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Ultrasound-assisted extraction of R-phycoerythrin from *Grateloupia turuturu* with and without enzyme addition

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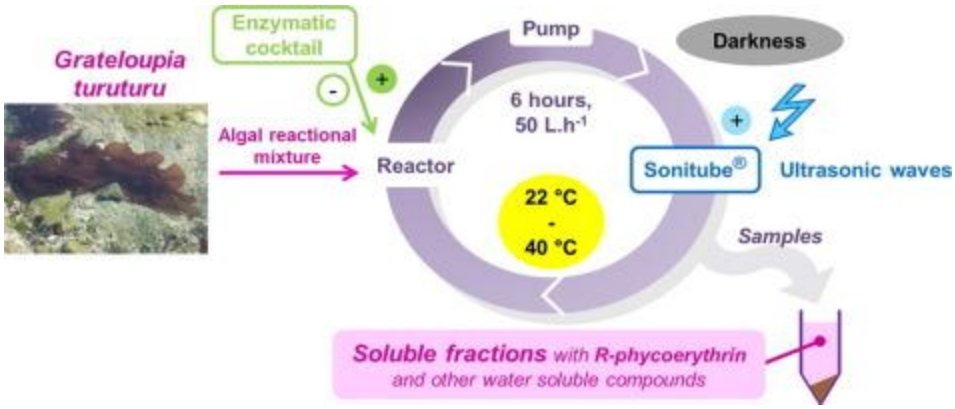
Abstract :

The aim of this study was to compare two processes for the extraction of R-phycoerythrin (R-PE) from the red seaweed *Grateloupia turuturu*: ultrasound-assisted extraction (UAE) and ultrasound-assisted enzymatic hydrolysis (UAEH). Process efficiencies were both evaluated by the yield of R-PE extraction and by the level of liquefaction. Experiments were conducted at 40 and 22 °C, for 6 h, using an enzymatic cocktail and an original ultrasonic flow-through reactor. R-PE appeared very sensitive to temperature, thus 22 °C is strongly recommended for its extraction by UAEH or UAE. However, the higher processing temperature (40 °C) clearly increased the extraction of water-soluble compounds (up to 91% of liquefaction).

These two new processes are thus promising alternatives for the extraction of water-soluble components including R-PE, from wet seaweeds, with extraction yields at least similar to conventional solid–liquid extraction.

Graphical abstract



Keywords : Seaweed, *Grateloupia turuturu*, R-phycoerythrin, Ultrasound-assisted extraction, Ultrasound-assisted enzymatic hydrolysis, Liquefaction

1. Introduction

The concept of biorefinery continues to make progress with many studies dealing with the development of new technologies for the total recovery of biomass. Among vegetal biomasses, marine substrates like seaweeds are of interest due to their content in a wide range of biomolecules and their worldwide distribution, making them a promising feedstock [1]. Although several species have been used for hundreds of years for various applications, many remain underexploited, notably in European countries. Among these, the red seaweed *Grateloupia turuturu* (Yamada), introduced 45 years ago, is now proliferative and abundant on the French Atlantic coast [2].

Red seaweeds are interesting notably for their richness in proteins, polysaccharides and lipids, and also due to some biomolecules such as the valuable R-phycoerythrin (R-PE), a water-soluble pink-purple pigment [3]. R-PE is the main light-harvesting pigment of Rhodophyta and belongs to the phycobiliprotein family [4,5]. In fact, it is the most abundant phycobiliprotein in *Grateloupia turuturu*, representing up to 0.30 % dw [6]. Such a pigment has a number of potential applications, like as a natural colorant, a fluorescent probe (these 2 are already available on the market), an antioxidant, antitumoral and antidiabetic compound [3].

The more conventional R-PE extraction method is based on a solid-liquid extraction, in sodium phosphate buffer, from a seaweed powder obtained after freeze-drying and grinding in liquid nitrogen. Such a process is very time-consuming and expensive, making it difficult to upscale, thus extraction alternatives are welcomed. Among these, enzymatic hydrolysis appears promising to extract R-PE [7] as well as other valuable components [1,8]. However, regarding the enzymatic extraction of R-PE, despite promising data on *Palmaria palmata* [7],

no successful results have been obtained on *G. turuturu* [9]. According to this study, the use of combined polysaccharidases did not improve R-PE extraction, but was useful for the extraction of oligosaccharides [9].

In the last decade, the use of ultrasound (US) for extracting natural products from different vegetable biomasses has been validated and increasingly applied. This method is usually named ultrasonic/ultrasound-assisted extraction (UAE), and is already a proven technology for large-scale extraction [10]. However, only very few studies, most of them recent, have been conducted on seaweeds [11–13]. According to Wang et al. [13], the use of US enhanced the extraction efficiency of taurine from the red seaweed *Porphyra yezoensis*, compared to the conventional solid-liquid procedure. They also noticed that the UAE process required a shorter extraction time and lower operating temperatures, which is interesting regarding the weak stability of R-PE [4]. Ultrasound has already been used to extract phycobilin but from microalgae; Benavides and Rito-Palomares [14] reported that the extraction of B-phycoerythrin was improved by sonication, due to a better disruption of microalgal cells.

