



# Enhanced Liberation of Soluble Sugar, Protein, and R-Phycoerythrin Under Enzyme-Assisted Extraction on Dried and Fresh *Gracilaria gracilis* Biomass

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This study aims to investigate the bio-refinery process through an enzyme-assisted extraction (EAE) on freeze-dried and fresh macroalgae *Gracilaria gracilis* for the release of water-soluble components (R-phycoerythrin, proteins, and sugar). Three enzymes, cellulase, protease, and enzyme cocktail (mixture of cellulase and protease), were applied in the study. Results showed that freeze-dried biomass yielded the highest target components in the presence of enzyme cocktail while a single enzyme was better with fresh biomass, either protease for the release of R-PE and protein or cellulase for sugar. The extraction of protein and sugar was improved by 43% and 57%, respectively, from fresh biomass compared to dried biomass. The difference of biomass status was shown to affect the required enzyme and recovery yield during the extraction process. Employing an enzyme cocktail on freeze-dried biomass boosted the extraction yield, which was probably due to the complementary effect between enzymes. On the other hand, single enzyme worked better on fresh biomass, giving economic benefits (enzyme limitation and drying stage) for further implementation of the bio-refinery process. Thus, biomass treatment (fresh or freeze-dried) and enzyme-type determined the efficiency of enzyme-assisted extraction according to the target components.

**Keywords:** *Gracilaria gracilis*, R-phycoerythrin (R-PE), enzyme-assisted extraction (EAE), enzyme cocktail, bio-refinery

## INTRODUCTION

Rhodophyta, one of the three main phyla of marine macroalgae, is reported to contain various biologically active compounds, that is, polysaccharides, sterols, phycobiliproteins, and vitamins (Francavilla et al., 2013; Bedoux et al., 2014). Polysaccharides are often the main extracted product derived from macroalgae and have become a high-value raw material of industrial interest (Wu et al., 2014; Buschmann et al., 2017; Kazir et al., 2019; Khan et al., 2019).

**Abbreviations:** EAE, Enzyme-assisted extraction; R-PE, R-phycoerythrin; BL, Freeze-dried biomass; AFM, Fresh biomass; ME, Enzyme cocktail; CE, cellulase; PE, protease.

**TABLE 1** | Operating conditions of enzyme-assisted extraction.

Enzyme	Codes	pH Buffer	Biomass	Enzyme ratio (mg/g dw)
Cellulase	CE	5.0	BL	47
			AFM	47
Protease	PE	7.5	BL	47
			AFM	47
Cocktail (cellulase + protease)	ME	5.0	BL	94
			AFM	94

mg/g dw: mg of enzyme per 1 g biomass dry weight E1: 47 mg/g dw, E2: 94 mg/g dw.

The *Gracilaria* genus is recognized as one of the main resources for macroalgae polysaccharides and is reported with 62–63% of polysaccharides per dry weight (Rioux et al., 2017). The genus is widespread and can be found in tropical to temperate latitudes (Iyer et al., 2004; Torres et al., 2019). Polysaccharides of red algae cell walls gather agar, carrageenan, xylans, water-soluble sulfated galactans, and porphyrins (mucopolysaccharides) (Charoensiddhi et al., 2017), as well as cellulose, xylanase, or mannan fibrils (Mittal and Raghavarao, 2018). These polysaccharides serve as food emulsifiers, gelling agents, and can be used as media for bacterial growth identification, or used as therapeutic agents (Cicinskas et al., 2019). In South-East Asian countries, agar-agar (extracted from *Gracilaria*) is used in food preparation as a gelling and thickening agent (Souza et al., 2012; Chan and Matanjun, 2017).

In addition to the high content of polysaccharides, the Rhodophyceae family is reported with phycoerythrin, especially R-phycoerythrin (R-PE), which can be found to make up to 50% w/w of total protein (Pimentel et al., 2019). This R-phycoerythrin is classified as phycobiliproteins and is of great interest in the food industry (natural colorant), research sector (fluorescent dye, immunology, cell biology, and flow cytometry), and cosmetic industry (Dumay et al., 2013, 2014; Bedoux et al., 2014; Munier et al., 2014; Mensi, 2019). The purified powder of R-PE has a high market value (between US\$ 180 and 250 per milligram), which varies according to the purity level (Nguyen, 2017; Mittal and Raghavarao, 2018; Wang et al., 2020).

Several extraction methods such as the conventional method (solid–liquid extraction using phosphate buffer), hydrothermal, acid, base, and organic solvent, as well as the physical methods (freeze-thaw cycles, freeze grinding, ultrasound, and liquid nitrogen grinding), have been used to extract bioactive compounds from macroalgae (Sudhakar et al., 2015; Wang et al., 2020). However, these techniques are mostly time-consuming (several days), costly, and unsuitable for an industrial setup (Le Guillard et al., 2015; Wang et al., 2020). In addition, protein extraction from macroalgae is challenging due to the complexity of the cell wall structure with strong cohesion and mechanical properties with the complex ionic and hydrogen-bonding interactions (Deniaud et al., 2003; Wang et al., 2020). Moreover, various polysaccharides in algal cell walls serve as a barrier that prevents biomolecule release as well as act as anti-nutritional factors to limit the digestibility of protein fractions (Hardouin et al., 2016; Wang et al., 2020). Many studies have confirmed the complexity of protein extraction due to the cell membrane and the entrapment with polysaccharides

(Kazir et al., 2019). It is highlighted that unwanted protein–polysaccharide interactions can limit the efficiency of water-soluble protein in red algae. As a result, protein extraction in red algae *Gracilaria gracilis* can be improved by degrading polysaccharides in the algal main cell walls (Fleurence et al., 1995; Wang et al., 2020).

