International Food Research Journal 24(4): 1660-1666 (August 2017)
Journal homepage: *http://www.ifrj.upm.edu.my*

Enzyme aided extraction of sulfated polysaccharides from *Turbinaria turbinata* brown seaweed

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Article history

Received: 30 July 2016
Received in revised form: 22 August 2016
Accepted: 24 August 2016

Keywords

Sulfated-polysaccharide
Enzyme-aided-extraction
Brown seaweed
Optimization
Fucoidan

Abstract

This study involves extraction of sulfated polysaccahride (SP) from brown seaweed (*Turbinaria turbinata*). Eight processing conditions affecting enzyme aided extraction (EAE) were screened using Plackett-Burman design. Three significant factors (hydrolysis time, enzyme concentration and extraction stage) were optimized using Faced Centred Central Composite Design in Random Surface Methods. Micrograph obtained using Field Emission Scanning Electron Microscopy revealed that cellulase degradation ruptured the seaweed cell matrix thus caused increase in the release of SP. The optimum conditions for extraction of SP from *T. turbinata* are: extraction stage of 2, hydrolysis time of 19.5 h and enzyme concentration of 1.5 µl/ml to produce 25.13% yield. The SP obtained from cellulase treated *T. turbinata* is a suitable anti-inflammatory agent for pharmaceutical applications.

Introduction

Marine organisms contain sulfated polysaccharides (SPs) of therapeutic potentials (Jaswir and Monsur, 2011). Sulfated fucans extracted from Lobophora variegate inhibited migration of leucocyte to inflammatory site (Cumashi *et al*., 2007) and sulfated fucans obtained from Laminaria saccharina inhibited recruitment of leucocyte and adhesion of neutrophils to platelets (Croci *et al*., 2011). In-vivo studies have revealed that fucoidans of various brown seaweeds exhibited anti-inflammatory property by inhibiting secretion of nitric oxide (NO) in lipopolysaccharide (LPS) induced mammalian cell lines (Hwang *et al*., 2011; Kang *et al*., 2011).

Cell wall of brown seaweed contains cross links of polysaccharides for mechanical support. SPs are embedded within the polysaccharide-polymer matrix of the cell wall which contains cellulose microfibril (Mackie and Preston, 1974; Mabeau and Kloareg, 1987; Bilan *et al*., 2004). Efficiency of conventional aqueous extraction of SPs from brown seaweed is challenged by presence of cell wall polymers. Therefore, research efforts have been channelled toward development of extraction aided methods. Among various extraction aiding methods, enzyme aided extraction (EAE) has high potential because it is a green process. Enzymatic hydrolysis of the cell wall polymers has been stated to enhance biocompounds extraction (Puri *et al*., 2012; Wijesinghe and Jeon, 2012).

Use of EAE to obtain bio-compounds from algae has numerous advantages including increase in yield, release of novel biocompounds, retain structure and biological properties of extract (Hammed *et al*., 2013). As a bioprocess technique, EAE is affected by numerous factors that require thorough investigation. Siriwardhana *et al*. (2004) studied effect of type of enzymes, hydrolysis time and enzyme concentration on EAE of antioxidative compounds from Hizikia fusiformis, Heo *et al*. (2003), Kang *et al*. (2011) and Athukorala *et al*. (2009) studied effect of type of enzymes on EAE of anti-oxidative, anti-inflammatory, and anti-proliferative extracts from Ecklonia cava while Park *et al*. (2009) studied effect of type of enzyme on anti-oxidative extracts from Laminaria japonica. However, other conditions (agitation speed, extraction stages, solvent-substrate ratio, pH and temperature) have not been investigated.