In recent years, growing number of studies have focused on the simultaneous combination of enzymes and sonication in plants, which was notably developed to enhance the extraction and hydrolysis of polysaccharides [15–18]. It appears that ultrasound irradiation can act as a tool of hydrolysis intensification, sometimes with synergistic effects between the enzymes and US leading to a lower enzyme consumption [16,17]. The mechanism of this positive interaction, ultrasound-enzymes, is not well understood, although it could be due to an increase in the mass transfer, through the implosion of cavitation bubbles, enhancing the accessibility of the substrate to the enzyme [16,18]. Moreover, the ultrasound might act by the induction of structural transformations which may affect the active site (secondary structure). The

ultrasound irradiation might confer more stability to the enzyme and they might modify the affinity between the enzyme and the substrate [19].

This relatively new process is known as ultrasound/ultrasonic-assisted enzymatic hydrolysis (UAEH) or ultrasound/ultrasonic-assisted enzymatic extraction (UAEE).

On seaweeds, a study demonstrated the value of UAEH in speeding up enzymatic hydrolysis (12-fold) [20]. Recently, Korzen et al. [21] highlighted the interest of ultrasound to produce bioethanol from the macroalgae *Ulva rigida*, using an ultrasound-assisted saccharification and fermentation process. As seaweed cell walls are mainly composed of polysaccharides, such a process could be very helpful in improving access to valuable compounds such as R-PE.

To the best of our knowledge, no study has been carried out on the extraction of R-PE under sonication (UAE), or with the simultaneous combination of enzymes and ultrasound (UAEH) on seaweed.

2. Materials and methods

2.1 Materials

Seaweed, *G. turuturu*, was harvested on 24th May 2013, in the intertidal zone of Batz-sur-Mer on the Atlantic coast, France. Epiphytes were removed by hand and algae were partially dewatered with a spin-dryer, then vacuum-packed (Boulanger INV 40) and immediately frozen. The algae were stored at -20 °C in darkness. Four industrial carbohydrases were used and combined according to their similar pH and temperature optima and their complementarity (Table 1). Regarding our target, all these enzymes do not work at temperatures higher than 40 °C. The enzymatic cocktail was thus composed of Sumizyme TG and Sumizyme MC produced by SHIN NIHON CHEMICAL and kindly provided by Takabio

100 (Beaucouzé, France); Multifect® CX 15 L kindly provided by DSM; and Ultraflo® XL kindly
101 provided by Novozymes®.

102 **Table 1.** Enzymatic cocktail composition: activities, pH and temperature ranges. Minimum
103 (Min), maximum (Max) and optimal (Opt) values are given.

	Activities		**pH**			**Temperature (°C)**		
	Primary	***Secondary***	***Min***	***Max***	***Opt***	***Min***	***Max***	***Opt***
Sumizyme TG	ß-1,3-glucanase Botrytis glucanase		3.5	8	4	40	50	50
Sumizyme MC	Polygalacturonase	Protease Amylase	5	6	5	40	45	45
Multifect® CX 15L	Cellulase ß glucosidase		4	6	5	35	65	55
Ultraflo® XL	ß glucanase (endo-1,3(4-))	Xylanase α amylase	Nd*	Nd*	6	40	65	Nd*