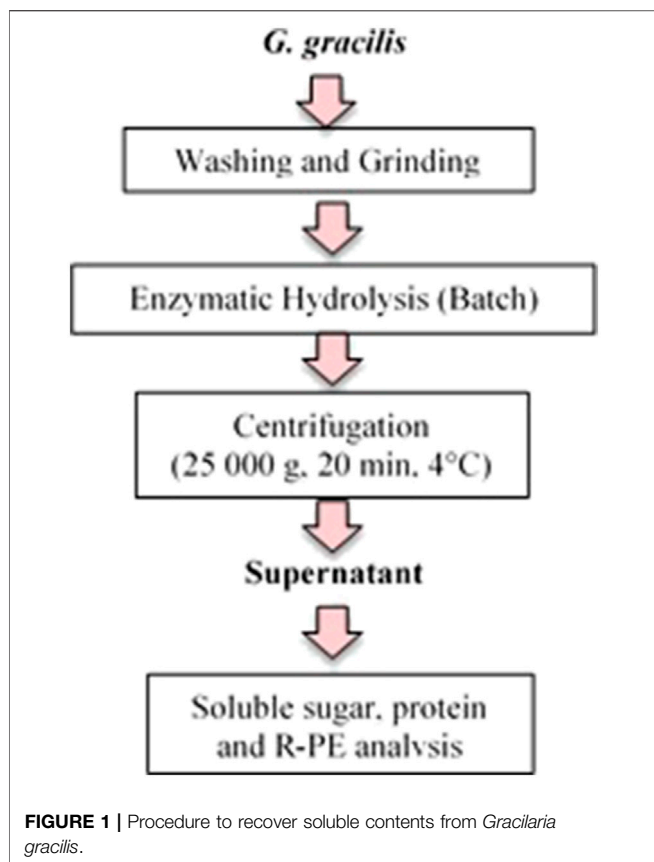
In this regard, the use of enzymes would allow the penetration of extraction solvents into the cell to increase the release of bioactive compounds (Mittal and Raghavarao, 2018) and act as a surfactant (Vandanjon et al., 2016). Furthermore, enzyme-assisted extraction could prevent any degradation of the targeted compounds (Hardouin et al., 2016; Maehre et al., 2016). This type of extraction takes place in mild conditions and provides strong substrate specificity, high yield, and less by-products (Zhang et al., 2019). Given the dominance of polysaccharides present in red algal cell walls, polysaccharidase enzymes are mostly investigated. Some enzymes/enzyme consortia have been tested. Cellulase was demonstrated to deliver the best results for R-PE and protein extraction from dried *Gracilaria gracilis* compared to xylase and  $\beta$ -glucanase (Nguyen, 2017). Enzymatic pre-treatment followed by alkaline extraction resulted in high protein extraction yield from *Palmaria palmata* (Mæhre et al., 2016). The use of enzyme consortia composed of agarose, cellulase, and xylanase also improved the extraction yield of R-PE by 26% from *Gelidium pusillum* (Mittal and Raghavarao, 2018).

To our knowledge, only few studies have worked with fresh biomass with respect to dried biomass. In addition, the use of combination of enzymes is still in the investigation phase. This work aims to investigate the effect of enzyme types (cellulase, protease, and combination of both—cocktail) and enzyme concentrations in enzyme-assisted extraction of fresh and dried *Gracilaria gracilis* seaweed biomass. Here, the extraction yields of aqueous soluble compounds (R-PE, protein, and sugar) were determined.

## MATERIALS AND METHODS

### Sample Collection and Preparation

*Gracilaria gracilis* (*G. gracilis*) was collected in Piriac-sur-Mer (47°22'38.0" N, 2°33'20.2" W), Atlantic coast in France in mid-April 2019. The biomass was rinsed with tap water two times to remove sand, epiphytes, and foreign matter, and then finally rinsed one more time with distilled water. A salad spinner was