Literature search revealed that optimization studies on extraction of SPs from brown seaweed have been conducted. Zhu *et al*. (2010) optimized crude polysaccharide extraction from Hizikia fusiformis using conventional aqueous extraction method, Ale *et al*. (2012) optimized extraction of fucose-containing sulfated polysaccharide from Sagassum sp. using acid-hot water extraction method, Rodriguez-Jasso *et al*. (2011) optimized sulfated polysaccharide extraction from Fucus vesiculosus using microwave assisted extraction method, Wang *et al*. (2010)

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optimized sulfated polysaccharide extraction from Eucheuma striatum using ultrasonic-assisted extraction method and Rodríguez-Jasso *et al*. (2013) optimized extraction of sulfated polysaccharide from Fucus vesiculosus using autohydrolysis method. However, there is no work on optimization of EAE of SPs from *T. turbinata*.

In this study, eight extraction conditions that affect EAE were screened using Plackett Burman design and three extraction conditions, with positive effect on yield, were optimized using Faced Centred Central Composite Design in Random Surface Methods (FCCCD-RSM). Anti-inflammatory property of extracted SPs was also investigated.

Materials and Methods

Design of experiments

Plackett-Burman design was applied to determine the important factors among eight factors that potentially affect EAE. Three factors (Hydrolysis time, enzyme concentration and extraction stages) that exhibited positive significant effects on extract yield were then optimized using FCCCD-RSM. The FCCCD was fitted with second order model Equation 1.

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i^2 + \sum_{i<j}^{k} \beta_{ij} X_i X_j \quad (1)$$

Where, Y is the predicted response (Yield, %); Xi and Xj are input variables that influence the response Y; k is the number of variables; β0 is the constant term; βi is the ith linear coefficient; βii is the ith quadratic coefficient and βij is the ijth interaction coefficient.

Enzyme aided extraction of crude extract

EAE was carried out according to previous method (Kang *et al*., 2011) with slight modification. The enzymes (cellulase, amyloglucosidase and vicozyme) used in this study were obtained from Sigma Aldrich, USA. Fresh seaweeds, harvested from Straits of Malacca near Port Dickson, Malaysia, were kept in black plastic bag and transported to the laboratory in ice box. The plant materials were washed with tap water (x3) followed by distilled water (x1) to remove salt and shrubs, and then freeze dried. Dried samples were ground into powdered with the aid of an electric blender and then sieved through 500 µm size sieve. A mass of 2.5 g or 5 g of dried seaweed samples was homogenized with 100 ml or 200 ml, respectively, distilled water to make a ratio of 1:40 (w/v) and mixed with 25 µl enzyme of enzyme solutions. After hydrolysis, the samples were boiled for 10 min to inactivate the enzymes. Thereafter, the samples were clarified by centrifugation (3000 rpm, for 10 min at 4°C) to remove the residue (Kang *et al*., 2011). The supernatant was concentrated and subjected to ethanol precipitation by slow addition of 95% ethanol under continuous stirring until concentration reached 80% ethanol. The precipitate was washed with 95% ethanol and then acetone and dried for 48 h.

Infrared spectral analysis of the purified polysaccharides

IR spectrum of SPs was obtained using Fourier transform infrared spectrometer (FTIR, Nicolet 8700 Thermo Scientific) equipment. The SPs was ground with KBr and pressed to form pellet disc. The frequency range used was between 4000 - 400 cm^{-1}.

Micrograph using FESEM

Residues of non-enzyme and cellulase-hydrolysed seaweed, obtained after centrifugation, were air dried. Dried samples were mounted separately on stubs and sputter-coated with gold. The samples were examined using a Field Emission Scanning Electron Microscope (FESEM, JSM 6700F; JEOL, Tokyo, Japan). Photographs of samples were taken at x10,000.

Cell culturing and treatment with sulfated polysaccharides

Cell culture (100 µL of RAW 264.7) was plated into each well of the 96 well plate for 12 h to make a population of 1.0 x 10^5 cells/well. Then 50 µL of DMEM containing various concentrations of the extracts was added into each well, followed by incubation for 2 h at 5% CO_2 and 37°C. Cells were induced with 50 µL of Dulbecco's Modified Eagle Medium (DMEM) containing lipopolysaccharide (LPS) and further incubated for 24 h. The final concentration of LPS in the culture media was 1 µg/mL.

