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Optimisation of hydrolysis conditions for extraction of R-phycoerythrin from *Gracilaria gracilis* by enzyme polysaccharidase and response surface methodology

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Abstract

R-phycoerythrin has been widely used in food, cosmetic, pharmaceutical, and analytical reagent industries. The present work reports an effective procedure for the extraction of R-phycoerythrin from *Gracilaria gracilis*. Firstly, the algae pre-treatment is a significant stage, and the highly effective extraction of R-phycoerythrin from *G. gracilis* was carried out with freeze-dried seaweed and grinding with liquid nitrogen. Secondly, two different methods for the extraction of this pigment were tested: (i) by using the extraction solutions (tap water, pure water, phosphate buffer 100 mM with pH 6.5, and phosphate buffer 20 mM with pH 7.1); and (ii) by using polysaccharide-degrading enzymes (xylanase, cellulase, and β-glucanase). Cellulase enhanced the extraction yield of R-phycoerythrin from *G. gracilis* (12.76 mg g^{-1} dw). Finally, under the optimal hydrolysis conditions obtained with cellulase according to a response surface methodology study of temperature (32°C), hydrolysis time (286 min), and enzyme/dry seaweed ratio (47 mg g^{-1}), maximum yield of R-phycoerythrin (15.75 mg g^{-1} dw) was obtained.

Introduction

Gracilaria are a red macroalgae species, and considered economically valuable resources for food and cosmetic industries, as well as in biotechnological applications (Capo *et al.,* 1999). *Gracilaria* species are a major source of agar, which is an important material for the food industry. In addition, approximately 60% of all agars are produced from this genus (Santelices and Doty, 1989). *Gracilaria* species are farmed on a large scale in several countries including China, Taiwan, Chile, and Vietnam.

Gracilaria gracilis are a marine macroalgae which has been indicated as an inconvertible material in several exciting applications. For instances, the agar polymers were used as mesoporous material and sacrificial bio-templates for nanoparticles production (Francavilla *et al.,* 2013a; 2014); the phycobiliproteins were studied and used as natural colorants for foods and cosmetics, and particularly in conducting medical diagnoses; or for producing proteins in foods and feeds (Francavilla *et al.,* 2013b). Nevertheless, *Gracilaria* is not currently utilised for its phycoerythrin content. Since G. gracilis is one of the only four red seaweeds authorised for human consumption in Europe, this species could be a promising source of R-phycoerythrin (Francavilla *et al.,* 2013b).

R-phycoerythrin (R-PE) is an oligomeric protein of 240 kDa, with 6 α (about 16 kDa), 6 β (about 21 kDa), and 1 γ (about 39 kDa) subunits (Senthilkumar *et al.,* 2013). R-PE has been applied in immunology and cell biology, as well as in flow cytometry. The main applications of this oligomeric protein are as the fluorescent markers of cells, and as natural colorants in foods and cosmetics (Dumay *et al.,* 2014). The product of this pigment has a high price. For example, the cost of R-PE lyophilised powder from *Porphyra tenera* (commonly known as "Nori") is €408/mg. Thus, finding new seaweed sources has become a challenge for human application usage, as well as in an economical point of view.

Extraction is an important preliminary step in separating different types of bioactive compounds from seaweeds. Nevertheless, its efficiency is reduced and depended on complex cell wall polysaccharides such as cellulose, hemicellulose, agars, alginates, and carrageenan. Therefore, different extraction methods have been studied and employed to enhance and optimise the extraction efficiency of bioactive compounds (Wijesinghe and Jeon, 2012).

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The classic R-phycoerythrin extraction method is based on maceration in water, distilled water (Fleurence and Guyader, 1995; Denis *et al.,* 2009a), or sodium phosphate buffer (Nguyen *et al.,* 2017). According to several recent studies, enzymatic hydrolysis was used to extract R-PE, proteins (Dumay *et al.,* 2013), and other valuable compounds (Wijesinghe and Jeon, 2012). In addition, they provided effective extraction yield improvement. The red seaweed cell walls are mainly composed of polysaccharides, which are the reason why several polysaccharidases were utilised in order to improve access without denaturation of the valuable proteinaceous compounds such as R-PE (Denis *et al.,* 2009a).

