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Effect of cellulase and xylanase enzyms mixed with the pigment R-phycoerythrin extraction process from *Gracilariaverrucosa*

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Abstract: R-phycoerythrin is an oligomeric protein and a major photosynthetic pigment in red seaweed. R-phycoerythrin used in food industry, in cosmetic, in biology and pharmacy. In this study, enzyme mixture cellulase-xylanase was used to hydrolyze and extract the pigment R-phycoerythrin from red algae *Gracilariaverrucosa*. Different algal treatments prior to digestion were first investigated. Freeze-dried *Gracilariaverrucosa* and grinding in liquid nitrogen can improve the R-phycoerythrin extraction. For hydrolysis by enzyme cellulase-xylanase, proteins and R-PE yields obtained 25.17± 1.59 mg. g^{-1}dw and 10.32 ± 0.36 mg. g^{-1} dw, respectively. R-PE concentration isabout three times greater than without enzyme treatment in water and phosphate buffer. Enzymatic digestion appears to be an effective treatment for proteins and R-phycoerythrin extraction.Purifying by ammonium sulfate gives the interesting results in terms of extract, purify quality and economic cost.Ammonium sulfate precipitationsenhance the purity index up to 2.9 times.

Keywords: *Gracilariaverrucosa*; Enzyme; Extraction; Red seaweed; Hydrolysis; Cellulase-Xylanase.

1. Introduction

Red algae have an important role in primary production and are used in food industry, cosmetic industry, pharmacy,… [1]. *Gracilariaverrucosa*(Huds.) Papenf is one of species of the genus Gracilaria which were a rich source of agars. Besides, this red seaweed was cultured worldwide for the agar extractionbegan in the mid-1980s. Physico-chemical analyses of the thalli of *Gracilariaverrucoas* presented the high content of carbohydrate [2]. Besides, proteins and R-phycoerythrin were extracted from this red algal. Biomass productivity of *Gracilariaverrucosa* has the highest value in winter, especially in Decembre [1]. *Gracilariaverrucosa*was cultivated in Chile, China, Taiwan, Namibia, Venezuela, Malaysia, Vietnam … [1].

Phycobiliproteins (PBPs) are water-soluble light harvesting chromoproteins exist in red algae. Phycobiliproteins have four classes: phycoerythrins (PE), phycocyanins (PC), phycoerythrocyanins (PEC) and allophycocyanins (APC) [3].Phycobiliproteins have four phycobilins such asyellow phycourobilin (PUB), red phycoerythrobilin (PEB), blue phycocyanobilin (PCB) and purple phycobiliviolin (PXB). Phycourobilinand phycoerythrobilin were found in phycoerythrin (R-PE). R-phycoerythrin is an oligomeric protein which has a pink color. R-Phycoerythrin displayed a double peak of absorbance at 498 nm and 565 nm, and a shoulder at 540 nm [3]. This pigment used for fluorescence applications in clinical and immunological analysis [4]. Especially, R-PE was also used as colorant in food and cosmetic industries [5]. This pigment cost depends on the purity index (PI), the highest cost is around 400 euros per mg (Sigma Aldrich 2018). For R-phycoerythrin extraction, this compound was extracted by the different solvents such as: water, phosphate buffer, acetate buffer, hydrolysis by the different enzymes such as cellulase, xylanase, glucanase, … or used ultrasound assisted extraction,…[3][5][6]. R-phycoerythrin obtained from fresh algae, dry algae, freeze-dried seaweed,…[5][6].In this study, we used enzyme mixtures cellulase-xylanase for extracting the pigment R-PE from *Gracilariaverrucosa*.

Ammonium sulfate precipitations have been developed to purify R-phycoerythrin from the seaweed [3]. Depending on the species, different saturation percentages (25% to 100%) have obtained [3][5]. With this method, the purity index of the pigment R-PE has been increased and enhanced for *Grateloupiaturuture*, *Polysiphoniaurceolata*, *Portieriahornemannii*,…Using ammonium sulfate precipitations is simple and less expensive. Besides, other methodshave applied to purify R-PE from the algae such as chromatographic method, preparative electrophoresis,… [3][5].