* Non-defined values

104

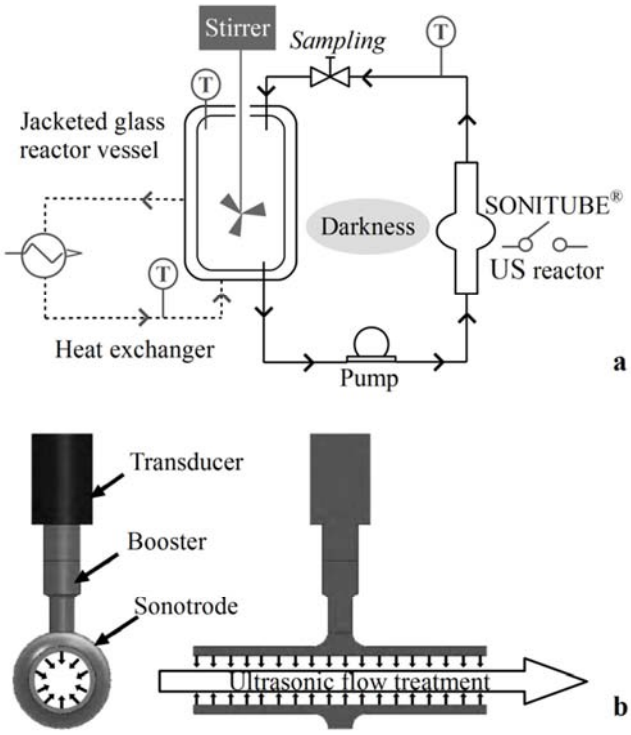
105 ## *2.2 R-PE temperature stability*

106 A portion of the seaweed was freeze-dried and ground in liquid nitrogen to give an algal
107 powder, stored at -20 °C in darkness. Following the conventional R-PE extraction method, the
108 resulting powder was suspended in tap water, with a 1/20 ratio (w/v) for 20 min at 4 °C; then
109 the suspension was centrifuged (25,000 g, 20 min, 4 °C). The soluble phase, called the water
110 extract, was maintained in darkness at 4 °C, 25 °C, 30 °C or 40 °C. The R-PE concentrations
111 ([R-PE]) were monitored over 6 hours.

2.3 Extraction methods

A portion of the seaweed was cut into small pieces (about 5 - 7 mm^2) using a cutting mill (Microcut Stephan MC 15). These were subsequently stored at -20 °C. All the experiments were performed in a jacketed glass reactor vessel (5 L) containing around 3 kg of reaction mixture, composed of 20 % wet and cut seaweed homogenized in tap water (corresponding to the minimal water quantity to obtain an effective circulation of the reaction mixture, with the pump, in our conditions) with the pH adjusted to 5.5 by addition of 6 M HCl (Radiometer analytical TitraLab® 854). Homogenization was conducted continuously, at 100 rpm (Stuart® Overhead Stirrer SS20), and the reaction mixture was circulated using a peristaltic pump (Leroy® Somer) at a flow rate of 50 $L.h^{-1}$. An external circulation system (Hitema® ESE 010 and Memmert) was used to control and adjust the temperature (22 ± 1 °C or 40 ± 1 °C) in the reactor during the 6 hours of the process. To ensure R-PE preservation, the whole system was kept in darkness (Figure 1a).

Regular sampling (± 30 mL) was carried out throughout the experiment. Samples were immediately centrifuged (15,500 g, 30 min, 20 °C, Beckman Coulter Avanti® J-E Centrifuge) providing supernatant and sludge fractions that were weighed and then freeze-dried. The temperature was regulated (22 ± 1 °C or 40 ± 1 °C) and pH was monitored inside the reactor during the whole experiment.



130 *(single column)*

131 **Figure 1.** a: Diagram of the extraction system. b: Schematic illustration of the SONITUBE®,

132 an ultrasonic flow-through reactor (SYNETUDE, France)

133 ***2.3.1 Ultrasound-assisted extraction (UAE)***

134 The reaction mixture was sonicated for 6 hours using an ultrasonic flow-through reactor

135 (SONITUBE® 35 kHz, 200 to 400 W), manufactured and kindly provided by SYNETUDE

136 (Chambéry, France) (Figure 1b). At the amplitude of 100 %, the power delivered during the

137 experiments in the reaction mixture varied between 300 W and 340 W. No enzyme was added

138 during these experiments.

139 ***2.3.2 Ultrasound-assisted enzymatic hydrolysis (UAEH)***

140 The UAEH process is a combination of enzymatic hydrolysis (EAE) and the UAE extraction

141 method. The UAEH was initiated by the addition of the enzymatic cocktail and the

simultaneous application of US. After preliminary tests (data not shown), 1 % w/w of each enzyme related to the weight of wet seaweed was added, corresponding to a concentration of 0.2 % w/w of each enzyme in the reaction mixture. Experiments were monitored during 360 min.

2.4 Analyses

2.4.1 Determination of seaweed liquefaction

For all experiments, the liquefaction of the material was calculated over time. The proportion of soluble material was obtained by calculating the ratio between the weight of the freeze-dried supernatant (m_1) and the weight of the freeze-dried supernatant (m_1) added to the weight of the sludge (m_2), expressed in percentage, according to Equation (1):

$$\text{Solubilized material} = m_1/(m_2 + m_1) \times 100 \qquad \text{Eq. (1)}$$

Thus, for each time, the gain in liquefaction was calculated as the proportion of soluble material without the proportion of soluble material at the beginning of the process.

2.4.2 R-phycoerythrin (R-PE)

Absorption spectra were monitored from 200 to 800 nm, using a UV-VIS spectrophotometer (Shimadzu UV-1800). R-PE concentrations were determined spectrometrically, using the Beer and Eshel [22] equation (Equation (2)), where A_{565} , A_{592} , A_{455} and A_{492} are the absorbances at 565 nm, 592 nm, 455 nm and 492 nm:

$$[\text{R-PE}] = [(A_{565} - A_{592}) - (A_{455} - A_{492}) \times 0.20] \times 0.12 \qquad \text{Eq. (2)}$$

R-PE extraction yield was expressed as $mg.g^{-1}$ seaweed dried weight (dw).