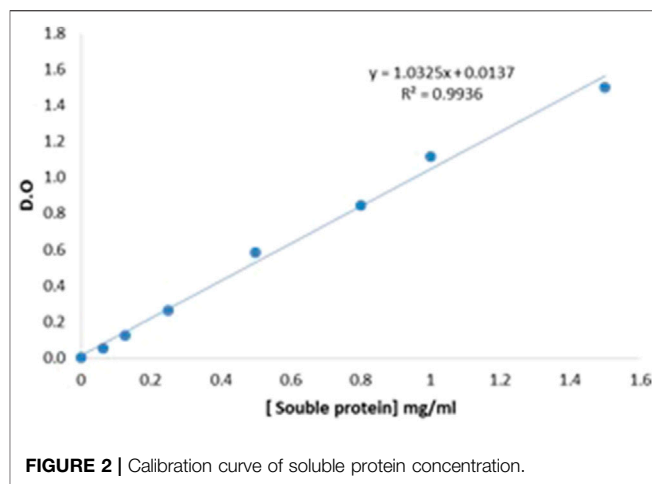
used to remove surface water before storage at  $-20^{\circ}\text{C}$  prior to further analysis. Freeze-dried biomass (BL) was ground with liquid nitrogen using mortar and pestle. The fresh biomass (AFM) was ground with a mixer (Moulinex FP542110, French) to cut down the long filaments into smaller sizes. Afterward, a mixer (Retsch GM 200) was used at 7,000 rpm for 1 min to grind into uniformly sized and homogeneous samples.

### Biochemical Components

The dry weight content was determined by heating at  $105^{\circ}\text{C}$  for 24 h in an oven and the ash content was determined by heating at  $550^{\circ}\text{C}$  for 24 h in a furnace. Extraction of total carbohydrate was performed according to Francavilla et al. (2013). Total carbohydrate was determined by the phenol-sulfuric acid method (Dubois et al., 1956). Total protein was determined by multiplying the total nitrogen (Kjeldahl method) with a conversion factor of 6.25. All extractions were performed in triplicate and the results were expressed in percentage per algae dry weight.

### Chemicals and Enzymes

Analytical grade sodium acetate ( $\text{CH}_3\text{COONa}$ ) and concentrated glacial acetic acid ( $\text{CH}_3\text{COOH}$ ) were used to prepare acetate buffer solution at various pH to function as the working solutions for enzymes. *Aspergillus niger* 22,178) cellulase and *Bacillus*



*licheniformis* (P5459-5G) protease were used. All consumables were purchased from Sigma Aldrich.

Cellulase was chosen following the previous study of Nguyen (2017). Algal cell wall is known to contain a portion of protein; hence, protease was selected to catalyze the cell wall (protein-peptide bonds) more efficiently (Vandenberghe et al., 2020).

### Enzyme-Assisted Extraction

Enzyme-assisted extraction (EAE) was conducted in a batch mode following a method optimized in the study of Nguyen (2017). The mixture of seaweed sample, enzyme, and buffer solution was agitated under a controlled temperature ( $32^{\circ}\text{C}$ ) in the dark for 286 min. The supernatant was collected after the separation process ( $4^{\circ}\text{C}$ ; 25,000 g; 20 min) with a centrifuge (Sorvall Lynx 6,000, United States). The ratio of algae to solvent (acetate buffer) was set at 3 g per 100 ml. A control sample (without enzyme) was always carried out in each condition simultaneously. Triplicate experiments were conducted to ensure reproducibility. A summary of operating conditions is given in Table 1. The procedures to recover soluble compounds are summarized in Figure 1.

### R-Phycocerythrin Determination

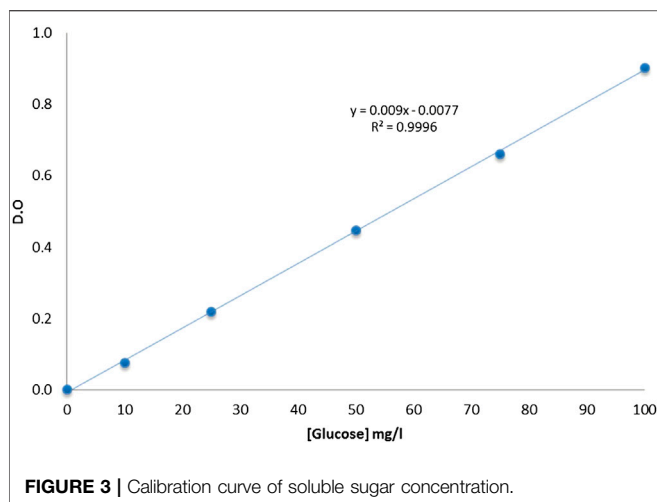
The measurement of spectral absorbance was conducted with a UV-visible spectrophotometer (Jasco V-630, Germany). The concentration of R-PE was determined by spectral analysis adapted from Beer and Eshel from the following equation (Beer and Eshel., 1985):

$$[R - PE] \text{ (mg/ml)} = [(A_{565 \text{ nm}} - A_{592 \text{ nm}}) - (A_{455 \text{ nm}} - A_{592 \text{ nm}}) * 0.20] * 0.12$$

The final R-PE contents were determined and expressed as mg/g dw (dry algae).

