

An evaluation of methods for quantifying the enzymatic degradation of red seaweed *Grateloupia turuturu*

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Abstract For better exploitation of the red seaweed *Grateloupia*, enzymatic digestion of the thallus may be a way to increase access to metabolites of industrial interest. With this aim, we have tried to find a method to quantify the efficiency of enzymatic digestion. Vegetative algal material was treated with polysaccharidases (Onozuka R-10 cellulase, agarase, and Ultraflo L mixture). The proportion of degraded surface area was determined by microscopic measurement of the residue surface using imaging software and compared with the analysis of carbohydrates and R-phycoerythrin released in the incubation solution. Both the reducing carbohydrate concentration and percentage of degraded surface area appeared the most reliable methods to study enzymatic efficiency. The amount of solubilized total carbohydrates, and particularly that of R-phycoerythrin, showed non-specific variations, so no conclusions could be drawn. The application of this procedure to the screening of the efficient digestion of *Grateloupia* material demonstrated that cell walls were only partially digested by polysaccharidase enzymes alone. The Ultraflo L mixture and Onozuka R-10 cellulase produced a greater degradation of *Grateloupia* tissues and a higher release of reducing carbohydrates, whereas agarase did not display any specific action. Thus, the proposed procedure based on the quantification of residue surface area seems to be an accurate method to analyze enzymatic digestion. Other tests using different concentrations and combinations of enzymes are now required.

Keywords Red algae · *Grateloupia turuturu* ·
Enzymatic hydrolysis · Polysaccharidases ·
Degradation efficiency · Histological quantification

Introduction

Enzymatic hydrolysis of the seaweed cell wall is a procedure used in many applications. It has been described for protoplast production (Cheney et al. 1986; Chen and Chiang 1994), in cell wall composition studies (Lahaye and Vigouroux 1992; Deniaud et al. 2003a, b), and for improving protein digestibility by removing anti-nutritional factors such as polysaccharides (Fleurence 1999; Nikolaeva et al. 1999; Fleurence et al. 2001). This type of approach has also been reported as a mild method to increase the extraction yield of major compounds such as proteins, especially R-phycoerythrin (Fleurence 2003), and DNA (Joubert and Fleurence 2005).

However, the efficiency of the enzymatic process differs according to cell wall composition and should be adapted to each species (Fleurence et al. 1995; Fleurence 1999). In addition, variations in the chemical composition of algae are known to occur depending on life-cycle stage, physiological status, and seasonal and environmental parameters of culture (Morgan et al. 1980; Kloareg and Quatrano 1988; Craigie 1990), largely complicating the routine application of the enzymatic process. Moreover, some parameters, such as the nature of the enzymatic preparation (enzyme type and combination) and reaction pH or temperature, can also greatly change the efficiency of cell wall enzymatic hydrolysis. Optimization of cell wall enzymatic degradation requires specific and reliable parameters. Generally, yields of seaweed hydrolysis are determined by using indirect methods according to the application concerned, e.g.,

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amounts of extracted sugars and proteins (Fleurence et al. 1995, 2001; Deniaud et al. 2003a, b; Fleurence 2003). In this study, we carried out a comparison between biochemical analyses of solubilized sugars and the water-soluble pigment R-phycoerythrin, and a microscopic analysis of the final degradation residues. Three methods were tested on the red seaweed *Grateloupia turuturu* Yamada (Halymeniaceae, Rhodophyta), an invasive alga found along the shorelines of Brittany (Simon et al. 2001). In France, *Grateloupia* may represent an all-year-round substantial source of red algal material that is not being exploited. Its bioconversion could be facilitated by adjusting the methodology of the enzymatic liquefaction.

Materials and methods

The *Grateloupia turuturu* samples were collected in May 2005 in the intertidal zone at Piriac-sur-Mer (47°22'N, 2°33'E, Atlantic coast, France). Epiphytes were removed and samples were successively rinsed with seawater, tap water and distilled water. Subsequently, the algae were freeze-dried and homogenized before enzymatic treatment.