2.4.3 Soluble carbohydrates

The water-soluble carbohydrates were analyzed using a phenol-sulfuric acid method. Glucose was used as a standard (range from to 15 to 150 $mg.L^{-1}$). Absorbance was measured at 490 nm (Shimadzu UV-1800, UV-VIS Spectrophotometer) [23]. The extraction yield of soluble carbohydrates was expressed as $mg.g^{-1}$ seaweed dried weight (dw).

2.4.4 Elemental composition: carbon and nitrogen

The elemental C and N composition was determined on dehydrated samples (algal powder and freeze-dried supernatants), weighed (1.5-5 mg) and placed in small tin capsules that were carbonized by flash combustion at 1,800 °C. The C and N contents were oxidized and converted into a gaseous form, at 950 °C in a combustion column and at 750 °C in a reduction column. The gases formed were transferred by carrier gas (helium) and analyzed by gas chromatography (FLASH 2000 NC Organic Elemental Analyzer - Thermoscientific). The results were integrated using the Eager Xperience for Flash software. Carbon and nitrogen extraction yields were expressed as a percentage of the initial carbon and nitrogen seaweed content (%).

2.4.5 Statistics

All the extractions were carried out in three independent replicates (n=3). Means and standard deviations (SD) are given for three independent experiments. Analyses were performed using the software Sigmastat 3.1. Multiple comparison tests were carried out using the Holm-Sidak test following the ANOVA procedure ($p < 0.05$).

3. Results and Discussion

3.1 R-PE temperature stability

The R-PE stability according to temperature is depicted in Figure 2a as the percentage of preserved R-PE over time.

Whatever the processing temperature, the level of preserved R-PE decreased with time without any plateau, even after 6 hours. The shape of the curves was similar with the highest denaturation occurring during the first 30 min followed by a slower one. However, a temperature impact was clearly noticeable. Indeed, after 30 min, at 4 °C, 94 % of R-PE was preserved but only 61 % at 40 °C while intermediate values were found at 25 °C and 30 °C (92 % and 86 %, respectively). After two hours, 91, 86, 76 and 35 % of preserved R-PE was quantified at 4, 25, 30 and 40 °C, respectively, while at the end of the experiments (6 hours), the level of preserved R-PE was 86, 79, 63 and 26 %. These results clearly illustrate a negative influence of temperature on the preservation of R-PE. An increase of 10 °C (30 to 40 °C) led to a large reduction in the amount of preserved R-PE after 6 hours, from 63 to 26 %.

A recent study demonstrated that R-PE was stable for up to 60 min at 40 °C while a consequent denaturation occurred at 60 °C [4]. The lower stability observed here (45 % of preserved R-PE after 1 hour at 40 °C) could be due to temperature but also to pH as this influences thermal stability [24].

As depicted in Figure 2b representing the absorption spectra, a noticeable effect of temperature was observed after 360 min for the three main R-PE characteristic peaks (498 nm, 540 nm and 565 nm), with a regular decrease in absorbance according to the temperature

increase. However, among these three, the peak at 498 nm demonstrated a greater stability toward temperature than the 540 and 565 nm peaks, corresponding to the chromophores phycourobilins (PUB) and phycoerythrobilins (PEB), respectively [5]. This is in accordance with previous studies dealing with the thermal stability of bilins [4,25].