2. Material and Methods

2.1 Material

Gracilariaverrucosa was collected on the Atlantic coast, France. The red seaweed was washed and cleaned in seawater, tap water and distilled water. Then *G.verrucosa*was pretreatment: one part was cut into small pieces (wet algae); another part was freeze-dried and homogenized in liquid nitrogen (dry algae or algal powder). Dry weight of the seaweed was determined by water loss estimation after 12h at 105^{o}C. Dry weight

corresponded to 19% of the wet algae.

Enzyme cellulase and enzyme xylanase from *Trichodermalongibrachiatum* purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

2.2 R-phycoerythrin extraction

Maceration - Extraction

The samples were soakedin water, phosphate buffer (20 mM, pH 7.1) in 500 mL glass reactor, darkness. Wet algae or dry algae were homogenized with 200 ml. The experiments were performed at 150 rpm during the 6 h, at 35°C and stirred. After suspended, the samples were centrifuged at 25,000 x g for 20 min, 4°C to separate into pellets and supernatants. Then theywere determined the water- soluble protein, R-phycoerythrin concentration, reducing sugar.

Hydrolysis –Extraction

Hydrolysis experiments were performed using a 500 mL glass reactor under in darkness. The samples was homogenized with 200 mL acetate buffer 50 mM, pH 6. Cellulase-Xylanase (16.5 mg. g^{-1}dw) was added to the mixture and stirred at 150 rpm during the 6 hours hydrolysis, at 35°C [5][6]. After hydrolysis, the samples were centrifuged at 25,000 x g for 20 min, 4°C to separate into pellets and supernatants. Then theywere determined the essential components.

R-phycoerythrin determination

By using the method of Beer and EshelEq (Beer and Eshel 1985) [7], R-phycoerythrin concentration and purity index were determined spectrophotometrically. The R-PE absorption spectra displayed three peaks: : two at 495 and 545 nm and one main peak at 565 nm; 455 and 592 nm . R-PE yield was expressed as mg g^{-1}dw (dry algae)[7].

$$[\text{R-PE}]\ (\text{mg.mL}^{-1}) = [(A565-A592) - (A455-A592) \times 0.20] \times 0.12 \quad (1)$$

$$\text{Purity Index or PI} = A565/A280 \quad (2)$$

Water-soluble proteins determination

After centrifuged, water-soluble proteins were determined by the method of Bradford (Bradford 1976). 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 50 mg L^{-1}) enabled the protein content to be determined[8].

Reducing sugars

The reducing sugar concentrations were determined the modified colorimetric phenol-sulfuric acid method [3]. In each assay, glucose was added as a standard. Phenol at 5% (200 µL) was added to sample or glucose solution (200 µL) followed by concentrated sulfuric acid (1 mL). The tubes were left to stand for 10 minutes at room temperature before vortexing (10 sec at 500 g), then for 15 min at room temperature and 30 min at 35°C before absorbance was measured at 490 nm.

Precipitation and dialysis

The supernatant or the crude extract was fractioned with ammonium sulfate from 70% to 100%. The sample and salt allow precipitate to homogenized and stirred for 2 hours at 4°C. Then, recover precipitate by centrifugation, remove supernatant, respin briefly to clear remaining ammonium sulfate. Finally, the precipitate was dissolved in phosphate buffer 20mM pH 7.1 and desalted overnight by dialysis using a Spectra/Por Regenerated Cellulose 3.5 kDa cut-off membrane against phosphate buffer (20 mM, pH 7.1) [3].

3. Results and Discussion

3.1. Effect of treatment of material and R-phycoerythrin extraction

In this study, wet algae and dry algae were extracted with three types of extraction solution: water, phosphate buffer 20mM pH 7.1 and mix enzyme cellulase-xylanase. After centrifuged and obtained the crude extract, we determined proteins concentration, R-phycoerythrin yield, Purity Index of R-PE, reducing sugars.

Proteins concentration

The concentration of proteins of crude extract were determined in Figure 1.The proteins extraction significantly increased for all samples of dry algae (from 14.64 ± 1.98 to 25.17± 1.59 mg. g^{-1}dw) compared to wet algae(from 1.58 ± 0.03 to 5.51± 0.08 mg. g^{-1}dw). For wet algae and dry algae, *Gracilaria verrucosa* incubated with phosphate buffer 20mM pH 7.1, no enzyme did not differ from the water and no enzyme. The quantity algae using the mix enxyme cellulase-xylanse was determined (25.17± 1.59 mg. g^{-1}dw) which was significantly higher than the remainings samples.