Enzyme assays

The digestion was performed in the presence of three commercial β -glucuronidases: Onozuka R-10 cellulase (Ce) (Yakult Pharmaceutical, Japan), agarase (Ag) from *Pseudomonas atlantica* (Sigma A-6306, USA), and the multi-enzyme preparation Ultraflo L (Uf) (containing arabanase, cellulase, pentosanase, xylanase activities) (Sorensen et al. 2003) which displays a wide range of activity was kindly supplied by Novozyme (Denmark). These enzymes were used separately at two concentrations: 0.08 (Ce) and 0.16 U mL⁻¹ (Ce2) for cellulase; 1.1 (Ag) and 2.2 U mL⁻¹ (Ag2) for agarase. Units of cellulase and agarase are expressed as the amount of enzyme producing, respectively, 1 μ mol of glucose and 1 μ g of glucose from agar per min at 40°C. Hydrolysis with Ultraflo L was performed at 3.0 (Uf) and 6.0 μ L mL⁻¹ (Uf2).

The enzymatic hydrolysis protocol was adapted from the procedure of Fleurence et al. (2001). To avoid the influence of reproductive and seasonal status, screening of the efficient digestion of cell walls was carried out on a single very large sample of vegetative mature thallus.

The freeze-dried algae (0.077 g dry weight) were suspended in 5 mL of 0.1 M sodium phosphate buffer (pH 7.0) containing the appropriate enzymes or without enzymes as a control. The mixture was gently stirred at 40°C for 6 h. After enzymatic hydrolysis, undigested reaction residues and solubilized compounds were separated by centrifugation (3,500 g and 9,000 g for 10 min at

4°C). The supernatants were collected and used to determine the total sugars, reducing sugars, and R-phycoerythrin content. The residual pellets were collected and stored at 4°C. For each condition, triplicate digestions were carried out simultaneously and repeated at least 3 (3–5) times independently.

Determination of supernatant content

Total carbohydrates were analyzed in each enzymatic extract using the colorimetric phenol-sulfuric acid method (Dubois et al. 1956). The tubes were allowed to stand for 2 h in concentrated sulfuric acid before absorbance measurement at 485 nm. The reducing sugar concentrations were assayed using the ferricyanide method adapted from Kidby and Davidson (1973). In each assay, glucose was used as a standard.

R-phycoerythrin concentration was determined spectrophotometrically using the Beer and Eshel (1985) equation and standardized to thallus dry weight.

Residual pellet analyses

The degradation residues were examined quickly after centrifugation with a light microscope (Olympus AX70/provis) without staining. Images were collected with a digital camera (Dmx 1200F, Nikon, Japan) and analyzed using Lucia G imaging software (Laboratory Imaging, CZ). For each pellet the surface area of undigested residues was determined for at least 15 areas. For every image, the contrast was adjusted for optimal detection of fragments by the image analyzer. Areas were then adjusted to take into account the initial fragmentation (93.2% \pm 1.3) that thalli displayed before the enzymatic incubation. Finally, data were expressed as the percentage of degraded surface compared to the surface of the control condition without enzyme.

Statistics

All results are expressed as means \pm SE, where the sample size is at least 3. Significance was tested by means of one-way ANOVA and for R-phycoerythrin content with the non-parametric Kruskal-Wallis test. Probabilities $p < 0.05$ were considered significant.

Results

The digestion of *Grateloupia* thallus using various polysaccharidic enzymes led to different levels of partial cell wall degradation. The cells and residues were dispersed in a viscous, pink-colored reaction mixture.

Composition of hydrolysis medium

The digestion supernatants were analyzed for total and reducing carbohydrates, which were solubilized from cell cytoplasm and walls. The quantity of total and reducing sugars released by agarase after 6 h of treatment at 40°C did not differ from the control treatment (Fig. 1A, B). In contrast, hydrolysis in the presence of Onozuka R-10 cellulase or Ultraflo L mixture was effective at degrading cell walls, as confirmed by the significant increase in reducing sugars released (Fig. 1B, Table 1). Total sugars were released to a similar extent but only Ultraflo L significantly increased their extraction (Fig. 1A, Table 1). Moreover, cellulase and Ultraflo L displayed a dose-dependent effect. The use of a two-fold quantity of enzyme increased significantly the level of reducing sugar extraction (Fig. 1A, Table 1).

Overall, the quantity of reducing sugars released in the medium could be useful for evaluating the degradation of *Grateloupia* cell walls, whereas total carbohydrate content displayed non-significant variations. The results also demonstrate the greater release of carbohydrates using Ultraflo L mixture rather than cellulase alone.