### Soluble Protein Determination

The concentrations of soluble proteins were analyzed by the bicinchoninic acid (BCA) assay (Shen, 2019). This method combines the well-known reduction of  $\text{Cu}^{+2}$  to  $\text{Cu}^{+1}$  by protein in an alkaline medium (the biuret reaction) with the highly sensitive



and selective colorimetric detection of the cuprous cation ( $\text{Cu}^{+1}$ ) using a unique reagent containing bicinchoninic acid. Reactant came from Thermo Fisher (Ref. 23225). The calibration curve (Figure 2) was obtained from seven concentrations of common protein BSA (bovine serum albumin): 1.5, 1.0, 0.8, 0.5, 0.25, 0.125, and 0.063 mg/ml. To start, 200  $\mu\text{l}$  of working reagents were added to 25  $\mu\text{l}$  of the prepared sample and incubated at ambient temperature for 45 min in the absence of light. The absorbance was measured at a wavelength of 570 nm. The concentration was calculated using the equation obtained from the calibration curve and was expressed as mg/g dw (dry algae). Samples were normalized by subtracting the total detected protein content with enzyme proteins in order to only count the proteins extracted from biomass.

### Soluble Sugar Determination

Soluble sugar was determined by the phenol-sulfuric acid method (Dubois et al., 1956). Simple sugar and oligosaccharides, including methyl ethers with free or potentially free reducing groups, give an orange-yellow color when treated with phenol and concentrated sulfuric acid. The obtained monosaccharides are dehydrated and rearranged to form furfural (from pentose) or hydroxymethyl furfural (from hexose). These compounds lead to a characteristic yellow coloration by condensation with phenol. The reaction is sensitive and the color is stable. The calibration curve (Figure 3) was prepared at different glucose concentrations (100, 75, 50, 25, and 10 mg/l) as the standard. Briefly, 500  $\mu\text{l}$  of the prepared sample was introduced into a flask, and then 500  $\mu\text{l}$  of 5% phenol solution was added. Then, 2.5 ml of concentrated sulfuric acid (95%–98%) was added. After 10 min of reaction, the samples were vortexed (Heidolph Reax 2000; Germany) for 10 s followed by 15 min of incubation at ambient temperature. Next, the flask was placed in a water bath at 35°C for 30 min. The reading wavelength was set at 490 nm. The results were obtained from the equation provided by the calibration curve and were expressed as mg/g dw (dry algae).

### Statistical Analysis

Results were assessed using one-way ANOVA test using Minitab 19 software. A significant difference was considered when the  $p$ -value was smaller than 0.05 ( $p < 0.05$ ).

## RESULTS

### Biochemical Contents

The biochemical compositions of *G. gracilis* are summarized in Table 2. According to the literature, *G. gracilis* contains around 16–31% dw (dry matter) of protein while the carbohydrate varies from 18–34% dw (Sfriso et al., 1994; Francavilla et al., 2013; CEVA, 2015; Rodrigues et al., 2015; Mensi, 2019). Some studies have shown that the content of polysaccharides of this algal genus could reach up to 62–63% dw (Rioux et al., 2017). In this study, the amount of carbohydrates and proteins was reported at 43.68% and 24.81%, respectively, in accordance to quantities already observed in these seaweed species. Ash content was reported at 2.85%, which was lower compared to values reported in previous studies (17–24%) (CEVA, 2015; Rodrigues et al., 2015). Therefore, the amount of mineral content is of little significance in our biomass. The variation of these contents is strongly related with the preparation methods and environmental factors, which vary according to season, geographical location, water quality, climate, tidal movement, etc.

### Enzyme-Assisted Extraction

#### Soluble Contents Released From Freeze-Dried Biomass

The first objective of the study was to determine the best enzyme, single or cocktail, for freeze-dried biomass that would allow the best recovery of soluble components, that is, R-phycoerythrin (R-PE), sugars, and proteins. The results of enzyme-assisted extraction (EAE) using cellulase (CE), protease (PE), and enzyme cocktail (ME) are presented in Figure 4.

The addition of either protease or cellulase resulted in a significant increase in R-PE extraction (Figure 4A) compared to the control without enzyme ( $p < 0.05$ ). The use of a single enzyme released a similar amount (~0.70 mg/g dw) of R-PE against 0.27 mg/g dw for the control condition. In addition, applying enzyme cocktail (mixture of cellulase and protease) released almost three times higher of R-PE (4.34 mg/g dw) compared to the release from single enzyme. Based on this phenomenon, synergistic relationship between enzymes seemed to affect R-PE liberation from dried biomass.