Therefore, it is clear that enzymatic hydrolysis should be individually considered for each species (Denis *et al.,* 2009b). Response surface methodology (RSM) is a combination of mathematical and statistical techniques used in an empirical study of relationships and optimisation, in which several independent variables influence a dependent variable or response (Alauddin *et al.,* 1997). By optimising a process, its production costs can be reduced (Sangkharak and Prasertsan, 2007). Optimisation of the hydrolysis conditions for the extraction of proteins and R-PE from marine macroalgae by enzyme has already been researched, and demonstrated real benefits (Guerard *et al.,* 2007; Nguyen et al., 2017).

In the present work, different methods to extract R-PE from dry and wet *G. gracilis* were screened to establish the most efficacious extractant. Various glycosidases were tested on dry and wet conditions, while focusing on the extraction yield improvement of R-PE from *G. gracilis*. Key parameters such as hydrolysis time, temperature, and enzyme/dry seaweed ratio were also studied and optimised using the RSM. The aim of the present work was to obtain an effective method for the extraction of R-PE from *G. gracilis*.

Materials and methods

Material

Red seaweed, *G. gracilis* was collected at the Atlantic coast, France. Firstly, the algae were washed, and the epiphytes were removed in seawater, tap water, and distilled water. Secondly, they were immediately stored at -20°C in the dark until further use. Next, one part of the frozen algae was defrosted and then cut into small pieces (≈1 cm; wet algae). Another part of the frozen algae was freeze-dried, and homogenised in liquid nitrogen (dry algae). Dry weight of the biomass was determined at 105°C after 12 h. The dry weight corresponded to 19.19% of the wet algae.

Digestion was performed using three polysaccharidases from *Trichoderma longibrachiatum*, namely cellulase (Ce), xylanase (Xy), and β-glucanase (Glu), which were purchased from Sigma-Aldrich (France).

Extraction procedures for R-phycoerythrin using classic maceration

Wet algae and dry algae were suspended in the buffers. Four types of extraction solutions were investigated: tap water (for its economic and industrial interest), pure water (Denis *et al.,* 2009b), phosphate buffer 100 mM with pH 6.5 (Nguyen *et al.,* 2017), and phosphate buffer 20 mM with pH 7.1 (Denis *et al.,* 2009a). The extraction was performed with a 1/20 ratio (w/v) for 20 min at 4°C, then the suspension was centrifuged (25,000 *g*, 20 min, 4°C) (Nguyen *et al.,* 2017). The supernatant or crude extract (CE) was stored until further use. For each condition, triplicate extractions were simultaneously carried out, and independently repeated at least three times.

Extraction procedures for R-phycoerythrin using enzymatic hydrolysis

Enzymatic hydrolysis experiments were performed by using a 500 mL glass reactor under controlled conditions (temperature and stirring speed), in the dark. Wet algae and dry algae were homogenised with 200 mL acetate buffer 50 mM with pH 5. The enzyme was added to the mixture with 16.5 mg g^{-1} dw of enzyme, and the system was continuously stirred at 150 rpm during the 6-h hydrolysis (Dumay *et al.,* 2013).

All experiments were conducted at 35°C (compatible temperature with the activity of the enzymes). Triplicate digestions were fulfilled, and a control (sample without enzyme) was simultaneously performed under similar conditions (Dumay *et al.,* 2013).

After hydrolysis, the samples were centrifuged at 25,000 *g* for 20 min at 4°C. Next, the CE were stored until analyses of total sugar, water-soluble protein contents, and purity of R-PE.

R-phycoerythrin determination

R-PE yield and purity were determined spectrophotometrically using the classic Beer and Eshel equation (Eq. 1), and the A_{565}/A_{280} ratio (Purity Index or PI), respectively (Dumay *et al.,* 2013). R-PE concentration was expressed as mg g^{-1} dw (dry algae).

$$[\text{R-PE}] = [(A_{565} - A_{592}) - (A_{455} - A_{592}) \times 0.20] \times 0.12 \quad \text{(Eq. 1)}$$

The R-PE absorption spectra displayed three peaks: one main peak at 565 nm and two peaks at 495 and 545 nm.

Water-soluble proteins

The total water-soluble proteins in the CE were analysed using the method adapted from Bradford. Bradford reagent (Sigma; 200 µL) was added to 800 µL of sample solution. The absorbance measurement at 595 nm (read immediately after the reaction) and the use of BSA (Sigma) as a standard (from 0 to 100 mg/L) enabled the protein content to be determined (Dumay *et al.,* 2013).