The amount of extracted R-phycoerythrin was determined after each enzymatic treatment (Fig. 1C, Table 1) and demonstrated a strong heterogeneity between the extracts and the conditions. The presence of high molecular weight proteinaceous pink material between the cellulase- and Ultraflo L-digested residues (Fig. 2C, H) demonstrated the retention of R-phycoerythrin in pellets after centrifugation and its low extractability in enzymatic supernatants. Thus, the quantification of R-phycoerythrin release appears inappropriate for estimating the digestion yield.

Light microscope investigation of remaining enzymatically-digested thallus

Figure 2 shows representative micrographs of non-degraded residues after each enzymatic treatment. They demonstrate that, even if none of the enzymes completely dissociated the *Grateloupia* thallus, each enzymatic treatment had a specific action (Fig. 2B–H). The *Grateloupia* thallus incubated with agarase did not differ from that in control conditions; the final non-degraded residues still showed intact thallus organization and cell structure (Fig. 2E, F). In contrast, the remaining cellulase- and Ultraflo L-digested fragments were found to be smaller, more transparent and thinner than the agarase-digested and control fragments (Fig. 2C,D,G,H). This was especially observed for the double concentration of cellulase (Fig. 2D) and Ultraflo L (Fig. 2H). In these conditions, a higher magnification enabled us to observe some single cells between thallus