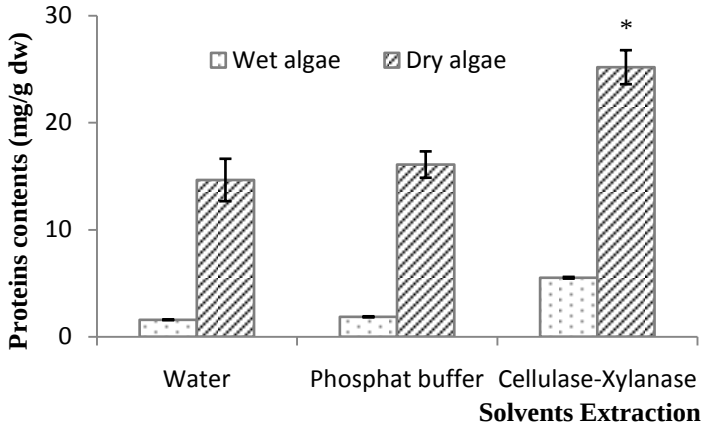

Figure 1: Proteins of *Gracilaria verrucosa*after extraction in water, phosphate buffer 20 mM, pH 7.1, mix enzyme cellulase-xylanase. Values are expressed as the mean ± SD (n=3).
NB: Anova one way significantly different results with p<5% are indicated by *.

R-phycoerythrin yield and Purity Index (PI)

The R-PE PI results obtained for each treatment are showed in Figure 2 and Figure 3.The results obtained were very similar to those for proteins. Firstly, R-PE yield significantly increased for dry algae (from 3.76 ± 0.23 to10.32 ± 0.36 mg. g^{-1}dw) compared to wet algae (from 0.42 ± 0.02 to1.44 ± 0.03 mg. g^{-1}dw) in the three conditions. The mix enzyme cellulase-xylanase was effective in degrading polysaccharides of wet algae as well as dry algae, as confirmed by the significant increase in R-PE yields 1.44 ± 0.03 mg. g^{-1}dw and 10.32 ± 0.36 mg. g^{-1}dw, respectively. Besides, PI values did not differ for all samples (from 0.02 to0.08).

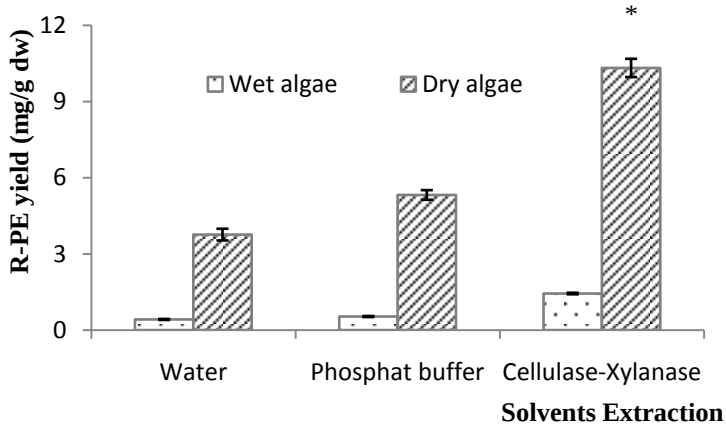

Figure 2: R-phycoerythrin of *Gracilaria verrucosa*after extraction in water, phosphate buffer 20 mM, pH 7.1, mix enzyme cellulase-xylanase.Values are expressed as the mean ± SD (n=3).
NB: Anova one way significantly different results with p<5% are indicated by *.