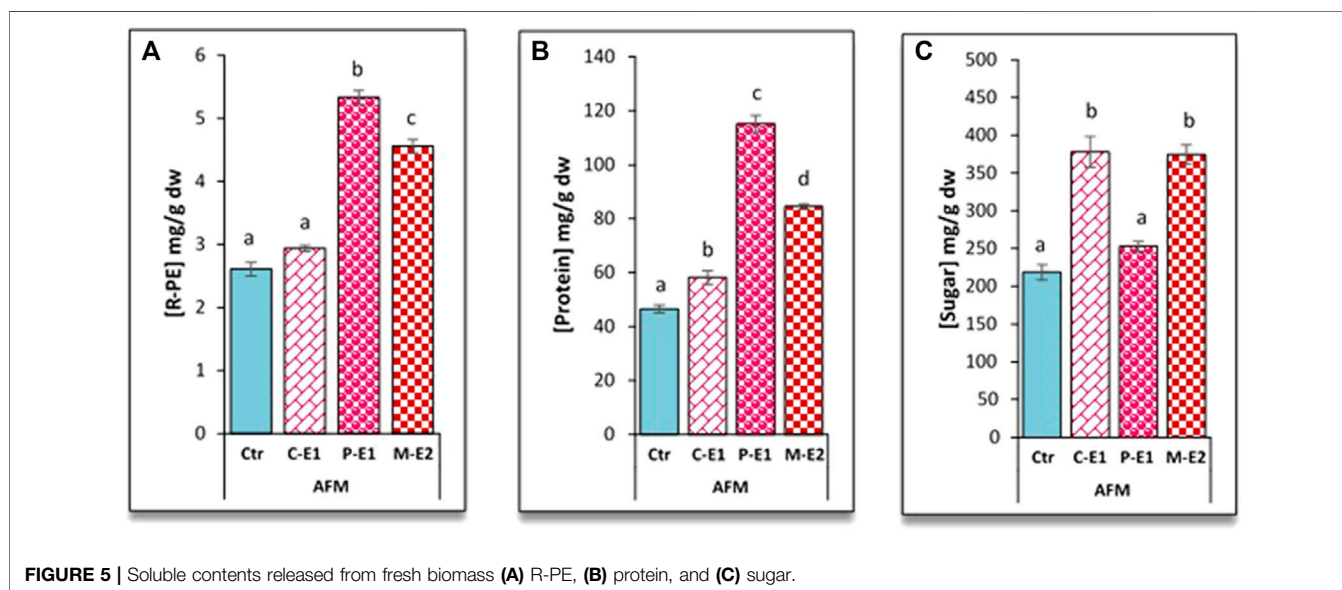
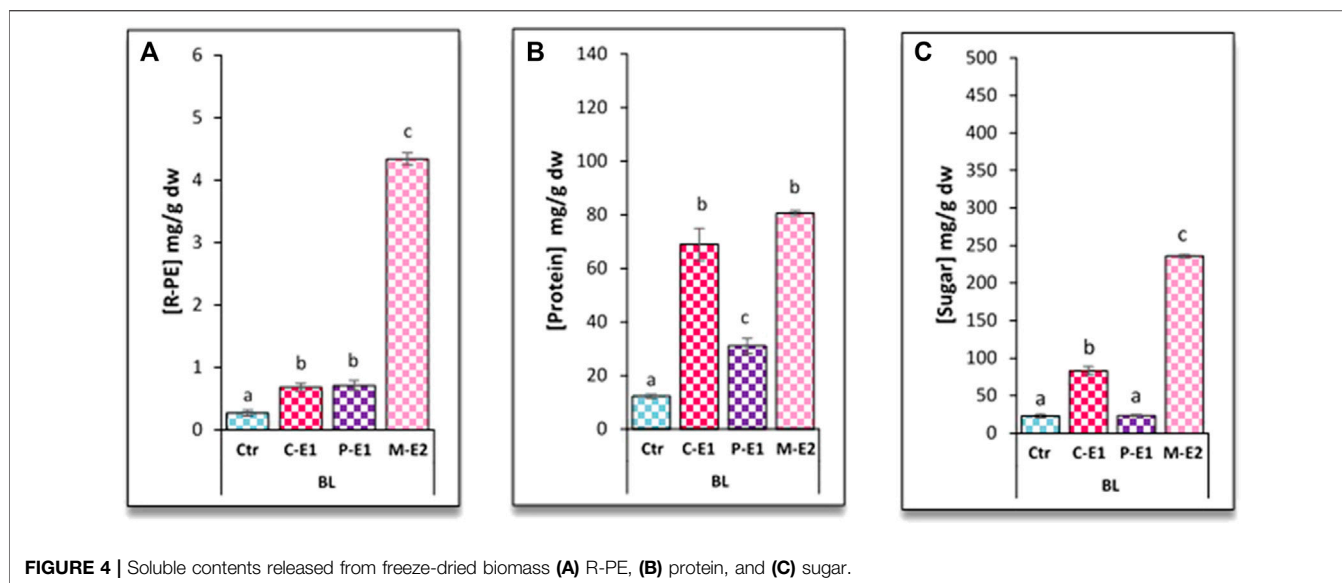
The quantities of soluble protein were also higher with enzyme cocktail than with only single enzyme. Among the three enzymes, CE and ME yielded significantly higher values compared to PE (Figure 4B). Maximum recovery was obtained from ME at 80.57 mg/g dw, followed by CE at ~60 mg/g dw compared to the control sample value of 12 mg/g dw. The use of PE also positively affects soluble protein yield, albeit half the value of cellulase at

**TABLE 2** | Biochemical content of *G. gracilis* biomass.

Compositions	Content (%)
Dry weight <sup>a</sup>	13.41 ± 0.20
Water <sup>a</sup>	86.59 ± 0.20
Ash <sup>b</sup>	2.85 ± 0.03
Carbohydrate <sup>b</sup>	43.68 ± 0.20
Protein <sup>b</sup>	24.81 ± 0.50

<sup>a</sup>Contents are expressed per fresh weight.