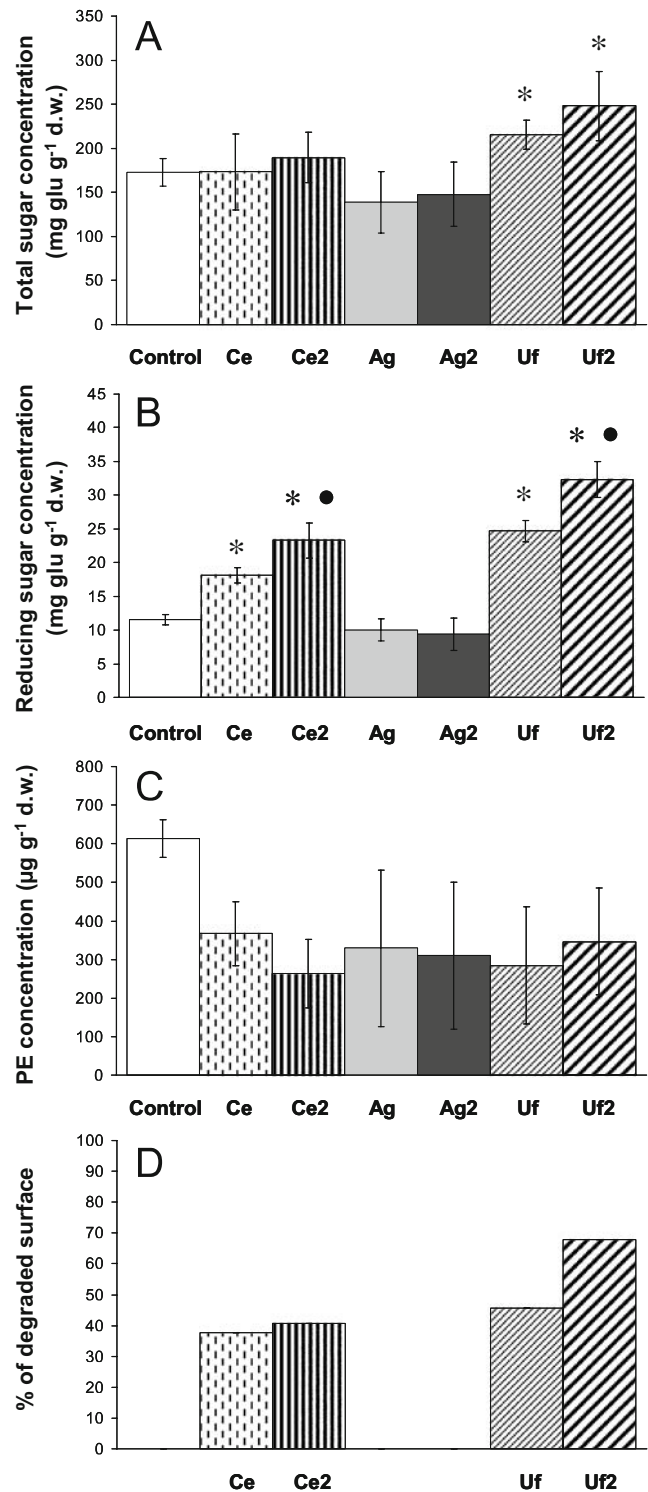


Fig. 1 Comparison between the level of tissue degradation and the quantity of (total and reducing) sugar and R-phycoerythrin released from *Grateloupia* thallus hydrolyzed by cellulase (Ce, 0.08 U mL⁻¹; Ce2, 0.16 U mL⁻¹), agarase (Ag, 1.1 U mL⁻¹; Ag2, 2.2 U mL⁻¹), and Ultraflo L (Uf, 3.0 µL mL⁻¹; Uf2, 6.0 µL mL⁻¹). Mean and standard deviation (n=3). Statistical significance compared with control conditions is indicated by *p*-value (* *p*<0.05), that between both enzyme concentrations (● *p*<0.001)

Table 1 Component analysis of the medium and residues after the enzymatic hydrolysis of *Grateloupia* thallus using various polysaccharidic enzymes

Enzyme	Concentration (abbreviation)	Supernatant components			Indigestible residues	
		Total sugars (mg glu g ⁻¹ d.w.)	Reducing sugars (mg glu g ⁻¹ d.w.)	R-phycoerythrin (μg g ⁻¹ d.w.)	% surface	% degraded surface
Control		172.3±15.5	11.5±0.8	614.3±48.8	75.3±2.6	
Cellulase	0.08 U mL ⁻¹ (Ce)	173.2±43.4	18.1±1.2 *	366.8±84.2	46.8±3.8	37.8
	0.16 U mL ⁻¹ (Ce2)	189.2±28.7	23.3±2.6 *	264.6±88.5	44.7±3.7	40.6
Agarase	1.1 U mL ⁻¹ (Ag)	138.5±35.2	10.0±1.6	329.6±202.3	77.1±3.0	0
	2.2 U mL ⁻¹ (Ag2)	147.6±36.4	9.4±2.4	309.5±190.2	78.3±3.2	0
Ultraflo	3.0 μL mL ⁻¹ (Uf)	215.1±16.6 *	24.7±1.5 *	284.8±151.4	40.8±3.9	45.8
	6.0 μL mL ⁻¹ (Uf2)	247.9±39.3 *	32.3±2.6 *	346.7±139.4	24.3±3.0	67.7

Mean ± IC_{95%} (n=3–5). Statistical significance compared with control conditions is indicated by *p*-value (* *p*<0.05), that between both enzyme concentrations

fragments associated with degraded material (data not shown).

Thallus fragmentation was quantified as the percentage of residue surface area (Fig. 2A) and then as the percentage of degraded surface area in relation to the control (Fig. 1D, Table 1). Data on agarase-digested thallus confirmed the lack of specific enzymatic hydrolysis for this enzyme on *Grateloupia* tissues. In contrast, cellulase and Ultraflo L produced a significantly higher percentage of degradation. It was especially the case for the 6.0 μL mL⁻¹ Ultraflo L treatment which increased the fragmentation to 67.7% in comparison with the control (Fig. 1D, Table 1). Interestingly, the degradation percentage increased between both Ultraflo L concentrations (45.8% at 3.0 μL mL⁻¹ vs 67.7%) while it did not differ significantly between both cellulase concentrations studied (37.8% at 0.08 U mL⁻¹ versus 40.6% at 0.16 U mL⁻¹) (Table 1).

In summary, except for the 0.16 U mL⁻¹ cellulase treatment (Ce2) which showed a higher non-specific relation between the determination of degraded surface area and the amount of reducing sugar (Fig. 1B vs Fig. 1D), our microscopic quantitative investigation of the remaining enzymatic residues revealed comparable results with those of reducing sugar measurement in enzymatic supernatants. They demonstrated that the greatest degradation of *Grateloupia* thallus was achieved using the Ultraflo L mixture.

