Title:

Enhancement of R-phycoerythrin extraction from *Mastocarpus stellatus* by the use of enzymatic hydrolysis

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Abstract

R-phycoerythrin (R-PE) is the major phycobiliprotein in the red algae. R-PE can be exploited for pigment extraction and utilization such as natural colorant. *Mastocarpus stellatus*, abundantly found in French Brittany coasts, is known as a rich source of carrageenan and protein. In this study report, *Mastocarpus stellatus* is also investigated as a potential source of R-PE. The algae pretreatment is one of the important stage of the extraction procedure and determine the final extraction yields. In this study, the highest algal conditioning is achieved from freeze-dried seaweeds and grinding with liquid nitrogen. Indeed, R-PE extraction from the algal freeze-dried increased more three times than the wet algae. R-PE extraction from most seaweed is difficult due to the presence of large amounts of anionic cell-wall polysaccharides. Based on the algal cell wall degradation, enzymes are able to improve the extraction of R-PE. According to our results, action of enzymes degrading these polysaccharides is effective for the extraction R-phycoerythrin from *M.stellatus*. Using algal freeze-dried and different cell-wall-degrading enzymes have brought about the most interesting results for R-PE yield than algal thawed, especially using the enzyme xylanase. This preliminary step is then followed by the optimization of hydrolysis condition (enzyme substrate ratio, temperature and pH) by the mean of experimental design. After using the method response surface methodology, the R-phycoerythrin of algal freeze-dried extraction yields is 2.2 times greater than without enzyme treatment, 1.8 times greater than without optimization which could be considered as a good potential for the valorization of this biomass.

1. Introduction

R-phycoerythrin (R-PE) is the major phycobiliprotein in the marine red algae(Glazer, 1984). R-PE is made up of at least three different subunits and varies according to the species of algae that produces it. The subunit structure of the most common R-PE is $(\alpha\beta)_6\gamma$. R-phycoerythrin can be exploited for pigment extraction and utilization such as natural colorant and applied as a natural food dye (Dufossé et al., 2005; Sudhakar et al., 2014). R-PE has some biological activities, such as, antioxidant, antidiabetic, antitumoral, immunisuppressive and antihypertensive (Dumay et al., 2014).

Mastocarpus stellatus (Stackhouse) is a morphologically similar intertidal red macroalgae (Guiry & West, 1983). *Mastocarpus stellatus* is a rich source of carrageenan (Venugopal, 2011) and also represent a rich source protein from 7.7% to 21.3% dw (Gómez-Ordóñez et al., 2010; Mathieson & Tveter, 1976). Protein and R-PE extraction from most seaweed are difficult due to the presence of large amounts of anionic cell-wall polysaccharides (Denis et al., 2009). The benefits of using enzymatic degrading these polysaccharides on the extraction of protein and R-phycoerythrin were well known (Dumay et al., 2014). The algal cell wall degradation enzymes are able to great facilitate for the extract R-PE (Denis et al., 2009; Dumay et al., 2013).

Response surface methodology is a useful technique for investigating complex processes such as enzymatic hydrolysis. The production cost of any biotechnological process can be considerably reduced by optimizing the process (Sangkharak & Prasertsan, 2007). In our study, we will optimize the conditions of enzymatic hydrolysis for R-PE such as pH, temperature, enzyme concentration.

2. Material and method

2.1. Materials

Mastocarpus stellatus samples were collected at Batz sur mer (47°16′33.2″N-2°29′39.8″O) in the mediolittoral (Atlantic coast, France). Algae were cleaned of epiphytes and washed in seawater, tap water and distilled water. Subsequently, the algae were immediately stored at -20^0C until use. For algal pre-treatment and study hydrolysis, one part of the frozen algae was freeze-dried and homogenized in liquid nitrogen (dry algae). Another part of the wet samples was cut with an average size $<1cm^2$ (wet algae).

These enzymes used for hydrolyses were purified cellulase C9748, β-glucanase G4423 and xylanase X2629 from *Trichoderma longibrachiatum* purchased from Sigma-Aldrich (Saint Quentin Fallavier, France).

2.2. Methods

2.2.1. R-phycoerythrin determination

R-PE concentration and purity were determined spectrometrically using the classic Beer and Eshel equation (1) (Beer & Eshel, 1985) and the A_{565}/A_{280} ratio (= Purity Index or PI), respectively (Galland-Irmouli et al., 2000; Liu et al., 2009). R-PE yield was expressed as mg g^{-1}dw.

$$[\text{R-PE}] = [(A565-A592) - (A455-A592) \times 0.20] \times 0.12$$

2.2.2. Water-soluble proteins

Total water-soluble proteins in the soluble fraction were analyzed by the method adapted from Bradford (Bradford, 1976).