<sup>b</sup>Contents are expressed per dry weight.



~30 mg/g dw. The difference between extracted protein yield from CE and ME did not differ much although total enzyme content in ME was twice CE. It is worth noting that the presence of protease in ME did not boost the recovery of protein by a large margin. Cellulase presented an interesting insight into the optimization of protein release in the function of enzyme availability.

For soluble sugars (Figure 4C), similar to the results on protein, cellulase and enzyme cocktail presented a significant increase compared to the control (~23 mg/g dw). Protease did not affect the recovery yield of soluble sugars. On the other hand, the highest sugar concentration was obtained with the enzyme cocktail (236 mg/g dw) followed by cellulase at 80 mg/g dw. The difference between both enzymes is clear as enzyme cocktail resulted in 3 times the value obtained with cellulase.

### Soluble Contents Released From Fresh Biomass

After working with freeze-dried biomass, enzyme-assisted extraction was continued on fresh biomass. The results are summarized in Figure 5. R-PE liberation varied from 2.8 to 5.50 mg/g dw (Figure 5A). The addition of protease (PE) and enzyme cocktail (ME) resulted in a significantly higher R-PE yield compared to the control ( $p < 0.05$ ). In contrast to the freeze-dried sample, the highest liberation was obtained from protease ~5.50 mg/g dw in comparison to enzyme cocktail ~4.5 mg/g dw against the control sample ~3 mg/g dw. Interestingly, enzyme cocktail did not induce more R-PE than single enzyme albeit it contains twice as much enzyme. The synergy effect between enzymes did not seem to occur for the liberation of R-PE on fresh biomass. Moreover, the addition of cellulase (CE)

**TABLE 3** | Comparison the release of protein from different enzymes and red algae.

Algae	Enzyme	Protein extraction yields	References
<i>Gracilaria gracilis</i>	Enzyme cocktail (cellulase and protease)	32%	Current study
	Cellulase	24.18%	
<i>Gracilaria gracilis</i>	Cellulase	10.31%	Nguyen, (2017)
<i>Gracilaria gracilis</i>	Enzyme mixture agarase and cellulase	Protein yields improved 3 times than control condition	Fleurence et al. (1995)
<i>Solieria chordalis</i>	Endo-peptidase	15.20%	Hardouin et al. (2014)
<i>Chondrus crispus</i>	Novozyme-cellulase	7.1%	Kulshreshtha et al. (2015)

did not generate higher R-PE release compared to the control (without enzyme) condition.

The recovery of soluble protein with the addition of enzyme showed a significant increase compared to the control as indicated in **Figure 5B**. Similar to R-PE, protease produced the highest liberation of proteins (~115 mg/g dw), followed by enzyme cocktail (~84 mg/g dw) and cellulase (~58 mg/g dw). Following the release of R-PE and protein, the effect of protease was significantly higher among other enzymes on fresh biomass while it was less effective on dried biomass. The broken cell walls of fresh biomass seem to be more susceptible toward the presence of protease, which allowed better accessibility of the enzyme to the intracellular components during EAE. In this study, we did not see any evidence to show that protease degrades liberated proteins. However, investigation relying on qualitative protein properties will need to be done in the future.

The quantities of soluble sugar released in the presence of CE and ME were significantly higher compared to the control (**Figure 5C**). In contrast to the results of R-PE and protein, the presence of PE was less effective on the release of sugars (~252 mg/g dw), without significant difference against control (~218 mg/g dw). Similar values (~374 mg/g dw) were obtained for both CE and ME. These results were not in agreement to those obtained from freeze-dried biomass, where ME resulted in three times higher sugar release than CE. Synergistic relationships between enzymes in an enzyme cocktail were not observed in fresh biomass unlike in dried biomass. The exact reasoning behind these phenomena will need to be investigated further in future studies.

## DISCUSSIONS

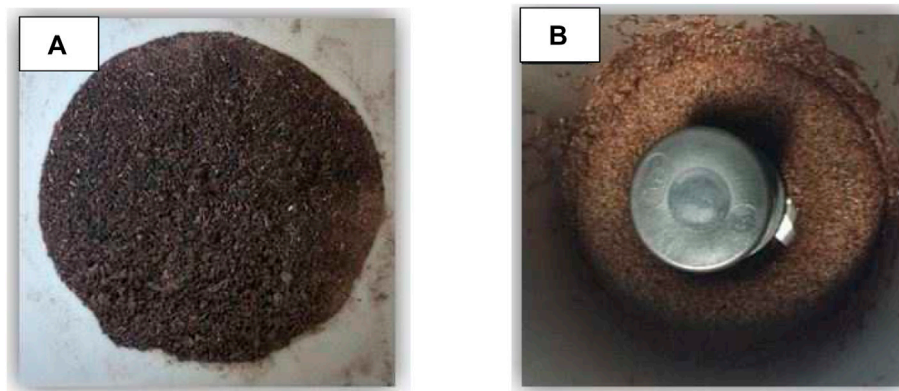
### Influence of Biomass Treatment on Extraction Yields