Discussion

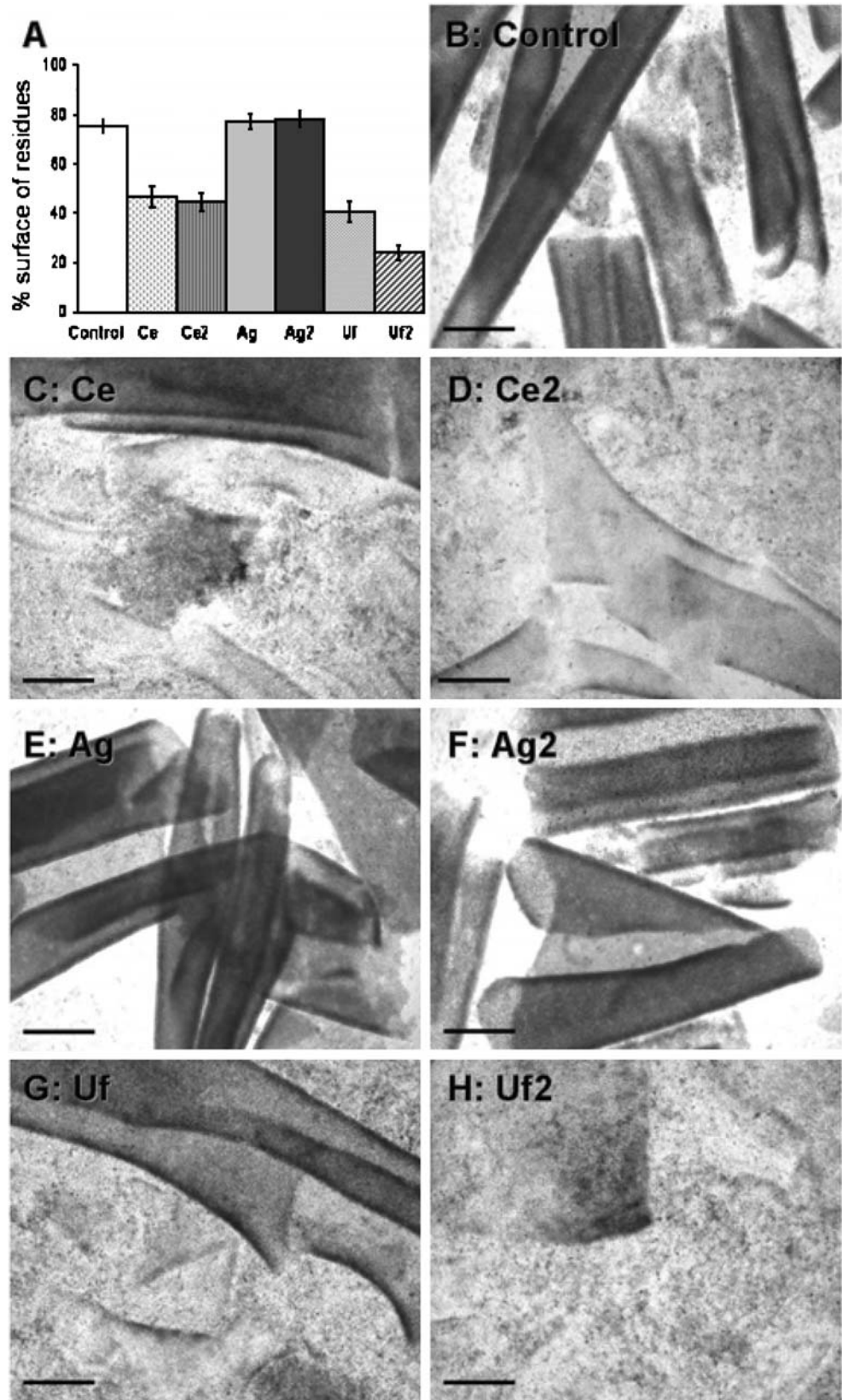
The present study investigated various methods for evaluating and quantifying the efficiency of enzymatic hydrolysis of *Grateloupia turuturu* thallus. Three important facts were found in screening for the efficient digestion of *Grateloupia*. The first was that the amount of water-soluble proteins, such as R-phycoerythrin, failed to follow the progress of enzymatic digestion. The second was that the

amount of solubilized reducing carbohydrates could be useful for assessing enzymatic digestion, but results may sometimes be overestimated. The last was that the measurement of the size of enzymatically-undigested parts could determine more precisely the efficiency of enzymatic degradation.