Determination of NO secretion LPS induced RAW 264.7 cell line

The secretion of NO in culture media was determined using Griess reagent. A volume of 100µL of supernatant from each cell culture well was reacted with 100 µL Greiss reagent (1% sulfanilamide in 2.5% phosphoric acid and 0.1% naphthylenediamine dihydrochloride in water). Absorbance at 540 nm was taken after 30 min at room temperature with microplate reader (Amersham Pharmacia Biotech, USA). The percentage NO inhibition was estimated according to Equation 2.

Table 1. ANOVA of response surface model

Source	Sum of Squares	df	Mean Square	F Value	p-Value Prob > F
Model	68.38561	9	7.598401	78.77493	< 0.0001
X_1	5.65504	1	5.65504	58.62752	< 0.0001
X_2	15.47536	1	15.47536	160.4378	< 0.0001
X_3	1.849	1	1.849	19.16915	0.0014
$X_1 \times X_2$	9.0738	1	9.0738	94.07084	< 0.0001
$X_1 \times X_3$	1.0658	1	1.0658	11.04947	0.0077
$X_2 \times X_3$	2.6912	1	2.6912	27.90049	0.0004
X_1^2	2.524809	1	2.524809	26.17546	0.0005
X_2^2	4.634509	1	4.634509	48.04737	< 0.0001
X_3^2	1.379184	1	1.379184	14.29842	0.0036
Residual	0.964571	10	0.096457		
Lack of Fit	0.698238	5	0.139648	2.621668	0.1568

Extraction stages (X1)
Hydrolysis time (X2)
Enzyme concentration (X3)

$$\% \text{ NO inhibition} = \frac{A-B}{C-B} \times 100 \quad (2)$$

Where, A = absorbance of treated culture media
B = absorbance of untreated culture media
C = absorbance of LPS induced culture media

Results and Discussion

Screening of enzymes

Cellulase, vicozyme and amyloglucosidase (singly or combined) were used to hydrolyse *T. turbinata* to obtained the water soluble extracts. The yield of ethanol insoluble of the extracts and their percentage nitric oxide (NO) inhibition in lipopolysaccharide (LPS) induced RAW 264.7 cell line were determined (Figure 1). The yields obtained from EAE processes were higher than their respective non-EAE processes. Cellulase assisted process gave highest yield (~20%) among the EAE processes.

All extracts exhibited dose dependent inhibition of NO secretion in LPS induced RAW 264.7 macrophage cell line. Extract obtained from enzymatic extraction method inhibited NO secretion than that of the control (non-enzymatic aided extracts) in LPS induced RAW 264.7 cell line. In agreement with previous work, crude enzymatic extracts obtained from Ecklonia cava reportedly down-regulate NO secretion in RAW 264.7 cell line in a dose dependent manner and that all enzyme aided extracts inhibited NO higher than non enzymatic extract (Kang *et al*., 2011). The results of yield and NO inhibition suggest that EAE enhanced release of anti-inflammatory SPs from *T. turbinata*.

Screening of processing conditions

Placket Burman design was used to screen the effect eight processing conditions on yield of extracts. The experimental result for the 12 trials in Plackett Burman design was that the yield varied widely ranging from 14.00 - 21.00%. This range is comparable with previous works. Zhu *et al*. (2010) reported a yield of 21.83% sulfated polysaccharide from Hizikia fusiformis using conventional aqueous extraction and Rodríguez-Jasso *et al*. (2013) reported a yield of ~16.5% of fucoidan from F. vesiculosus using autohydrolysis. However, Ale *et al*. (2012) reported lower value of 7.0% of fucose-containing sulfated polysaccharide from Sagassum sp. using acid-hot water.

The variation in the yield obtained in screening stage suggested the need for optimization study in order to improve the yield of SP using EAE. Statistical analysis of regression coefficient and t-value of 8 factors have shown that all the factors have positive effect except temperature and agitation speed (result not shown). Also, agitation speed, enzyme concentration, hydrolysis time and extraction stages were significant ($P<0.05$). The factors (enzyme concentration, hydrolysis time and extraction stages) with significant positive effects on the yield of SP were considered for the optimization study.