Water-soluble sugars

Water-soluble sugars were assayed in the CE by using the modified colorimetric phenol-sulphuric acid method. Phenol 5% (200 µL) was added to 200 µL of sample solution or glucose solution, followed by 1 mL of concentrated sulphuric acid. The tubes were allowed to stand for 10 min at room temperature before vortexing (10 s at 3000 rpm), then for 15 min at room temperature, and 30 min at 35°C before absorbance was measured at 490 nm. In each assay, glucose was added as a standard (from 0 to 100 mg/L) (Dumay *et al.,* 2013).

Experimental design and evaluation

After the hydrolysis studies, the R-PE content was optimised by the response surface methodology (RSM) procedure which determined the influence of the hydrolysis variables, including hydrolysis time (120 to 360 min), hydrolysis temperature (20 to 50°C), and enzyme-substrate (E/S) ratio (20 to 40 mg g^{-1} of dry algae) on the digestion of *G. gracilis*. A total of 19 experiments were conducted and designed including 2^3 orthogonal factorials, six-star points, and five central points. In the 2^3 central composite designs (CDD), the starting hydrolysis temperature, hydrolysis time, and E/S ratio were systematically varied at high and low levels. Nevertheless, the medium levels of all these three factors studied were used to check the linear relationship between the high and low levels of the variables tested. In this method, the measured variable responses were water-soluble proteins, total sugar, R-PE concentration, and the R-PE Purity Index (PI). The software Statgraphics Plus v.5 Experiment Design was utilised.

For the R-PE extraction by classic maceration as well as the algae pre-treatment, statistical analyses were conducted using Minitab 16 by means of an ANOVA procedure ($p < 0.05$).

Results

Extraction process for the R-phycoerythrin by the maceration buffer

The R-PE extraction concentration, water-soluble protein content, and PI of the crude extract are reported in Table 1.

The results showed that whatever the type of extraction solutions used, the best outcomes for R-PE as well as water-soluble protein yields were obtained with dry algae ($p < 0.05$), where on average, resulted in 3.27 and 4.73 mg g^{-1} dw, respectively. With wet algae, these values were half as much. Similar trend was observed for PI. The achieved value was around 0.028 with wet algae, and 0.108 with dry algae.

The best extraction of R-PE concentration and water-soluble proteins content from *G. gracilis* (3.54 ± 0.09 and 8.12 ± 0.30 mg g^{-1} dw, respectively) was gained from freeze-dried seaweed and grinding by liquid nitrogen to the phosphate buffer 20 mM with pH 7.1.

Table 1. R-PE extraction yield, R-PE Purity Index (PI), and water-soluble protein content of *Gracilaria gracilis* following maceration in different types of extraction solutions.

Seaweed	Extraction solution	R-phycoerythrin yield (mg g^{-1} dw)	R-PE Purity Index (PI)	Water-soluble proteins (mg g^{-1} dw)
Wet algae	Tap water	1.20 ± 0.01*	0.02 ± 0.00	4.33 ± 0.18
	Pure water	1.65 ± 0.01*	0.04 ± 0.00	4.86 ± 0.06*
	Buffer pH 6.5 (100 mM)	1.52 ± 0.00*	0.03 ± 0.00	5.20 ± 0.11*
	Buffer pH 7.1 (20 mM)	1.33 ± 0.00*	0.02 ± 0.00	4.51 ± 0.13
Dry algae	Tap water	3.21 ± 0.08	0.11 ± 0.01	7.75 ± 0.20
	Pure water	3.15 ± 0.07	0.10 ± 0.01	7.25 ± 0.31
	Buffer pH 6.5 (100 mM)	3.17 ± 0.07	0.10 ± 0.01	7.65 ± 0.22
	Buffer pH 7.1 (20 mM)	3.54 ± 0.09*	0.12 ± 0.01	8.12 ± 0.30*

Data are expressed as mean ± standard deviation of three replicates ($n = 3$). * indicates significant difference ($p < 0.05$).

Extraction process for R-phycoerythrin by polysaccharide-degrading enzymes

The algae cell walls are commonly composed of cellulose, hemicellulose, xylans, agar, and carrageenan (Nguyen *et al.,* 2017). The cell wall polysaccharides can limit the efficiency of general extraction procedures of active compounds from seaweed. Hence, the degradation of cell wall polysaccharide structures can be an essential step in the release of these active compounds (Wijesinghe and Jeon, 2012). The action of polysaccharide-degrading enzymes (xylanase, cellulase, and β-glucanase) on the extraction of R-PE from *G. gracilis* was thus investigated.