Only a few studies have been undertaken on the enzymatic liquefaction of red seaweeds such as *Palmaria palmata*, *Gracilaria*, and *Chondrus crispus* (Lahaye and Vigouroux 1992; Fleurence et al. 1995; Deniaud et al. 2003a, b; Fleurence 2003). They have particularly demonstrated the effect of polysaccharidases on protein extraction from the red alga *Palmaria palmata* (Fleurence et al. 1995; Fleurence 1999, 2004) and concluded that enzymatic treatment may represent an efficient process for facilitating access to R-phycoerythrin (Fleurence 2003). In this study, we have confirmed the release of R-phycoerythrin from *Grateloupia* using enzymatic degradation. However, the level of R-phycoerythrin recovery from the supernatants was very variable, even under the same hydrolysis conditions. These results correlated with microscopic observations showing red precipitates between enzymatically-digested residues. This seems to indicate an inefficient extraction of R-phycoerythrin and agrees with previous studies demonstrating the strong influence of large amounts of anionic polysaccharides on protein solubility (Fleurence 1999; Barbarino and Lourenço 2005). To conclude, the quantification of R-phycoerythrin release in the digestion solution would not be useful for monitoring the enzymatic digestion of red seaweeds that are rich in phycocolloids, such as *G. turuturu*.

Since algal cell walls are mostly carbohydrate in composition (Morgan et al. 1980; Kloareg and Quatrano 1988; Craigie 1990), enzyme activity has frequently been assessed based on the extraction of solubilized cellular carbohydrates (Fleurence et al. 1995; Deniaud et al. 2003a, b; Jam et al. 2005; Kasai et al. 2006). Although high

Fig. 2 Final enzymatic residues observed in light microscopy. **A** Measurement of the surface area of undigested residues after various enzymatic treatments. The area is expressed in each enzymatic condition as the percentage of the initial surface area and represents means \pm IC 95% ($n=3-5$). **B-H** Representative microscopic observations of the same conditions. Control (**B**) contained no enzyme. Onozuka R-10 cellulase at 0.08 U mL⁻¹ (**C**) and 0.16 U mL⁻¹ (**D**), agarase at 1.1 U mL⁻¹ (**E**) and 2.2 U mL⁻¹ (**F**), and Ultraflo L mixture at 3.0 μ L mL⁻¹ (**G**) and 6.0 μ L mL⁻¹ (**H**). Scale bars = 500 μ m for B-H



performance anion-exchange chromatography provides the most relevant information about how enzymes work (Farias et al. 2000; Deniaud et al. 2003a, b; Sorensen et al. 2003), the measurement of water-soluble carbohydrates is very appropriate for routine applications (Fleurence et al. 1995; Deniaud et al. 2003a, b; Kasai et al. 2006; Goulard et al. 2001). We selected two common and complementary biochemical procedures, which quickly quantify reducing and total carbohydrate sugars released in the supernatants. The approximately 10-fold higher yield of total carbohydrates measured by the Dubois method suggests that both reducing and neutral sugars were released by the enzymatic treatment. This result is in agreement with an earlier report, which described water-extracted carbohydrates from *G. indica* (Chattopadhyay et al. 2007). In addition, the present data show that the amount of total carbohydrates was more variable than the amount of reducing sugars. This discrepancy could result from the limiting step of acid hydrolysis, which characterizes the Dubois method. It may underestimate the amount of total carbohydrate content depending on the sample (Dubois et al. 1956; Mecozzi et al. 1999). In contrast, the yield of reducing sugars obtained from *Grateloupia* tissues seems to result from a specific enzymatic hydrolysis, the amount reflecting more accurately the enzymatic hydrolysis of the thallus.