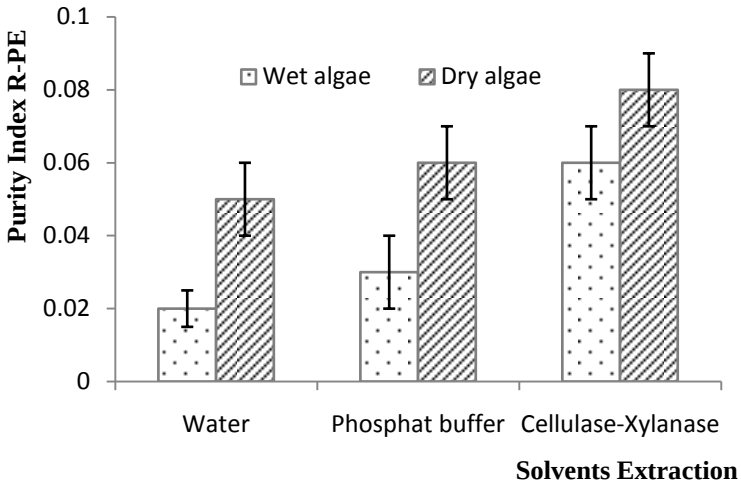

Figure 3: Purity Index of R-phycoerythrinfor*Gracilaria verrucosa*after extraction in water, phosphate buffer 20 mM, pH 7.1, mix enzyme cellulase-xylanase.Values are expressed as the mean ± SD (n=3).

Reducing sugars

After extracted and centrifuged, the reducing sugars of the samples presented in Figure 4.Reducing sugars are directly linked to the hydrolysis enzyme; the more osidic bonds are cut, the more reducing sugars appearand obtain [8].The contents of reducing sugars extracted ranged from 7.17 ± 0.48mg. g^{-1}dw (water, no enzyme) to 25.29± 0.91mg. g^{-1}dw (acetate buffer, cellulase-xylanase) from wet algae and from 15.86± 0.84mg. g^{-1}dw (water, no enzyme) to 51.07± 4.3 mg. g-1dw (acetate buffer, cellulase-xylanase) from dry algae.

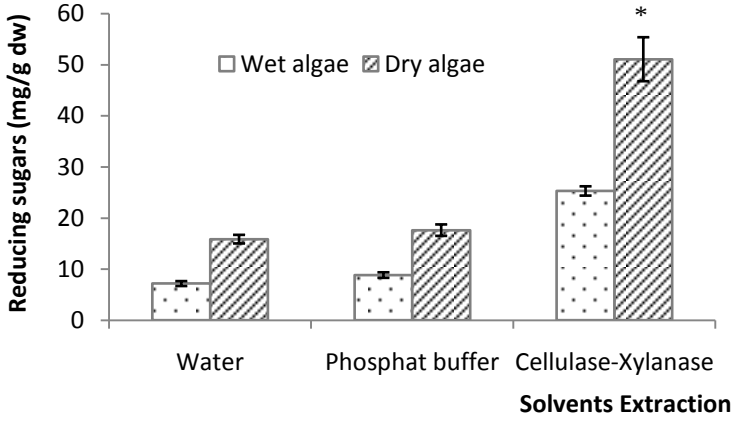

Figure 4: Reducing sugarsof *Gracilaria verrucosa* after extraction in water, phosphate buffer 20 mM, pH 7.1, mix enzyme cellulase-xylanase.Values are expressed as the mean ± SD (n=3).

From the results, the pretreatment of material was important for the extraction process. Dry algae or freeze-dried algae and grinding with liquid nitrogen may be useful for R-PE extraction for *Gracilaria verucossa*. According study of Nguyen et al. 2016 [5],grinding in liquid nitrogen can facilitate the destruction of the cell wall for red seaweed to extract R-phycoerythrin. In this study, the red seaweed had be destructed its cell wall after pretreated. Moreover, for proteins and R-phycoerythrin extraction from the red seaweed, the effective and benefits of using enzymes were confirmed by precedent studies [9][10]. By enzymatic hydrolysis, proteins and R-PE yields could increased and improved such as *Palmaria palmata, Mastocarpus stellatus, Chondrus crispus,*...[9][11][12]. Then, we continued teste the number of extract from the residue after hydrolysis with enzyme cellulase-xylanase.

3.2. The number of hydrolysis and extract

After hydrolyisis by cellulase-xylanase and obtained the crude extract, the residue will be extracted repeatedly. After three times hydrolysis and extraction, the concentration of R-phycoerythrin of CE were presented (Figure 5).