2.2.3. Experimental design and evaluation

Response surface methodology (RSM) was applied to determine the influence of the hydrolysis factors, including pH (5 to 7), temperature (20 to 50°C) and enzyme/substrate (E/S) ratio (20 to 40 mg/gdw). A total number of 19 experiments including 2^3 orthogonal factorial, six star point

and five central points were finished. A software statgraphics Plus v.5 Experiment Design was employed for design of these experiments.

2.3 Hydrolysis

Hydrolysis experiments were carried out using a 500mL glass reactor under controlled conditions (temperature and stirring speed) and in darkness to prevent R-PE degradation. Around 2 g of dry seaweed samples or 6g of wet algae was homogenized with 200 mL acetate buffer 50 mM, pH 5. Enzyme was added to the mixture and the system was continuously stirred at 150 rpm , 35°C during the 6 hours hydrolysis (Dumay et al., 2013).

After hydrolysis, the hydrolysate was centrifuged at 25,000g for 20 min at 4 °C (Denis et al., 2009). After centrifugation, crude extracts (CE) were stored and used to determine the water- soluble protein and R-phycoerythrin contents.

3. Results and discussion

3.1 Pre-treatment and extraction R-PE

3.1.1 Water – soluble proteins

In Fig.1, the water-soluble protein ranged from 0.76 mg g^{-1}dw to 1.72 mg g^{-1}dw with wet algae and from 1.64 mg g^{-1}dw to 2.94 mg g^{-1}dw with dry one. Regarding controls, the water-soluble proteins of wet algae allowed only 0.76 mg g^{-1}dw and one of dry algae determined approximately twice values (1.64 mg g^{-1}dw) ($p<0.05$). This study suggested that the differences in values could be due to the algae pretreatment before enzymatic hydrolysis.

In our study, the quantity of water-soluble protein obtained by enzymes after 6h treatment has increased from the control treatment (Fig. 1). Following these results, enzyme xylanase was effective at degrading cell walls for wet algae et also dry algae, as confirmed by the significant increase in water-soluble proteins 1.44 mg g^{-1}dw (wet algae) and 2.94 mg g^{-1}dw (dry algae) while these conditions of these controls with these lowers values 0.77 mg g^{-1}dw (wet algae) and 1.64 mg g^{-1}dw (dry algae). The results showed that the enzyme β- glucanase, cellulase did not seem to have any effect to destroy the cell wall of this species. Consequently, we have confirmed the water-soluble protein increase from *Mastocarpus stellatus* using enzymatic hydrolysis.

3.1.2. R-phycoerythrin extraction

As described similarity for water-soluble protein, R-PE extraction of the controls significantly increased for the dry algae (0.91 mg g^{-1}dw) than the wet algae (0.32 mg g^{-1}dw) (Fig. 2). In addition, all enzyme-treated samples of the dry algae had more values than the wet algae. The study of Denis et al., 2009 shows that the R-PE extraction from freeze-dried algae cryogenically ground provides better than a fresh biomass. In addition, the color of the crude extract from wet algal seems not rose. Therefore, the pretreatment of the samples will affect the extraction step and thus the R-PE yield.

For the wet algae, the *Mastocarpus stellatus* incubated with cellulase, β-glucanase did not different from that in control conditions of the samples. The results demonstrate the greater value of R-PE from wet algae using xylanase (0.36 mg g^{-1}dw). Besides, we also determined the quantities of R-PE of dry algae by using the enzyme xylanase (1.12 mg g^{-1}dw) which increased significantly than the samples of cellulase (0.74 mg g^{-1}dw) and β-glucanase (0.94 mg g^{-1}dw).

The R-PE Purity Index (PI) results obtained for each treatment are reported in Fig. 3. Regarding controls, PI values ranged from 0.06 (wet algae) to 0.35 (dry algae). The differences in values PI of wet algae and dry algae once again confirmed the importance of the pretreatment of seaweed before enzymatic hydrolysis. Regarding seaweeds disgested with xylanase, PI values increased from 0.09 (wet algae) to 0.34 (dry algae). Xylanase is known to exert beneficial effects on cellulose hydrolysis by degrading heterogeneous xylan polymers that shield cellulose fibers in terrestrial plants (Allen et al., 2001; Ishizawa et al., 2007). The ability extraction for R-PE seemed to

improve after combining freeze-drying material, grinding in liquid nitrogen and enzyme xylanase. Consequently, the following experiments (optimization procedure) were performed with enzyme xylanase and freeze-dried algae.