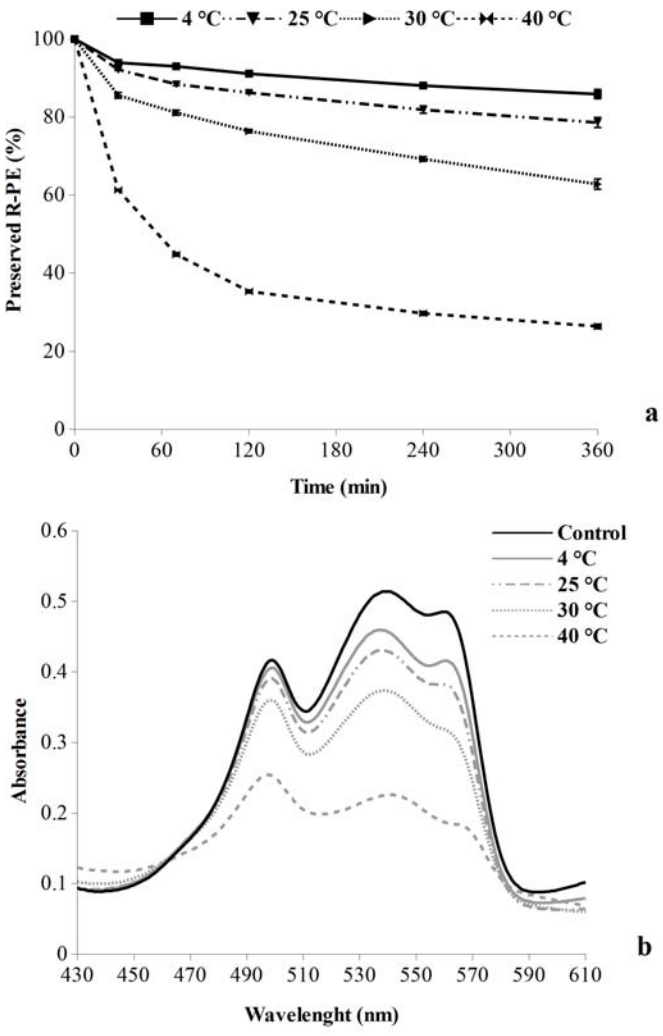


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Figure 2. a: Percentage of preserved R-PE over time (360 min) for the temperatures: 4 °C, 25 °C, 30 °C and 40 °C. Values are means ± SD from three independent experiments (n=3). b: Absorption spectra of R-PE extracts after 360 min at 4 °C, 25 °C, 30 °C and 40 °C; the extract at T0 was labeled Control.

3.2 Seaweed liquefaction

Whatever the process or the temperature, the rate of liquefaction increased with time (Figure 3a), which confirms that ultrasound can reduce the hydrolysis processing time [15].

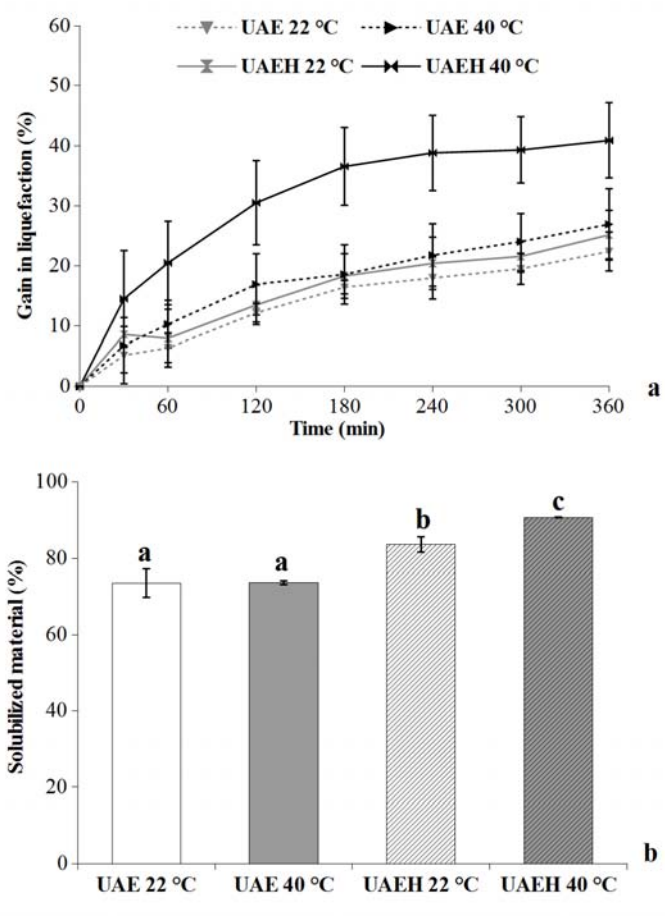
However, some discrepancies were found, as the gain in seaweed liquefaction over time at UAEH 40 °C was much higher than that observed with other treatments. In fact, a comparison of the kinetics for UAE 22 °C, UAE 40 °C and UAEH 22 °C revealed that the gain in liquefaction seemed to be similar over time, with the same regular and slight increase. Nevertheless, when the combined process (UAEH) was applied at 40 °C, the level of liquefaction increased rapidly during the first 3 hours, moderately for one more hour, and was then followed by a stationary phase until the end of the experiment (40 ± 1 %).

After 6 hours of treatment, no statistical differences were observed for UAE processes, as the final soluble contents were found identical (74 ± 4 % at 22 °C and 74 ± 0.5 % at 40 °C) (Figure 3b). Thus, in these conditions, seaweed liquefaction by sonication was not influenced by temperature. However, a recent study on grape marc demonstrated that the extraction yields under sonication were correlated to temperature (from 20 to 50 °C) but that too high temperatures could have a negative effect on the ultrasonic cavitation intensity due to the increase in vapor pressure [26].

In contrast, a clear impact of temperature was noticed when enzymes were used associated with sonication (UAEH). For example, while 83.6 ± 1.9 % of solubilized material was found at 22 °C, up to 90.7 ± 0.1 % of soluble compounds were recovered at 40 °C. This could be explained by the fact that 40 °C is closer to the optimal temperature of the enzymes used, thus leading to a better enzymatic efficiency. Moreover, some studies have demonstrated that the activation energy (temperature) of enzymes can be lowered in the presence of ultrasound, due

236 to their different relationship to pH and temperature parameters [27,28], although some
237 contradictory studies have revealed that the enzymes' optimal temperature does not change,
238 despite ultrasonic stimulation [19,29].