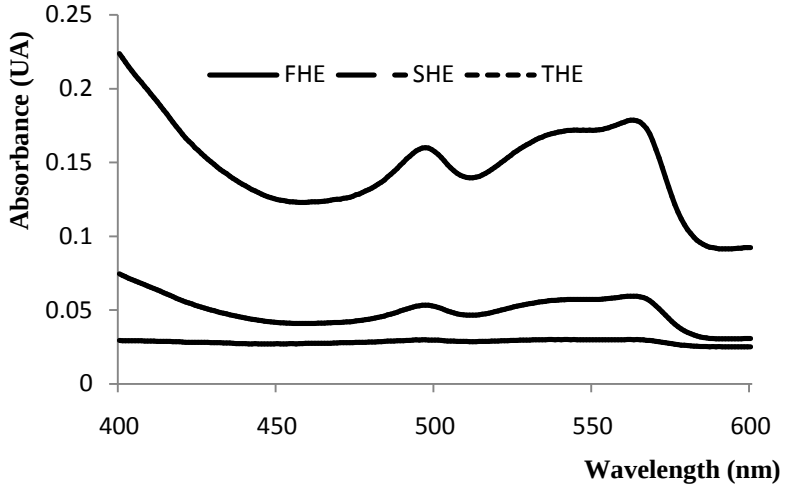

Figure 5: Absorbance spectrum of the crude extract of *Gracilaria verrucosa*after hydrolysis-extraction with cellulase-xylanase. The numbers of extract: 3 times. PHE: First hydrolysis extraction; SE: Second hydrolysisextraction; TE: Third hydrolysis extraction. Values are expressed as the mean ± SD (n=3).

The R-PE yield after first hydrolysis with enzyme cellulase-xylanase, acetate buffer50mM pH 6 obtained the highest yield, 10.32 ± 0.36 mg. g^{-1}dw. The R-phycoerythrin concentration (Figure 5) was a significant difference between the yields of the first hydrolysis-extraction step (FHE), and the contents of steps 2 and 3 (SHE, THE) in all threeextraction ($p<0.05$). The extraction concentration of the R-PE after the second and threehydrolysis are decreased. The supernatants of the first extraction presented a pink color. The samples of the extractions SHE and THE have a light pink, displaying a decrease of this pigment after be extracted repeatedly, as displayed by the data illustrated in Figure 5.

3.3. Precipitation ammonium sulfate and Dialysis

In this study, the precipitation ammonium sulfate was used to purify the pigment R-PE of *Gracilaria verrucosa*. Ammonium sulfate precipitation was used from 70-100% of saturation. Then, the samples were centrifuged and desalted overnight by dialysis using a spectra/Por Regenerated Cellulose 3.5 kDa cut-off membrane against phosphate buffer ((20 mM, pH 7.1).The purity and content of the R-PE were determined from the absorption spectrum after centrifuged and dialysis (Table 1).

Table 1: *Gracilaria verrucosa*R-phycoerythrin purity of the different fractions with the ammonium sulfate 70-100% saturation. Values are expressed as the mean ± SD (n=3).

	Fraction							
	CE	70%	75%	80%	85%	90%	95%	100%
PI	0.08± 0.01*	0.11± 0.01	0.12± 0.01	0.16± 0.01	0.22± 0.02	0.23± 0.01	0.23± 0.01	0.18± 0.02

NB: Anova one way significantly different results with p<5% are indicated by *.

The purity index of R-PE for the samples after precipitated by ammonium sulfate were higher than the crude extract.These values are significantly different ($p<5\%$). The concentration of ammonium sulfate precipitation (90%) has increased the purity index of R-PE about 2.9 times than the CE. However, the ratio A_{565}/A_{280} of the pigment R-PE by this method was still low. Consequently, the futher research can continue others methods such as the chromatography, ultra filtre to purify this pigment.

4. Conclusion

In this paper, the pretreatment of red algae *Gracilaria verrucosa* increased and improved R-phycoerythrin concentration, especially freeze-dried algae and grinding with liquid nitrogen. Moreover, using enzyme cellulase-xylanase can enhance proteins as well as the pigment R-PE yields. The results of study presented that the first hydrolysis-extraction obtained the high yield and the pink color of R-PE. After purified by ammonium sulfate, the purity index of R-PE also enhance but these value were still low. So, futher research need to study others purification methods.

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