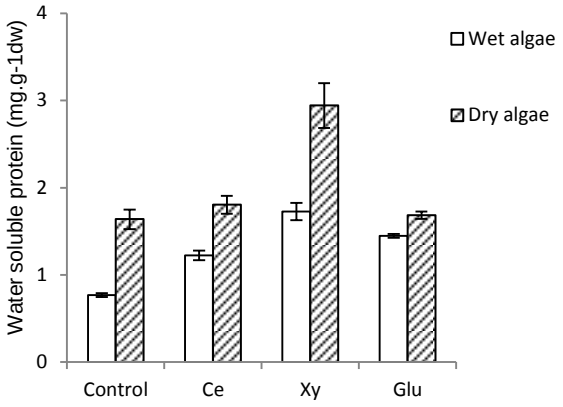

Fig.1. Effect of pretreatment methods and enzymatic hydrolysis on water-soluble protein content of *Mastocarpus stellatus*

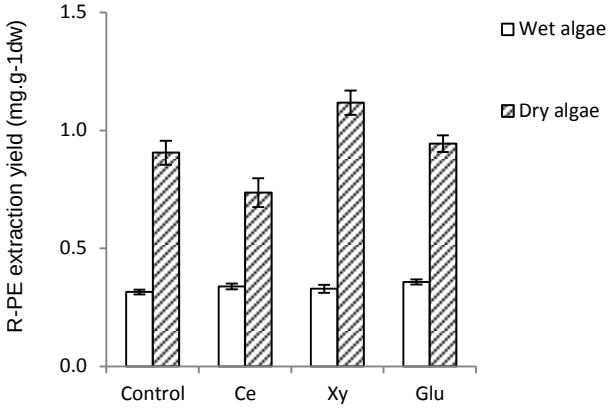

Fig.2. Effect of pretreatment methods and enzymatic hydrolysis on R-PE extraction yield of *Mastocarpus stellatus*

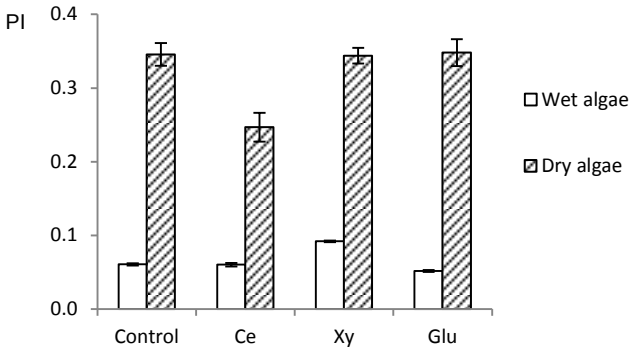

Fig.3. Effect of pretreatment methods and enzymatic hydrolysis R-PE Purity Index (PI) of *Mastocarpus stellatus*

3.2. Optimization of conditions hydrolysis enzymatic for R-PE extraction yield

With the experimental design, we performed a total number of 19 experiments including 2^3 orthogonal factorial, six star point and five central points. Based in the experimental results (data not shown), the analysis of the response surface in the investigated experimental design indicated that the highest of extraction R-phycoerythrin yield has obtained at the optimal conditions of independent variable are 12^oC (temperature), 6.45 (pH) and 13.18 mg g^{-1}dw (E/S ratio). The effect of the parameters for R-PE extraction yield have shown by three-dimensional graphs (Fig 4, A: pH and enzyme/substrate ratio, B: pH and temperature, C: temperature and enzyme/substrate ratio). This software indicates about 72.31% desirability. This mean that the high value of desirability demonstrates good reliability and indicates also here the cumulative robustness of all the factors studied (Dumay et al., 2013).