The use of carbohydrases and protease on enzyme assisted-extraction did improve the release yields of protein, neutral sugar, reducing sugar, and polyphenols from other red algae such as *Solieria chordalis*, *Palmaria palmata*, and *Grateloupia turuturu* (Denis et al., 2009a; Hardouin et al., 2014; Kulshreshtha et al., 2015). The comparison between our results with the literatures is summarized in **Table 3**. Endo-peptidase was shown to give the highest protein release (15.20%) from *Solieria chordalis* rather than endo-protease, cellulase, xylanase,  $\beta$ -glucanase, and arabanase (Hardouin et al., 2014). The study of Kulshreshtha et al., 2015 showed that Novozyme-cellulase allowed the highest protein recovery from red algae *C. crispus* (7.1%) among others enzymes ( $\beta$ -glucanase, ultaflo, and neutrase).

According to the study of Nguyen (2017), the yield of protein released at optimal condition was reported at 10.31% (per total content) under cellulase addition from freeze-dried *G. gracilis* biomass. Comparing to Nguyen (2017), our results showed that protein yield increased three and two times when using enzyme cocktail (32%) and cellulase (24.18%), respectively, on freeze-dried biomass. The improvement was probably related to the grinding method, which could be more efficient in our study. Furthermore, our results revealed that under enzyme addition, the release of protein improved by 6.7 (enzyme cocktail) and 5 (cellulase) times higher compared to the control (absence of enzyme) condition. This improvement was higher than the value reported by Fleurence et al. (1995), as only three times was improved in comparison to the control when an enzyme mixture of agarase and cellulase was used on *G. gracilis* for the release of protein. In this regard, our results confirmed the efficiency of using carbohydrase and protease enzyme for the release of biomolecule from algal biomass. The release efficiency depends on the enzyme type, which could vary according to the biomass species and operating conditions as well as the analysis methods.

The maximum extraction yields (per total content) of protein and sugar from dried biomass were 32% and 54%, respectively, with ME. Meanwhile, the yields from fresh biomass were higher at 46% for proteins (PE) and 85% for sugars (CE). Therefore, single enzymes seemed to work better for EAE from fresh biomass. On the other hand, an enzyme mixture may be needed to handle dried biomass.

It could be seen that biomass treatment (fresh or dried) plays an important role prior to the extraction methods. As shown in the current study, both types of biomass work better under different types of enzymes for a better release of target compounds. This was clearly due to the different levels of cell wall degradation, altering biomass susceptibility toward enzyme attacks. After grinding with liquid nitrogen, the grinded freeze-dried biomass (**Figure 6A**) was observed to be around 1–3 mm in length and the cell surface remained firm. Meanwhile, the grinded fresh biomass was a bit smaller in size at around 1–2 mm (**Figure 6B**). In addition, the cells of fresh biomass after grinding were already hydrated and seemed to be softer (due to the intracellular water), resulting in a better access of enzyme into the cell structure. From this phenomenon, an enzyme could act more efficiently as it can reach the substrates easily. The size of substrates (specific area) in addition to the temperature, pH, enzyme concentration, and extraction time are known as critical factors for the extraction process (Nadar et al., 2018). Although freeze-drying is a well-known process used to facilitate the extraction process on algal biomass (Denis et al., 2009b; Dumay et al., 2013; Munier et al., 2015; Nguyen et al., 2020),



**FIGURE 6** | Grinded *G. gracilis*: (A) freeze-dried biomass and (B) fresh biomass.

our study suggests that working on fresh biomass yielded better results. In this study, the recovery of soluble components increased by 43% for protein and 57% for sugar; yields superior than freeze-dried biomass under the condition that proper grinding method prior to EAE was applied to the fresh biomass.

### Influence of Enzymes on Freeze-Dried Biomass

Nguyen (2017) worked on the release of soluble R-PE and protein under the addition of cellulase on *G. gracilis* biomass with a maximum yield of 15.75 mg/g dw and 29.30 mg/g dw, respectively. In comparison to this study, the maximum yield of R-PE was lower albeit using an enzyme cocktail. However, the yields of protein were increased by 2.7 times and 2 times using ME and CE, respectively. Looking at the two different results from the same biomass and preparation methods, it appears that the efficiency of extraction has surely been affected by the compositions of biomass that vary according to the harvest season, location, tide, etc. The hypothesis is well supported when looking at the discrepancies for biochemical contents of our biomass and literatures, as discussed in *Biochemical Contents* previously.

Enzymes are known to be able to work synergistically. Based on the physiologically disintegrated state of the cells of freeze-dried biomass, the mixing of two enzymes allows a simultaneous attack on polysaccharides (cellulase) and proteins (protease) within the algal cell wall, resulting in a better breakdown of the cell–environment barrier. Cellulase and protease are usually employed to solubilize plant cell walls for the release of intracellular biomolecules (Nadar, et al., 2018). Fleurence et al. (1995) recovered three times more proteins from *G. gracilis* by using the enzyme mixture of agarase and cellulase. The yield of R-PE extracted from *Gelidium pusillum* was shown to improve by 26% with the enzyme consortia (agarose, cellulase, and xylanase) (Mittal and Raghavarao, 2018). Thus, the synergy between enzymes presented a strong effect on the physical disintegration of cell walls in red seaweed. In this study, it is shown that enzyme cocktail (protease and cellulase) applied in acetate buffer (50 mM, pH 5.0) is more efficient for the extraction of soluble contents from freeze-dried biomass than single enzymes. In this case, the

complementary effect of both enzymes allows a better alteration of the cellular walls of dried biomass.