To assess the efficiency of physical and chemical cell wall degradation, various markers may be used, e.g., arachidonic acid, sulfate, phosphate, water soluble antioxidant, total phenolic, and carrageenan contents, enzyme or biological activities, and intrinsic viscosity of the enzymatic extract. Most of these chemical analyses have been carried out on extracted solutions, whereas only a few other studies have described the status of the digested residues using light (Deniaud et al. 2003a; Kasai et al. 2006) and electron (Andrade et al. 2004) microscopy. Except for the estimation of residual biomass weight (Mishima et al. 2006), none of these studies have reported a direct and precise quantification of the amount of digestion residue. However, the study of indigestible residues could provide more useful knowledge for the investigation of the level of enzymatic hydrolysis, the supernatants being preserved for further utilization. In the present study, we have demonstrated that the yield of enzymatic hydrolysis may be measured using quantitative microscopic analysis and is in agreement with biochemical measurements of both reducing sugars. In detail, there is a discrepancy between the surface area of residual tissue fragments digested by cellulase and the amount of reducing sugar released in the supernatant. It is possible that the microscopic quantification might not take into account enzymatic degradation in the thickness of the thallus. However, microscopy of the thinner residues showed that they were more transparent and could not be quantified using the contrast adjustment. A second problem

could be the initial fragmentation of *Grateloupia* thallus, which increases the surface area in contact with the enzyme. However, it does not differ between the control and each enzymatic treatment and may not influence the specific enzymatic action. Finally, the calculation of degradation percentage from the comparison between control and enzymatic digestion conditions may avoid having to account for non-specific hydrolysis by mechanical breaking and/or intrinsic proteolytic enzymes during the 6 h of agitation, as shown in some studies (Duncan et al. 1956; Pérez-Lloréns et al. 2003).

The use of both microscopic analysis of digestion residues and release of reducing sugars leads to speculation about the action mechanism of polysaccharidic enzymes on *Grateloupia* tissues, even if we did not try to optimize the enzymatic hydrolysis with various combinations of cellulolytic enzymes. The fact that cell walls of *Grateloupia* were only partially digested by the carbohydrate enzymes supports the existence of a complex molecular structure of red algal cell walls (Morgan et al. 1980; Kloareg and Quatrano 1988; Craigie 1990).

Agarase did not display any hydrolytic activity, whereas it appears useful for protoplast isolation and liquefaction of various sulfated galactan-producing red seaweeds including *Grateloupia* (Cheney et al. 1986; Chen and Chiang 1994; Fleurence et al. 1995; Nikolaeva et al. 1999; Joubert and Fleurence 2005). In addition, most polysaccharides of the cell walls of Halymeniaceae (formerly Grateloupiaceae) appear to be carrageenan-agar hybrid polymers (Kloareg and Quatrano 1988; Craigie 1990). Moreover, protoplast production from *G. turuturu* tissues needs the effective degradation of cell walls and has been observed using an enzymatic combination of agar, cellulase and protease (Chen and Chiang 1994). Taken together, our results suggest that there is a limited access for agarase to its substrate in *G. turuturu*.

The action of Onozuka R-10 cellulase on *Grateloupia* cell walls was more evident, confirming the existence of cellulose in *Grateloupia* as in all red seaweeds (Kloareg and Quatrano 1988; Craigie 1990). Both cellulase concentrations produced different amounts of extracted sugars, whereas Onozuka R-10 cellulase alone did not produce protoplasts from *Gracilaria* (Cheney et al. 1986). Because the cellulase mixture also displays a hemicellulase activity, we can suppose there is a secondary degradation of solubilized hemicellulose in the supernatants.

Ultraflo L produced the highest amounts of reducing sugars and hydrolyzed cell walls most as shown by the quantification of indigestible residues. This yield may be related to the heterogeneous composition of Ultraflo L, which consists of heat-stable multi-active β -glucanases, such as endo-1,4- β -xylanase, β -glucosidase, and α -arabinofuranosidase (Sorensen et al. 2003). However, whatever

the enzymatic concentration used, total solubilization of *Grateloupia* was never reached showing that the enzymatic hydrolysis treatment was not fully optimized in this study. The efficiency of enzymatic digestion should be improved by using longer incubation times, higher concentrations of enzymes, and/or a combination of effective enzymes.

In conclusion, the above data provide a new accurate method to analyze the efficiency of enzymatic digestion of red seaweed based on the quantification of the size of digested residues. It might be completed by the biochemical measurement of reducing sugars released in the digestion solution. Further studies will be needed to optimize the enzymatic hydrolysis of *G. turuturu* thallus and to specify the exact action of polysaccharidases on the cell wall network.

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