Water-soluble protein extraction

Water-soluble protein extraction concentration for each treatment is reported in Figure 1A. It is apparent that the enzymatic treatment led to a significant increase of 1.5 to 2 times in quantities of water-soluble protein extracted from all experiments ($p < 0.05$). The results indicated that the pre-treatment of algae had a significant influence on the number of water-soluble proteins extracted. Their contents from wet algae ranged from 4.65 to 5 mg g^{-1} dw for control, and from 9 to 9.17 mg g^{-1} dw for enzymatic treatment; but these yields reached from 14.62 to 15 mg g^{-1} dw for control, and from 22 to 27.53 mg g^{-1} dw for enzymatic treatment with dry algae. The best result was obtained by using cellulase (27.53 mg g^{-1} dw) on dry algae.

R-phycoerythrin extraction

The R-PE extraction yields obtained for each treatment are indicated in Figure 1B. The result showed a significant difference between the controls and enzyme digestion ($p < 0.05$); R-PE concentrations without enzyme ranged from 1.69 mg g^{-1} dw (control, wet algae) to 6.97 mg g^{-1} dw (control, dry algae); whereas with cellulase, they ranged from 4.62 mg g^{-1} dw (cellulase, wet algae) to 12.76 mg g^{-1} dw (cellulase, dry algae). The present work demonstrated the increase in R-PE yield from *G. gracilis* using enzymatic hydrolysis by cellulase. The quantity from dry algae using cellulase (12.76 mg g^{-1} dw) was better than with xylanase (7.92 mg g^{-1} dw) or β-glucanase (9.44 mg g^{-1} dw).

Regarding the controls, the average R-PE concentration from dry algae (4.26 mg g^{-1} dw) were significantly higher than from wet algae (1.56 mg g^{-1} dw) ($p < 0.05$). For all enzymatic hydrolysis experiments, the R-PE values obtained from dry algae were also significantly higher than those obtained from wet algae ($p < 0.05$).

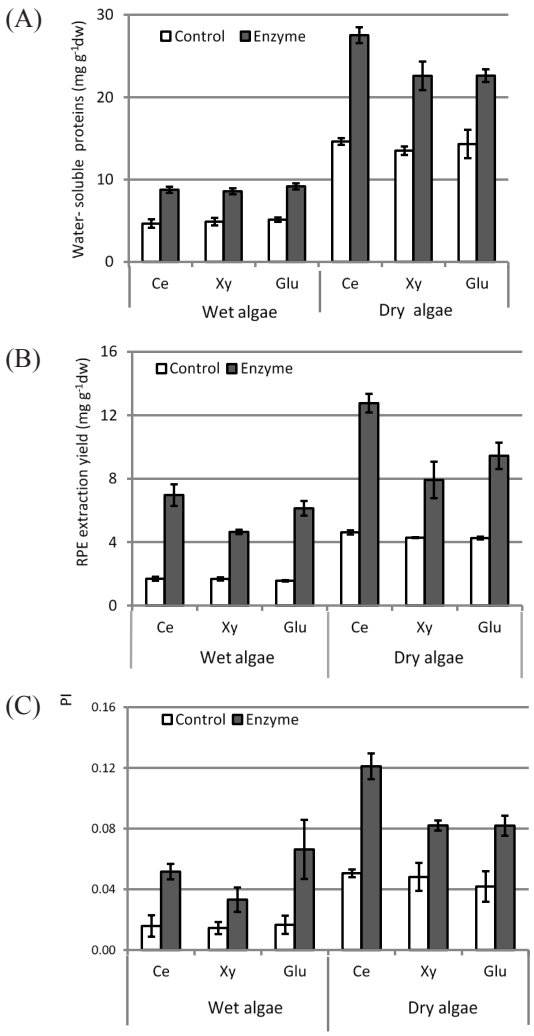

Figure 1. Effect of algae treatment and enzymatic hydrolysis on water-soluble protein (A); R-PE extraction yields (B); and Purity Index (PI) (C) of *Gracilaria gracilis* (n = 3, bars = standard deviation).

