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Purification and identification of polysaccharide derived from *Chlorella pyrenoidosa*

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Abstract

The conditions for extracting and purifying polysaccharides from *Chlorella pyrenoidosa*, including intensity and duration of ultrasound, the temperature and incubation time, and ethanol concentration, were investigated through an orthogonal design of $L_{16}(4^5)$ in this work. High performance liquid chromatography (HPLC) and gas chromatography (GC) were used to characterize the compounds in *C. pyrenoidosa*. The highest yield of 44.8 g kg^{-1} was achieved at 400 W of ultrasound for 800 s and then followed by incubation in water bath at 100 °C for 4 h in 80% ethanol. Two polysaccharide fractions (S1 and S2) were separated from the extracts of *C. pyrenoidosa* using Sepharose 4B column chromatography. The average molecular weights (M_w) of S1 and S2 were 81,877 Da and 1749 Da, respectively. Gas chromatographic (GC) traces of the hydrolyzed polysaccharides showed that most of the majority of monosaccharide in both fractions was mannose (78.0% and 76.5% of relative mass from S1 and S2, respectively) with low levels of glucose (13.2% and 8.4% of relative mass from S1 and S2, respectively). The Fourier-transform infrared spectra (FT-IR) of S1 and S2 revealed typical characteristics of polysaccharides. Both samples had the characteristics of hydroxyl groups, weak C–H band and α-pyranoses; however, only S2 had a carboxyl group.

Keywords: *Chlorella pyrenoidosa*; Polysaccharide; Chemical composition

1. Introduction

Studies on seaweeds have attracted more attention in recent years due to the potential biological functions. Edible seaweeds are nutritionally valuable as fresh or dried vegetables. *Chlorella pyrenoidosa* has been named a green healthy food by FAO and is an economic alga. It is also widely applied as an important ingredient for food industry (Robledo & Freile Pelegrin, 1997).

Purified seaweed polysaccharides, such as agar and carrageenan, are used extensively in industry (De Philippis & Vincenzini, 1998; Franz, 1989). In recent years, their biological activities, e.g. antitumor, antivirus, antihyperlipidemia and anticoagulant, have been found to be associated with polysaccharides in some species of seaweed which have been purified and developed as a new generation of drugs (Anderson, Beveridge, & Capon, 1994; Franz & Alban, 1995; Güven, özsoy, & Ulutin, 1991; Matsubara et al., 2001; Schaeffer & Krylov, 2000; Trento, Cattaneo, Pescador, Porta, & Ferro, 2001; Zhang, Zhou, Chen, Ling, & Hou, 2002). Studies are widely conducted on isolation, purification and structural analysis of polysaccharides (Girod, Baldet-Dupy, Maillols, & Devoisselle, 2002; Maksimova, Bratkovskaia, & Plekhanov, 2004; Ogawa, Ikeda, & Kondo, 1999; Pugh, Ross, ElSohly, ElSohly, & Pasco, 2001; Roberts, Zhong, Prodolliet, & Goodall, 1998; Zvyagintseva et al., 1999). Several reports have indicated that chemical components and molecular weight (M_w) of seaweed polysaccharides are among the major important factors responsible for biological activities (Wang et al., 2004; Zhou et al., 2004).

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However, use of polysaccharides is restricted for various reasons, including a lack of simple methods for isolating them from extracts. Although several methods for isolation of seaweed polysaccharides are reported, all of them are rather labour intensive and time consuming. The use of ultrasound results in increased component extraction in a shorter time and at lower temperatures. The aim of this study is to further define the conditions for extracting and purifying the polysaccharides from *C. pyrenoidosa* and ubiquitously improve polysaccharide production and shorten procedure time, providing a foundation theory for applying the technology to mass production. The parameters used in this work include the intensity and duration of ultrasound, the temperature and time of incubation, and the concentration of ethanol in extraction buffer. The molecular weights (M_w) and chemical compositions of purified polysaccharides were also measured.

2. Materials and methods

2.1. Materials

C. pyrenoidosa was provided by Tomorrow Biology & Technology Development Co., Ltd., Nanjing, China.

2.2. Chemicals

The following chemicals were used: trichloroacetic acid (TCA) (ca. 99%); ethanol (95%. Nanjing Chemical Industry, Nanjing, China); Sepharose 4B (Pharmacia, Sweden). Other reagents were of analytic reagent grade.