By comparing both enzymes used in this study, cellulase (in acetate buffer 50 mM, pH 5.0) is theoretically more promising since it attacks polysaccharides, which are the major component of the algal cell walls. Cellulase mostly randomly attacks the internal sites of the amorphous region of the polysaccharide chains, resulting in the generation of small oligosaccharides and facilitation of the release of entrapped molecules (Fernandes and Carvalho 2017; Nadar et al., 2018). Cellulase was reported to provoke microcracks on the surface of the cell wall, which led to increased extractability of soluble compounds (Nguyen, 2017). Prior enzyme-assisted extraction studies on *Chondrus crispus* (red algae) and *Codium fragile* (green algae) also revealed that commercial carbohydrase enzyme (Novozyme-cellulase,  $\beta$ -glucanase, and ultaflo) was much more efficient than commercial protease (neutrase) for the release of protein and sugar (Kulshreshtha et al., 2015).

The effect of protease (in acetate buffer 50 mM, pH 7.5) on dried *G. gracilis* biomass for extraction of soluble compounds was less effective for both protein and sugar as shown in the current results. The release of protein and sugar were reported to be lower compared to cellulase (in acetate buffer 50 mM, pH 5.0) and enzyme cocktail (in acetate buffer 50 mM, pH 5.0). This limitation possibly came from its action mechanism that differs from cellulase. In the case of freeze-dried biomass, protease seems to work as far as destabilizing algal cell wall, thus allowing only small quantities of either sugar or protein to be liberated.

### Influence of Enzymes on Fresh Biomass

When using cellulase (16.50 mg/g dw) on fresh biomass, Nguyen (2017) obtained the recovery of soluble R-PE and protein at  $\sim$  4.62 mg/g dw and 9 mg/g dw, respectively. In this study, enzyme concentration was three times higher, but only small improvements were made on the yield of extracted R-PE. On the other hand, the recovery of protein increased significantly by 6 folds (cellulase), 9 folds (enzyme cocktail), and 12 folds (protease).

It is worth noting that the release of R-PE and protein from fresh biomass was higher in the presence of protease. Enzyme cocktail (in acetate buffer 50 mM, pH 5.0), which contains both

protease and cellulase, should logically yield better results, due to the complementary effect between enzymes, than protease (in acetate buffer 50 mM, pH 7.5) alone. However, we observed the reverse in our current findings. The combination of enzymes did not seem to boost the extractability of soluble contents on fresh biomass. This limitation might come from the nature of the substrates, referring to fresh biomass that may not work best under the combination of both enzymes (cocktail). In the enzyme assay, it is noteworthy that the state, purity, and stability of the substrate are recognized as very important factors. Enzymes are widely accepted to have a high degree of substrate specificity according to its physiological function (Bisswanger, 2014). In addition, competition between enzymes could probably occur, limiting the efficiency of enzyme cocktail during the extraction process. The presence of cellulase provoked a higher release of small oligosaccharides that could slow down the attack of protease for the release of protein. This phenomenon hinders obtaining better protein and R-PE yields from enzyme cocktail than protease alone. Additionally, enzyme cocktail did not boost the release of sugar more than cellulase alone. These information, coupled with our observations, show that enzyme consortia (cocktail) was less effective for fresh biomass than single enzyme. In conclusion, the selection of enzymes applied in EAE for fresh biomass need to take into account the target components, either protease (protein and R-PE) or cellulase (sugar).

## CONCLUSION

The addition of enzymes under the most appropriate conditions and proportions enhanced the extractability of biomolecules from algal biomass. The type of enzyme depends on the state of biomass and target components. Our study showed that complementary effect (synergy) between enzymes (enzyme cocktail in acetate buffer 50 mM, pH 5.0) was suitable for freeze-dried biomass while single enzyme (protease in acetate buffer 50 mM, pH 7.5 or cellulase in

acetate buffer 50 mM, pH 5.0) works best with fresh biomass. This study shows that biomass treatment is a preceding step to EAE that will determine the degree and selectivity of enzymes. The extraction was improved by 43% and 57% for protein and sugar liberation, respectively, from fresh biomass compared to the dried biomass. From these results, it is suggested that working on fresh biomass under the condition of applying a proper grinding method could yield better results than working on freeze-dried biomass. This way, working on wet extraction is attractive, thanks to its benefits in terms of the elimination of the drying step, the use of a single enzyme, and lower overall operational costs to scale up for industrial applications. Lastly, the qualitative analysis of extracted liquid, as well as other polysaccharidase enzymes, should be investigated to further optimize the process for other interesting, valuable intracellular biomolecules.

## DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding author.

## AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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