239 Whatever the temperature, the addition of enzymes had a positive impact on the recovery of
240 soluble materials after 6 hours of treatment ($p < 0.05$). Recently, some research has been
241 carried out in order to understand how the combination of enzymes and ultrasonic waves can
242 improve extraction efficiency [15,18,30]. It has been demonstrated that, under sonication,
243 enzymatic activity could increase [28] due to some structural transformations (secondary
244 structure) of the active site, leading to an improved enzyme stability [19]. In contrast, it has
245 also been shown that US can reduce the specific activity of commercial enzymes, notably
246 cellulase; however, the resulting activity remained higher under sonication as the increased
247 mass transfer between enzymes and substrate could overcome this direct adverse effect on the
248 enzymes [31]. Nevertheless, it seems clear that each case is individual, depending on the type
249 of enzyme and the parameters of sonication [31,32].



250 *(single column)*

251 **Figure 3.** a: Evolution of the gain in seaweed liquefaction for each process (ultrasound-
252 assisted extraction (UAE) and ultrasound-assisted enzymatic hydrolysis (UAEH)) during 360
253 minutes at 40 °C and 22 °C. Values are means ± SD from three independent experiments
254 (n=3). b: Percentage of solubilized material at 360 min for ultrasound-assisted extraction
255 (UAE) and ultrasound-assisted enzymatic hydrolysis (UAEH), at 40 °C and 22 °C. Values are
256 means ± SD from three independent experiments (n=3). Significant differences ($p < 0.05$) are
257 indicated by different letters.

3.3 Biochemical composition of soluble fractions

3.3.1 R-phycoerythrin content

The R-PE extraction yields measured over time are presented in Figure 4, in which some kinetic differences are observed.

Whatever the treatment, during the first hour, two trends emerged: an increase in R-PE extraction yields at 22 °C and, conversely, a decrease at 40 °C. Such a reduction is consistent with the denaturation previously observed at 40 °C (***3.1***).

However, after these initial 60 min, different R-PE extraction kinetics were noticed: a regular reduction for UAE 22 °C and 40 °C throughout the processing time, a stagnation during one more hour followed by a decrease for UAEH 22 °C and a relative stability for UAEH 40 °C (1.81 ± 0.01 mg.g^{-1} dw). After 6 hours, UAE at 40 °C seemed to be the most denaturing treatment but the final amount of preserved R-PE extracted (39 ± 3 %) remained higher than that obtained by classic tap-water extraction (26 ± 0.6 %) (Figure 2a).

Regarding the extraction efficiency of R-PE from *G. turuturu* of the French Atlantic coast, some comparisons with previous studies can be made. With the conventional extraction method (in sodium phosphate buffer), yields varied between 1.2 and 4.4 mg.g^{-1} dw [2,6]. However, according to Figure 4, after 2 hours of extraction at 22 °C with UAEH or UAE, they reached 3.6 ± 0.3 mg.g^{-1} and 3.1 ± 0.1 mg.g^{-1} dw, respectively, which is in the range of the conventional solid-liquid extraction.

Whatever the temperature, the addition of enzymes always led to higher extraction rates ($p<0.05$). At the end of the processes (360 min), no significant differences were noticed between UAE 22 °C and UAEH 22 °C while at 40 °C, the R-PE yield with UAEH was higher

than with UAE, which could be associated with the positive action of enzymes at 40 °C, as previously mentioned.

Nevertheless, 22 °C is preferable as at this temperature, the thermal denaturation of R-PE is limited. This is in accordance with a previous study on the red seaweed *Palmaria palmata,* which demonstrated that the extraction of R-PE, by enzymatic hydrolysis, was improved by reducing the temperature to 25 °C [7].

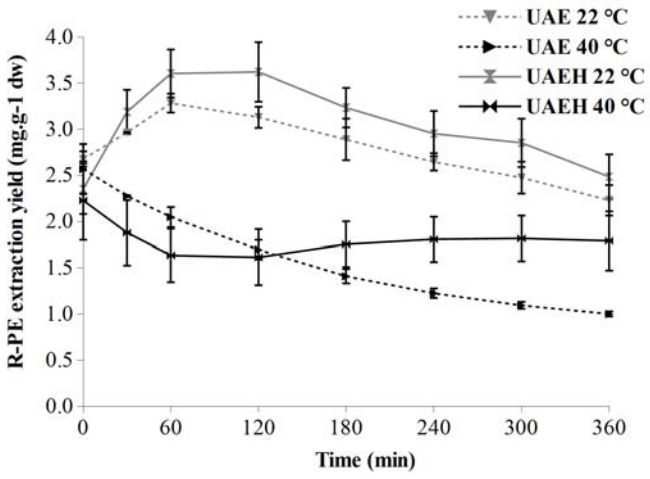
Based on these results, some assumptions can be formulated: **1**- R-PE would be more stable toward temperature in our soluble phase than in a tap-water extract due to the presence of other co-extracted compounds, such as polysaccharides or oligosaccharides (see below, Figure 5a); **2**- the extraction yield of R-PE was the difference between extraction and denaturation over time; **3**- soluble R-PE could be more sensitive to sonication than non-extracted R-PE, leading to its denaturation over time. Indeed, a recent study on the UAE of water-soluble pigments from *Bougainvillea glabra* flowers found that temperature was more influential than cavitation on the extraction of pigments, as they are temperature-sensitive [33]. Thus, temperature could have both positive and negative effects: it increased the solubility of solids (including pigments) from the biomass (Figure 3b) but, beyond a certain temperature, ultrasonic cavitation could be altered and pigments damaged by thermal denaturation.