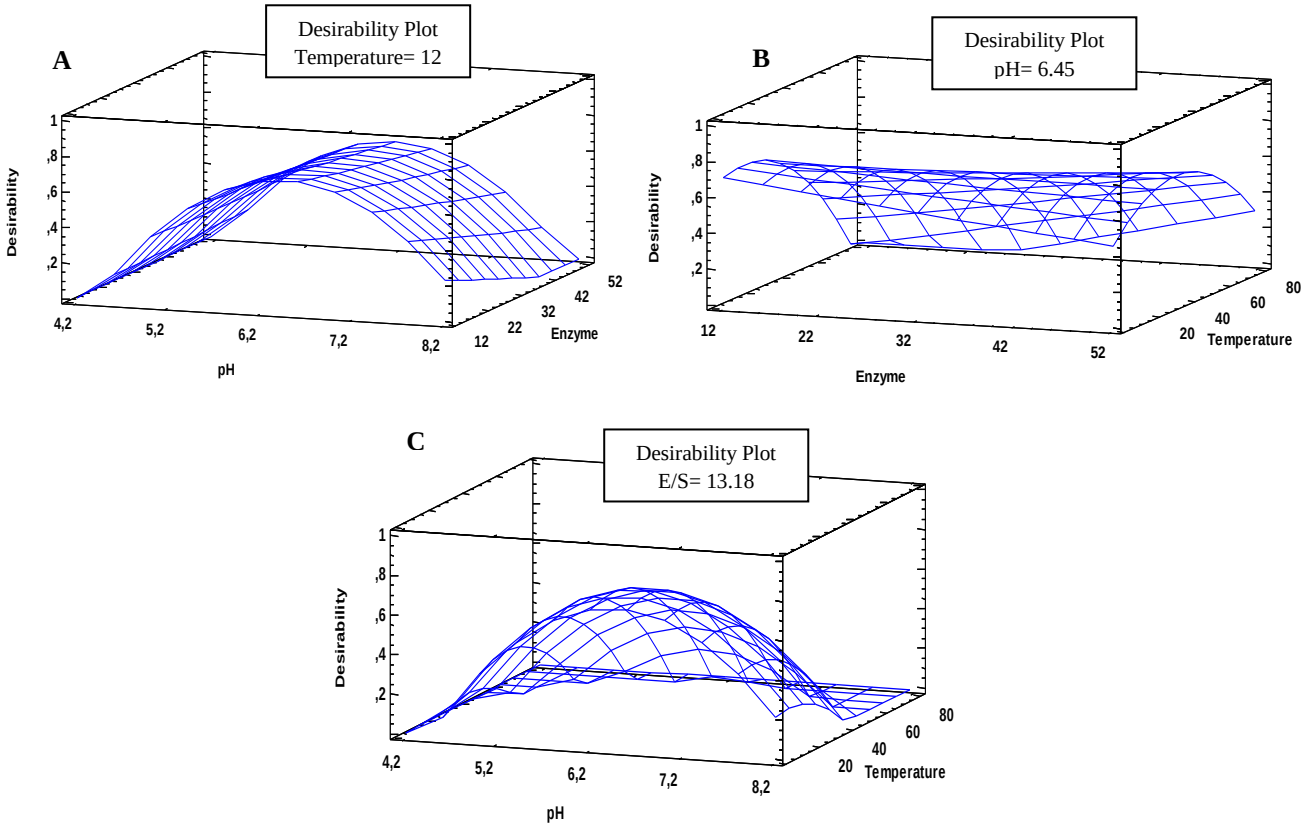

Fig.4. Estimated responses surfaces according to the pH, enzyme and temperature parameters

In the same conditions, at 12°C, pH= 6.45, with an enzyme /substrate ratio of 13.18 mg g^{-1}dw, water-soluble proteins, the optimized digestion led to a 3.07 ± 0.01 mg g^{-1}dw protein extracted yield. The R-PE yield after optimization reached 1.99 ± 0.01 mg g^{-1}dw. This result is necessary significant for industrial application we are always desirable to keep the enzyme dosage low to reduce enzyme production costs and to help also reduce products costs. Considering the R-PE extraction yield, the result using enzymes without optimization improved 1.2 times than the controls experiments (from 0,91 mg g^{-1} dw to 1,12 mg g^{-1} dw). After optimization, the R-PE yield increased about 1,8 times further (1.99 ± 0.01 mg g^{-1} dw) equipvalent to a total increased 2.2 times. Moreover, this yield was also increased more 6 times than wet algae treating enzyme xylanase. Considering the PI, the value of the control experiment obtained 0.35 while using the enzymatic digestion before and after optimization arranged from 0.34 to 0.36. Consequently, further studies will be needed to purify R-phycoerythrin and improve its quality.

