummy effect.