In accordance with our assumption 3, it is possible that too long an exposure of pigments to ultrasonic waves, even at 22 °C, induced their structural destruction leading to a lower yield [33]. For example, at 22 °C, the extension of the UAEH treatment from one to six hours led to the denaturation of 31 % of extracted R-PE.

It is important to keep in mind that sensitivity toward temperature and ultrasonic waves, depends on the type of molecules and the experimental parameters. As previously mentioned

303 by Roselló-Soto et al. [34], the UAE technique should be carefully used in the extraction of
304 unstable compounds (like carotenoids and chlorophylls) and the conditions optimized
305 accordingly.

306 In this study, one or two hours of ultrasonic treatment of ***G. turuturu*** with or without enzymes
307 at 22 °C appeared the most efficient to extract R-PE like the classic procedure. Furthermore,
308 with this new process, the steps of freeze-drying and grinding in liquid nitrogen were avoided,
309 saving time and energy. To an economical and trans positional point of view, and according to
310 the ultrasonic reactor used there, such process could be used with volume up to 50 L
311 moreover it could be upscaled to up to 200 or 300 L while with using the same technology
312 and powered by a SONITUBE® 20 kHz. As mentioned in the study of Denis et al. [35], a
313 membrane process dealing with the recovery of around 30 $L.day^{-1}$ with a cost of 1 $€.L^{-1}$ of pre
314 purified fraction of 0.245 g of $R\text{-}PE.L^{-1}$. Here, with a 3 L reaction mixture it's up to 0.306 g of
315 R-PE that could be extracted thus equivalent to 0.102 g of $R\text{-}PE.L^{-1}$ without any purification.



316 *(1.5 column)*

317 **Figure 4.** Evolution of the R-PE extraction yield for each process (ultrasound-assisted
318 extraction (UAE) and ultrasound-assisted enzymatic hydrolysis (UAEH)) during 360 minutes
319 at 40 °C and 22 °C. Values are means ± SD from three independent experiments (n=3).

3.3.2 Carbohydrate, carbon and nitrogen contents

In order to qualify the dry matter content, some biochemical analyses were carried out on the resulting soluble fractions. To ensure the accuracy of our values, enzymatic cocktail carbohydrates were estimated at 427.43 mg of carbohydrate per g of enzymatic cocktail. This is in accordance with data provided by the suppliers as: Sumizyme TG contains 50 % dextrins, Sumizyme MC is composed of 79 % maltodextrins and Ultraflo® XL by 30 % glycerol and 20 % sorbitol. No data were communicated for Multifect® CX 15L. In order to avoid an overestimation, these amounts were compared to dried seaweed, giving an overall value of 127.41 mg of carbohydrate per g of dried seaweed. This was removed from the biochemical composition of soluble fractions for UAEH processes. The biochemical compositions of the resulting fractions obtained after 6 hours of treatment are presented in Figure 5.

The comparison of soluble carbohydrates extracted by sonication (UAE) and ultrasound-assisted enzymatic hydrolysis (UAEH) demonstrated that the temperature did not affect significantly the extraction of carbohydrates by sonication alone (Figure 5a). However, at 40 °C, the combined process (UAEH) increased significantly the carbohydrate release (439 ± 16 $mg.g^{-1}$ dw) compared to sonication alone (296 ± 37 $mg.g^{-1}$ dw).

Thus, as previously noticed, the simultaneous combination of ultrasound and enzymes may have improved the process efficiency, leading to higher levels of polysaccharide extraction and hydrolysis (fermentable sugars) [15,16,18,30]. In our conditions, an increase in the temperature for the UAEH process significantly enhanced the extraction of soluble carbohydrates from 210 to 439 $mg.g^{-1}$ dw, which is in accordance with previous work [17]. A relationship can be made between the extraction of carbohydrates and the results of the R-PE

343 extraction yield (Figure 4). For example, as mentioned in assumption 1 (***3.3.1***), it is possible
344 that soluble carbohydrates contribute to R-PE preservation, as it seems that the presence of
345 uncharged glucan could prevent the thermal degradation of R-PE [25]. At least some side
346 effects of the released polysaccharides, such as antioxidant properties, could also contribute to
347 this preservation [17,36].