Optimization by response surface methodology

The result for the experimental design in FCCCD represented shows that the yield ranges from 17.30 to

25.10% in all the 20 runs. The data was analysed with multiple regression and a second-order polynomial equation and resulted in the model in Equation 2.

$$Yield = -2.49 + 7.45X_1 + 1.05X_2 + 12.75X_3 - 0.12X_1X_2 - 0.73X_1X_3 - 0.13X_2X_3 - 0.98X_1^2 - 0.02X_2^2 - 2.83 \quad (2)$$

Table 1 shows the analysis of variance (ANOVA) for FCCCD-RSM experimental design. All factors and their interactions are significant ($P<0.05$) and the model was also significant ($P<0.05$). There was only 0.01% chance that a "Model F-value" this large could occur due to noise. The adequacy of the model was checked with lack of fit (LOF). The LOF represents variation of experimental data around the model and was used to investigate the fitness of the model. The ANOVA result shows that LOF is not significant ($P<0.05$) with p-value of 0.1558. Non-significant LOF is good and that there is a 15.68% chance that a "LOF F-value" this large could occur due to noise.

The coefficient determination (R2) of 0.9861 indicates good agreement of experimental and predicted values, the adjusted coefficient of determination (Adj. R2) of 0.9736 is very close with the predicted coefficient of determination (Pred. R2) of 0.9223. Adequate precision measure the signal to noise ration and a value greater than 4 is desirable. Here adequate precision of 33.6986 indicates an adequate signal; thus, the model can be used to navigate the design space. A low value of coefficient of variation (CV) calculated as 1.33% demonstrated that the performed experiment was highly reliable.

Design-Expert software was used to obtained graphical representation; response surface plots (3-D view), and their corresponding contour plots (2-D view,) of the regression model (Figure 2). This is to investigate the interactive effects of the factors and to find the optimal level of each factor in order maximized the yield (Rui *et al*., 2009). The shape of the contour plots show if mutual interaction exists between the factors.

Figure 2a shows that the yield of SPs increased with increase in hydrolysis time (from 6 to 20 h) and extraction stage (1 to 2) and then gradually stabilized and tend to reduce. Figure 2b shows that increase in the enzyme concentration from 1 to 1.5 µl/ml caused increase in the yield of SP. Figure 2c shows that increase in enzyme concentration and hydrolysis stage resulted into gradual increase in the yield of SP. In overall, each response plot shows a clear peak for the response – yield – which suggested that the optimum point was within the boundary of design.

The model equation was used to solve the regression equation in order to generate optimum

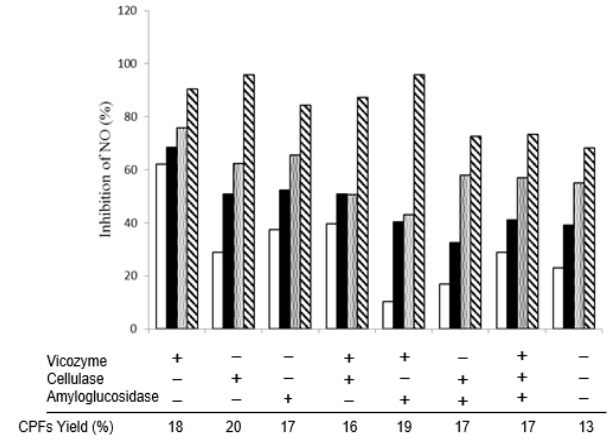

Figure 1. Inhibition of NO secretion in LPS induced RAW 264.7 macrophage by crude enzymatic CPFs extracts of *T. turbinata*. Yield (%) of enzymatic CPFs extracts of *T. turbinata*. + enzyme present, - enzyme absent. Error bars represent standard deviation and n = 3, 200 µg/ml, 100 µg/ml and 50 µg/ml and 50 µg/ml.

extraction condition. The optimum conditions suggested by the model are: extraction stage of 2, hydrolysis time of 19.5 h and enzyme concentration of 1.5 µl/ml to produce 25.13% yield of sulfated polysaccharide. The yield obtained after optimization was ~24% increment compared to un-optimized condition from Plackett-Burman design.