The R-phycoerythrin purity index

The R-PE Purity Index (PI) results are presented in Figure 1C. Pre-treatment of algae and enzymatic treatment also led to a significant increase in PI values ($p < 0.05$). For the control samples, PI values obtained were from 0.02 (wet algae) to 0.04 (dry algae). With cellulase, PI increased from 0.05 (wet algae) to 0.12 (dry algae). The present work demonstrated that the enzymatic hydrolysis was clearly suitable for degrading the cell wall of *G. gracilis*, and provided favourable conditions for the extraction of the water-soluble proteins, as well as R-PE. Consequently, in the following experiments, cellulase and dry algae were used to study the factors affecting the extraction.

R-phycoerythrin extraction optimisation using response surface methodology

Experimental design

The experimental design levels of independent variables used in the hydrolysis of *G. gracilis* with cellulase and the responses (R-PE, PI, proteins, and sugars) obtained for each experiment are presented in Table 2.

Concerning sugars, the results showed that they were directly linked to the enzymatic action. The more glycosidic bonds broken and separated, the more sugar appeared in the CE after hydrolysis (Dumay *et al.,* 2013). The sugar contents varied from 14.31 mg g^{-1} dw (Experiment No. 10) to 182.27 mg g^{-1} dw (Experiment No. 8). The highest sugar extraction (182.27 mg g^{-1} dw) was obtained after treatment with the highest concentration of enzyme (46.82 mg g^{-1} dw, Experiment No. 8), while the lowest sugar content was obtained from 38.19 min in Experiment No. 10.

Regarding proteins, their concentrations ranged from 18.15 mg g^{-1} dw (Experiment No. 4) to 29.22 mg g^{-1} dw (Experiment No. 1). The best protein extractions were obtained during Experiment No. 1, 3, 8, 13, and 14, with values ranging from 27.43 to 29.22 mg g^{-1} dw, and corresponding to the hydrolysis conditions with a high enzyme concentration. The lowest protein extraction (18.15 mg g^{-1} dw) was achieved at high temperature (60°C) in Experiment No. 4.

R-PE extraction varied from 6.68 mg g^{-1} dw (Experiment No. 19) to 15.57 mg g^{-1} dw (Experiment No. 1). As for the water-soluble proteins, Experiment No. 1 yielded the highest R-PE concentration at 20°C, 46.82 mg g^{-1} dw of cellulase during 360 min of hydrolysis. Central points also gave interesting R-PE yields (around 12 mg g^{-1} dw). Low R-PE release (approximately 6.68 mg g^{-1} dw, Experiments No. 4, 10, and 19) during hydrolysis was obtained when conditions involved a high hydrolysis temperature (60°C) and a short hydrolysis time (38.19 min).

Finally, regarding the PI responses, values varied from 0.06 (Experiment No. 4) to 0.18 (Experiment No. 1). The lowest PI value (0.06) was obtained during Experiment No. 4, which led to the highest sugar extraction. The PI responses of central points presented no significant differences (around 0.13).

Response surface methodology

After obtaining the responses from the experiments (Table 2), we continued the analysis by the response surface in the investigated experimental design. Considering the responses and the effect of the variable on the estimate, the optimal hydrolysis conditions to extract and obtain R-PE were analysed. Three-dimensional graphs presenting the influence of the parameters on the overall desirability for R-PE concentration are shown in Figure 2. The graphs are convex, meaning that the optimal hydrolysis conditions were determined for the R-PE response with a high desirability of 91.38%. The optimum R-PE extraction concentration obtained was around 15.61 mg g^{-1} dw when the values of independent

Table 2. Hydrolysis conditions and responses for R-PE contents, Purity Index (PI), water-soluble proteins, and total sugars obtained for the central composite design. Numbers in bold correspond to central point.