348 Regarding carbon and nitrogen, whatever the temperature, they were better extracted by
349 UAEH treatments compared to UAE ones (Figure 5b and c). However, a positive temperature
350 effect was only noticeable for carbon with UAEH as 83 % of the initial carbon was extracted
351 at 22 °C and 92 % at 40 °C. For nitrogen, no statistical differences were observed between
352 UAEH at 22 °C and 40 °C (Figure 5c).

353 All these results clearly demonstrate an enzymatic effect as the polysaccharidases, with an
354 optimal temperature close to 40 °C (Table 1), improved the extraction of carbohydrates and
355 carbon components at 40 °C rather than nitrogen components. Differences between
356 carbohydrates and carbon, for UAEH 22 °C, could be explained by the presence of carbon in
357 both proteins and carbohydrates.

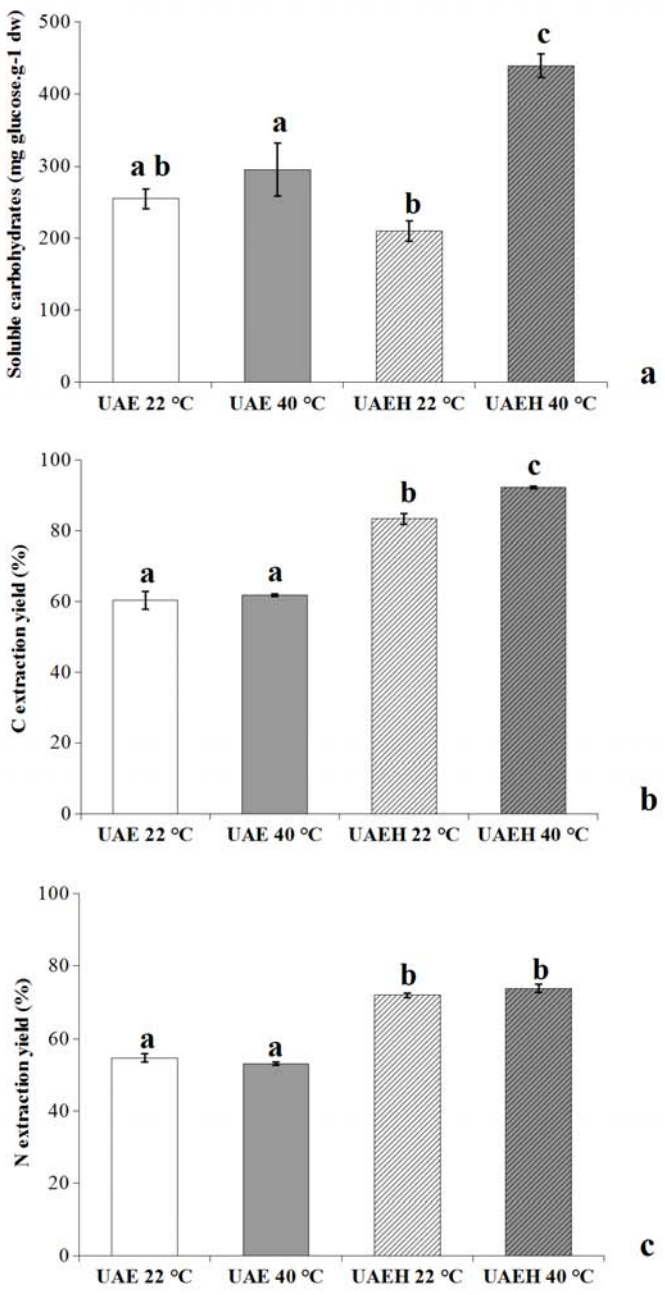


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Figure 5. Biochemical composition of soluble fractions at 360 min for ultrasound-assisted extraction (UAE) and ultrasound-assisted enzymatic hydrolysis (UAEH) at 40 °C and 22 °C. a: Carbohydrates, b: Carbon and c: Nitrogen. Values are means ± SD from three independent experiments (n=3). Significant differences ($p < 0.05$) are indicated by different letters.

4. Conclusions

These results highlight some differences between R-PE extraction and seaweed liquefaction. For liquefaction, the UAEH process at 40 °C for 6 hours (at least 4 hours) appeared to be the best condition with up to 91 % of solubilized material. For R-PE, both UAE and UAEH at 22 °C (60-120 min) were suitable, with extraction yields (around 3.6 $mg.g^{-1}$ dw for UAEH) close to conventional solid-liquid extraction. However, UAEH 22 °C also demonstrated a greater extraction of other water-soluble compounds. Thus, further work is needed in order to find the best compromise for R-PE extraction and *Grateloupia turuturu* liquefaction.

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