4. Conclusion

In this study, enzymatic treament for *Mastocarpus stellatus* bring effective and useful for R-PE extraction. The results have showed the best algal pre-treatment for the hydrolysis enzyme and the response surface methodology has improved the R-PE productivity. Further studies on the purification maybe an interesting way for improving the PI value of R-PE from *Mastocarpus stellatus*. Consequently, *Mastocarpus stellatus* can be used as the important resource seaweed for extraction R-Phycoerythrin.

Reference

Allen, S.G., Schulman, D., Lichwa, J., Antal, M.J. 2001. A Comparison of Aqueous and Dilute-Acid Single-Temperature Pretreatment of Yellow Poplar Sawdust. *Ind. Eng. Chem. Res*, **40**, 2352-2361.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**(1–2), 248-254.

Denis, C., Morançais, M., Gaudin, P., Fleurence, J. 2009. Effect of enzymatic digestion on thallus degradation and extraction of hydrosoluble compounds from Grateloupia turuturu. in: *Botanica Marina*, Vol. 52, pp. 262.

Dufossé, L., Galaup, P., Yaron, A., Arad, S.M., Blanc, P., Chidambara Murthy, K.N., Ravishankar, G.A. 2005. Microorganisms and microalgae as sources of pigments for food use: a scientific oddity or an industrial reality? *Trends in Food Science & Technology*, **16**(9), 389-406.

Dumay, J., Clément, N., Morançais, M., Fleurence, J. 2013. Optimization of hydrolysis conditions of Palmaria palmata to enhance R-phycoerythrin extraction. *Bioresource Technology*, **131**(0), 21-27.

Dumay, J., Morançais, M., Munier, M., Le Guillard, C., Fleurence, J. 2014. Chapter Eleven - Phycoerythrins: Valuable Proteinic Pigments in Red Seaweeds. in: *Advances in Botanical Research*, (Ed.) B. Nathalie, Vol. Volume 71, Academic Press, pp. 321-343.

Galland-Irmouli, Pons, L., M, L., Villaum, C., Mrabet, N., Guéant, J., Fleurence , J. 2000. One-step purification of R-phycoerythrin from the red macroalga Palmaria palmata using preparative polyacrylamide gel electrophoresis. *Journal of chromatography. B, Biomedical sciences and applications*.

Glazer, A.N. 1984. Phycobilisome a macromolecular complex optimized for light energy transfer. *Biochimica et Biophysica Acta (BBA) - Reviews on Bioenergetics*, **768**(1), 29-51.

Gómez-Ordóñez, E., Jiménez-Escrig, A., Rupérez, P. 2010. Dietary fibre and physicochemical properties of several edible seaweeds from the northwestern Spanish coast. *Food Research International*, **43**(9), 2289-2294.

Guiry, M.D., West, J.A. 1983. Life history and hybridization studies on gigartina stellata and petrocelis cruenta (rhodophyta) in the north atlantic 1. *Journal of Phycology*, **19**(4), 474-494.

Ishizawa, C.I., Davis, M.F., Schell, D.F., Johnson, D.K. 2007. Porosity and Its Effect on the Digestibility of Dilute Sulfuric Acid Pretreated Corn Stover. *Journal of Agricultural and Food Chemistry*, **55**(7), 2575-2581.

Liu, L.-N., Su, H.-N., Yan, S.-G., Shao, S.-M., Xie, B.-B., Chen, X.-L., Zhang, X.-Y., Zhou, B.-C., Zhang, Y.-Z. 2009. Probing the pH sensitivity of R-phycoerythrin: Investigations of active conformational and functional variation. *Biochimica et Biophysica Acta (BBA) - Bioenergetics*, **1787**(7), 939-946.

Mathieson, A.C., Tveter, E. 1976. Carrageenan ecology of Gigartina stellata (Stackhouse) Batters. *Aquatic Botany*, **2**(0), 353-361.

Sangkharak, K., Prasertsan, P. 2007. Optimization of polyhydroxybutyrate production from a wild type and two mutant strains of Rhodobacter sphaeroides using statistical method. *Journal of Biotechnology*, **132**(3), 331-340.

Sudhakar, M.P., Saraswathi, M., Nair, B.B. 2014. Extraction, purification and application study of R-Phycoerythrin from Gracilaria corticata (J. Agardh) J. Agardh var. corticata. 371-374.

Venugopal, V. 2011. *Marine polysaccharides*. Food applications, CRC Press.