Experiment No.	Hydrolysis conditions			Responses obtained			
	Temperature (°C)	E/S ratio (mg g^{-1} dw)	Time (min)	R-PE (mg g^{-1} dw)	PI (A_{565}/A_{280})	Proteins (mg g^{-1} dw)	Total sugars (mg g^{-1} dw)
1	20.0	40.0	360.0	15.57	0.18	29.22	117.37
2	20.0	20.0	360.0	11.21	0.14	23.09	106.52
3	**35.0**	**30.0**	**240.0**	**13.28**	**0.1**	**27.58**	**116.48**
4	60.0	30.0	240.0	6.75	0.06	18.15	174.78
5	50.0	40.0	120.0	10.21	0.1	26.39	25.95
6	50.0	20.0	120.0	7.29	0.09	21.96	21.17
7	50.0	40.0	360.0	12.83	0.11	23.67	21.95
8	35.0	46.82	240.0	13.27	0.15	28.04	182.27
9	**35.0**	**30.0**	**240.0**	**12.15**	**0.14**	**25.76**	**40.63**
10	35.0	30.0	38.19	6.80	0.11	23.91	14.31
11	50.0	20.0	360.0	9.48	0.11	20.46	49.34
12	35.0	13.18	240.0	11.40	0.13	24.62	137.62
13	**35.0**	**30.0**	**240.0**	**12.59**	**0.14**	**27.43**	**134.84**
14	**35.0**	**30.0**	**240.0**	**13.65**	**0.14**	**27.94**	**134.13**
15	20.0	40.0	120.0	8.74	0.13	26.30	70.06
16	**35.0**	**30.0**	**240.0**	**12.00**	**0.17**	**25.81**	**121.98**
17	35.0	30.0	441.82	11.41	0.13	24.55	132.28
18	20.0	20.0	120.0	7.27	0.11	22.57	45.07
19	10.0	30.0	240.0	6.68	0.1	24.59	49.91

variables performed at 32°C (temperature), 286 min (hydrolysis time), and 47 mg g^{-1} dw (E/S ratio).

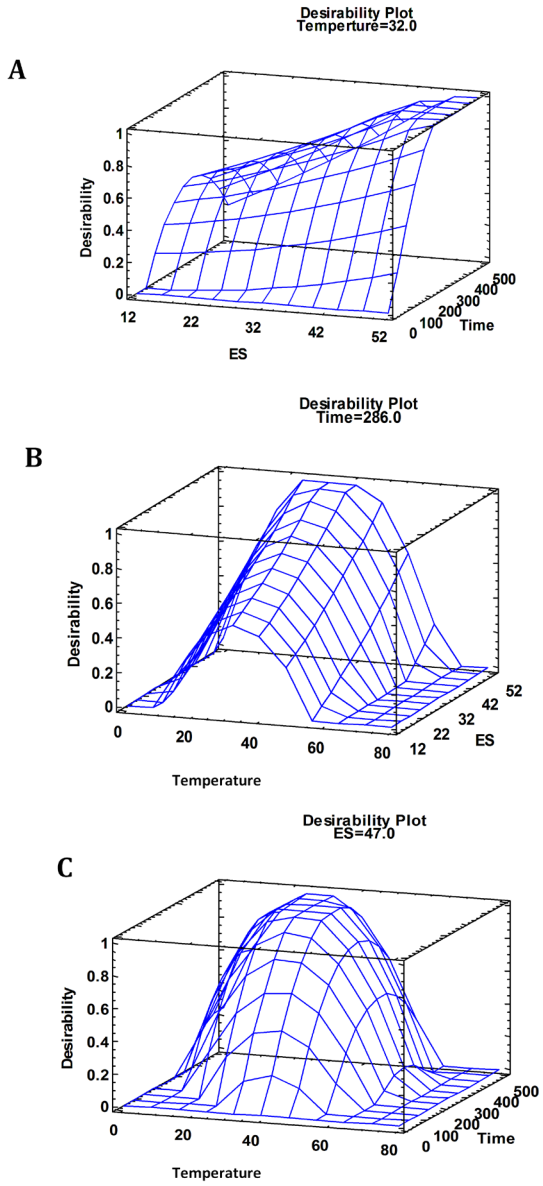

Figure 2. Estimated response surfaces according to hydrolysis time, enzyme, and temperature parameters. (A) = R-PE yield (mg g^{-1} dw) as a function of hydrolysis time and enzyme/substrate ratio. (B) = R-PE yield (mg g^{-1} dw) as a function of temperature and enzyme/substrate ratio. (C) = R-PE yield (mg g^{-1} dw) as a function of temperature and hydrolysis time.

R-phycoerythrin extraction yields after hydrolysis performed under optimised conditions

The results obtained after hydrolysis are reported in Table 3. Under such conditions, for a 286 min hydrolysis at 32°C with an enzyme/substrate ratio of 47 mg g-1 dw, the water-soluble protein concentration reached 29.30 mg g^{-1} dw, which was equal to the highest value obtained in the previous part of the study (29.22 mg g^{-1} dw, Experiment No. 1). The same conclusions can be drawn for the R-PE yields, where the result obtained after optimisation was 15.75 mg g^{-1} dw, which was close to that of Experiment No. 1 (15.57 mg g^{-1} dw). However, the protein and R-PE extraction concentrations of the experiments control without enzyme after optimization (16.51 and 6.72 mg g^{-1} dw, respectively) were lower than these yields in Experiment N0. 1 (19.09 and 8.42 mg g^{-1} dw, respectively). Besides, considering the PI, the optimisation yielded a value of 0.17 ± 0.01, which was close to the values achieved by the central points conducted during the 19 experiments (0.14).