In order to validate the predicted model conditions, another set of extractions experiments were carried out using the optimum condition and their yields were averaged. The results from validation experiments give an average yield of 25.05±0.33 which is very close to the predicted value. This indicated that the response model was adequate for the optimization.

FTIR analysis

Figure 3 shows the FT-IR spectra of SPs obtained from *T. turbinata* using EAE. The spectra exhibited major absorption band at 3445.91 cm^{-1} which correspond to O-H stretching. Other absorption bands and their corresponding bonds exhibited by the spectra are: 1624.25 cm-1 for C-H of carboxyl groups of uronic acid, 1260.75 cm^{-1} of S=O stretching vibration of sulfate ester, 2958.3 cm^{-1} of C-H stretching, 1418.77 of scissoring vibration of CH_2 and 817.94 for C-O-S secondary equatorial sulfate. The spectra is similar to that obtained for SP in numerous reports (Zvyagintseva *et al*., 1999; Kang *et al*., 2011; García-Ríos *et al*., 2012; Dore *et al*., 2013), thus supporting that the SP was not affected throughout the extraction procedure.

Micrograph analysis

According to Figure 4, the seaweed residue

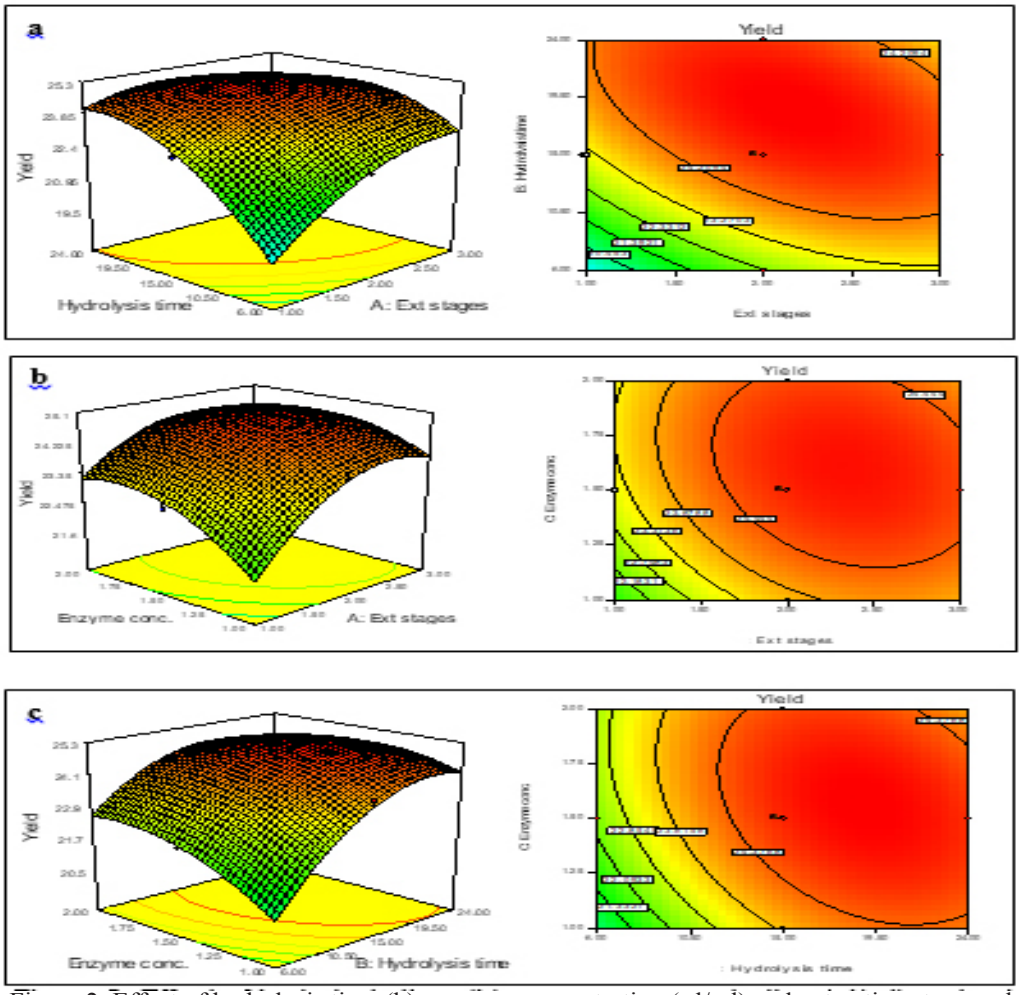

Figure 2. Effect of hydrolysis time (h), enzyme concentration (µl/ml) and extraction stage and their reciprocal interaction on extraction yield in 3D response surface and 2D contour plots).

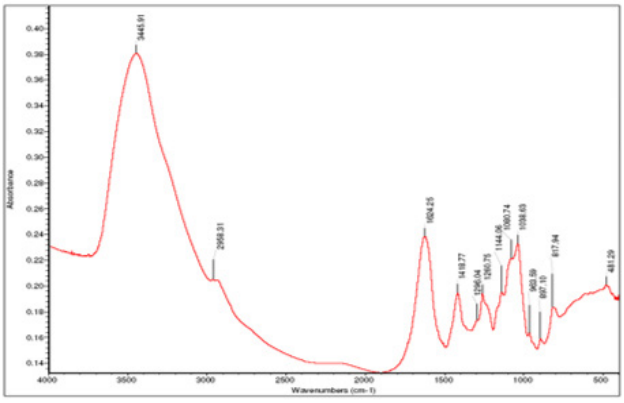

Figure 3. FTIR spectra of sulfated polysaccharide extracted from *T. turbinata* using EAE

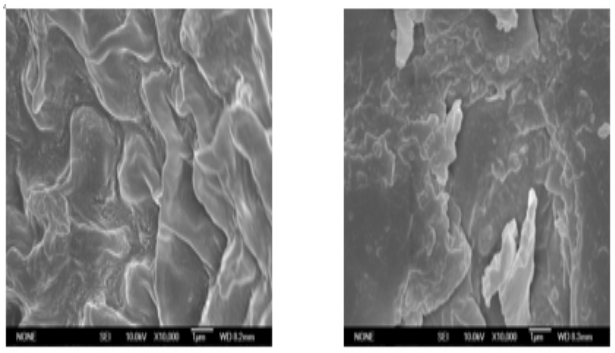