The values obtained for the extraction yields of water-soluble protein, R-PE, and PI throughout the whole study are presented and summarised in Table 3. Using cellulase, the water-soluble protein content obtained was 1.88 times higher than that of the control (without enzyme). After optimisation by RSM, the water-soluble protein concentrations increased by 1.77 mg g^{-1} dw than that without optimisation, but the hydrolysis time significantly decreased (286 min instead of 360 min). Regarding the R-PE concentration, the result obtained during the control experiment increased by 2.8 times (from 4.62 to 12.76 mg g^{-1} dw) using enzymes without optimisation. After optimization, this yield increased 1.23 times further (to 15.75 mg g^{-1} dw) equivalent to a total increase of 3.41 times (from 4.62 to 15.75 mg g^{-1} dw). Finally, considering the PI, enzymatic digestion led to a 2.4-fold increase in the PI value without optimisation (from 0.05 to 0.12), and a

Table 3. Summary of the results for R-PE extraction yield, R-PE Purity Index (PI), and water-soluble protein extraction yield using different extraction methods from dry algae.

	Control	Hydrolysis	
		Without optimisation	After optimisation
Temperature (°C)	35	35	32
Time (min)	360	360	286
pH	5 (50 mM)	5 (50 mM)	5 (50 mM)
E/S (mg g^{-1} dw)	0	20	47
Proteins (mg g^{-1} dw)	14.62 ± 0.42	27.53 ± 0.97	29.30 ± 0.56
R-PE (mg g^{-1} dw)	4.62 ± 0.93	12.76 ± 0.58	15.75 ± 0.11
PI (A_{565}/A_{280})	0.05 ± 0.00	0.12 ± 0.01	0.17 ± 0.01

Data are expressed as mean ± standard deviation of five replicates (n = 5).

3.4-fold increase (0.17) by means of the experimental design procedure.

Discussion

The present work proved that different results between extraction methods may be due to the pre-treatment step performed. Freeze-drying the thalli of *G. gracilis* led to the samples being easier to extract, especially when combined with grinding in liquid nitrogen for maceration. Freeze-dried macroalgae have been used extensively when investigating R-PE (Galland-Irmouli *et al.,* 2000; Denis *et al.,* 2009a; Munier *et al.,* 2013).

The extraction of proteins and R-PE with phosphate buffer 20 mM at pH 7.1 has been carried out in many species such as *Grateloupia turuturu* (Denis *et al.,* 2009a), *Palmaria palmata* (Galland-Irmouli *et al.,* 2000), and *Mastocarpus stellatus* (Nguyen *et al.,* 2017). The present work utilised *G. gracilis* with this buffer. However, high concentrations of various cell wall polysaccharides in *G. gracilis* can limit its access to the active compounds (Nguyen *et al.,* 2017).

The present work confirmed that enzymatic hydrolysis is effective for protein and R-PE extraction from *G. gracilis*. There was a significant increase in the concentration of water-soluble proteins extracted in the enzymatic hydrolysis experiments. From several previous studies, the effect of polysaccharidases on protein extraction from red alga has been demonstrated, and it was also concluded that enzymatic treatment may become an effective hydrolysis to assist the protein and R-PE extraction (Fleurence, 2003; Dumay *et al.,* 2013).

The experimental results presented a significant difference between controls (without enzyme) and enzyme digestion for obtaining a high-quality R-PE yield; the enzyme cellulase acting on dry algae gave the highest yield of R-PE. *Gracilaria* is considered as cellulose biomass resource which generate bioethanol products (Ahmad, 2014). A few studies have reported that *Gracilaria* sp. contains 4.11% (dw) (Kawaroe *et al.,* 2013), *G. sjoestedtii* contains 3.94% (Galan *et al.,* 2010), and *G. gracilis* contains 13.04 - 14.22% (Ahmad, 2014; Kawaroe *et al.,* 2015). In the present work, the content of cellulose was 12.94 ± 0.03% from *G. gracilis*. Our experiments confirm the occurrence of cellulose in the cell walls of *G. gracilis*, and suggest it has a critical structural role. This compound can be considered as a connecting compound between other polysaccharides. Martin *et al.* (1988) studied and indicated that an attack by the enzyme cellulase produced microcracks on the surface, probably due to cellulose breakdown in red seaweed (Martin et al., 1988). Thus, the use of cellulase may improve the extracting ability of water-soluble compounds such as proteins and R-PE from *G. gracilis*.

Based on previous studies, xylanase yielded the highest efficiency for the extraction of protein and R-PE for other algae such as wet algae *P. palmata* (Dumay *et al.,* 2013) or dry algae *Mastocarpus stellatus* (Nguyen *et al.,* 2017). Therefore, the effects of the digestion enzymes depend on the cell wall polysaccharides structure of each red algae.

By using the RSM, the maximum desirability D was calculated to be 91.38%; indicating that 91.38% of the variability in the response could be predicted by the design. According to the study of Dumay *et al.* (2013), the desirability was determined as 89% by RSM for the R-PE yield from the seaweed *P. palmata* (Dumay *et al.,* 2013); or from the study of Nguyen *et al.* (2017), where the maximum desirability was calculated to be 72.31% by this model for the R-PE of *Mastocarpus stellatus* (Nguyen *et al.,* 2017). In the present work, a high result was obtained for the desirability D owing to the cumulative total of all the factors studied. Each parameter had a certain influence on the responses obtained. Their interactions were not only in the hydrolysis, but also in the extraction that were aimed at recovering the R-PE with the best yield capacity. Analysing the influence of the variables on R-PE concentration revealed that the hydrolysis time and E/S ratio strongly influenced and had positive effects on this response.

Table 3 indicates the usefulness of enzymatic treatment to improve and enhance R-PE extraction from *G. gracilis*. In addition, RSM confirmed again that enzymatic hydrolysis increased R-PE yield from this red macroalgae. The RSM used established the feasibility and the reproducibility of such process, and the need to perform those work on every new species investigated. After optimisation by RSM, the water-soluble protein and R-PE yield increased as compared to that obtained without optimisation, but the hydrolysis time significantly decreased (286 min instead of 360 min). Cellulase was very effective against the algae cell wall, and the hydrolysis occurred strongly during the first hour of reaction (Martin *et al.,* 1988). The prolonged hydrolysis time may be due to the cellulase losing activity because the enzyme was attributed to reversible and irreversible adsorption during hydrolysis (Mensi *et al.,* 2012). It seems that an increase of hydrolysis

extraction from *G. gracilis*. The decreased hydrolysis temperature (32 instead of 35°C) may reduce the cost of heating and provide economic benefits. Based on other studies, the R-PE values obtained was in a range of 1.60 to 4.39 mg g^{-1} dw for *Grateloupia turuturu* (Denis *et al.,* 2009a; 2009b), 4 to 12.36 mg g^{-1} dw for *P. palmata* (Galland-Irmouli *et al.,* 2000; Dumay *et al.,* 2013), and 6.25 mg g^{-1} dw for *G. gracilis* (Mensi *et al.,* 2012). In the present wok, R-PE yields ranged from 1.20 to 15.75 mg g^{-1} dw, depending on pre-treatment of materials and the extraction conditions. In order to improve the R-PE extraction yield and reach highest values found previously on other seaweeds, enzyme cellulase could be used.

Conclusion

The present work demonstrates the specific efficiency of enzymatic assisted hydrolysis on red seaweed, *G. gracilis*. In the first step, freeze-dried macroalgae served as the best algae pre-treatment for the R-PE extraction from *G. gracilis*. Secondly, enzyme treatment has been proposed as an alternate stage to solvent extraction processes, in order to improve and enhance the R-PE extraction concentrations. Using the RSM, the importance of factors at various levels could be investigated. A high similarity was observed between the predicted and experimental results, which reflected the accuracy and applicability of this model to optimise the R-PE production from *G. gracilis*. Therefore, *G. gracilis*, which is commonly found worldwide, and available for human consumption, could be easily considered as a new profitable source of R-phycoerythrin. Extracts obtained from this process could benefit sectors such as medicine, cosmetic, or food. Nevertheless, further research is needed to improve its quality, such as purification of the fractions using chromatography or filtration. Once produced and purified, extracts could be incorporated in food or cosmetic formulation, as well as used in molecular probes. Finally, working on the purified R-phycoerythrin could give additional fundamental molecular characterisation (specific molecular weight and sub-unit identification).

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