Figure 4. FESEM Micrographs of seaweed samples (residue) after extraction at magnification of x1000.

without cellulase hydrolysis has wavy patterns which was absent in substrate hydrolysed with cellulose. Retention of wavy patterns might mean that the sample was not ruptured and that cellulose crystalline layers are present in seaweed which are responsible for maintaining cell wall integrity. Rough surface with star fish-like structure was previously observed in seaweed swelled with ionic liquid compared (Takahashi *et al*., 2013). Hydrolysis of cellulose crystalline components of cell wall polymers by cellulase might be responsible for loss of wavy-patterns. It is possible that seaweed samples are well ruptured/degraded by cellulase, resulting into un-wavy micrograph, allowing rapid extraction of biochemicals.

Conclusion

In conclusion, we established that the EAE enhance extractability of SP from brown seaweed – *T. turbinata*. Conditions affecting enzymatic extraction of sulfated polysaccharide from brown seaweed (*T. turbinata*) were optimized using statistical experimental designs (Plackett-Burman and FCCCD-RSM). The cell wall of *T. turbinata* was ruptured when treated with cellulase, thus, enhanced the release of SP. Use of cellulase during the EAE process did not degrade the SP. The SP obtained from cellulase treated *T. turbinata* exhibited anti-inflammatory potential for pharmaceutical applications. Future works will include purification, characterization and in-vivo study of the SP.

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