2.3. Preparation of crude polysaccharide

The polysaccharide extraction parameters are shown in Table 1. Five grammes of defatted *C. pyrenoidosa* powder were mixed with 100 ml of distilled water. The cells were disrupted by ultrasonic waves (JY98-III Ultrasound cell breakage apparatus). The resulting sample was incubated in a water bath at 100 °C and then centrifuged at 4500 rpm for 10 min. Trichloroacetic acid (TCA) was added to the samples, which were centrifuged at 7000 rpm for 15 min and the pellet was removed. The supernatant was dialyzed against deionized water for 24 h before concentration under vacuum evaporator at 55 °C. The crude polysaccharides were precipitated with absolute ethanol and then lyophilized for 48 h (Liu, Yang, & Lin, 2001; Oliveira, Marques, & Azeredo, 1999).

2.4. Purification of polysaccharide

Column chromatography was performed with Sepharose 4B (2×50 cm). Five milliliter of the extracts at 800 mg l^{-1} were subjected to chromatography. The column was eluted with deionized water at the flow rate of 30 ml h^{-1}. The isolated fractions were measured by the phenol–sulphuric acid method. Finally, two fractions of polysaccharide, S1 and S2, were obtained and lyophilized for 48 h for other assays.

2.5. Analysis of polysaccharide

The polysaccharide was determined by the phenol–sulfuric acid reaction, using glucose as standard (Cuesta, Suarez, Bessio, Ferreira, & Massaldi, 2003).

2.6. Determination of molecular weight

The molecular weight of samples were determined by HPLC on two Water Ultrahydrogel TM Linear 7.8×300 mm columns and eluted with 1 mol l^{-1} of sodium acetate solution at a flow rate of 0.9 ml min^{-1} at 45 °C. Elution was monitored by a refractive index detector. The column was calibrated with standard glucosans from Sigma (Molecular weight: 828, 2900, 10,000, 70,000, 188,000, 482,000, 2,000,000 Da) and a standard curve was then stablished (Zhang et al., 2004).

2.7. Spectroscopic methods

Fourier-transform infrared spectra of the polysaccharides were recorded with a Nicolet 5DXB FT-IR spectrometer in the range 3500–500 cm^{-1}, using the KBr disk method (Wang et al., 2004).

2.8. Neutral sugar analysis

Purified polysaccharide sample was hydrolyzed in 2 M HCl for 2.5 h at 105 °C in a sealed glass tube. The residual acid was removed under vacuum, followed by co-distillation with water. Then the hydrolyzates were converted to

Table 1
Main variables and their levels chosen in the extraction

Levels	Main parameters				
	Temperature of incubation (A, °C)	Intensity of ultrasound (B, W)	Duration of ultrasound (C, s)	Time of incubation (D, h)	Concentration of ethanol (E, v/v)
I	70	400	0	0	70%
II	80	600	400	4	75%
III	90	800	600	6	80%
IV	100	1000	800	8	85%

acetylated aldononitrile derivatives according to conventional protocols and analyzed by GC (Shimadzu, GC14A) using an OV-1701 capillary column (30 m × 0.32 mm ID). As references, the following neutral sugars were converted to their acetylated derivatives and analyzed: rhamnose, arabinose, xylose, mannose, glucose and galactose (Zhang et al., 2003).

2.9. Statistical analysis

The data were presented as means ± standard deviations of three determinations. Statistical analyses were performed using the student's *t*-test and one way analysis of variance. Multiple comparisons of means were done by the LSD (least significance difference) test. A probability value of <0.05 was considered significant.

3. Results and discussion

3.1. Effect of extracting conditions on polysaccharide yield of *C. pyrenoidosa*

An orthogonal design [$L_{16}(4^5)$] was applied to optimize the intensity and duration of ultrasound, the temperature and time of incubation, and the concentration of ethanol in extraction buffer (Table 1). Table 2 shows the yields of the extraction of polysaccharide under the indicated conditions. Basically, the stronger ultrasound for a longer time and higher temperature of incubation gave a higher yield of polysaccharide. A great increase in yield was observed at temperatures between 80 °C and 100 °C. A longer incubation in hot water produced a higher yield. However, after 4 h, the increase in yield became insignificant. The yield reached the peak of 22.7 g kg^{-1} at 80% ethanol. The extraction temperature and incubation time also had significant impacts on the overall yield of polysaccharide. The orders of impact of the different factors on the yield are: the temperature of incubation > the time of incubation > the intensity of ultrasound applied > the duration of cell breakage > the concentration of ethanol.

3.2. Purification of the polysaccharides

Chromatography results of crude *C. pyrenoidosa* polysaccharide are shown in Fig. 1. The concentrations of total saccharides in the eluate were determined and two peak

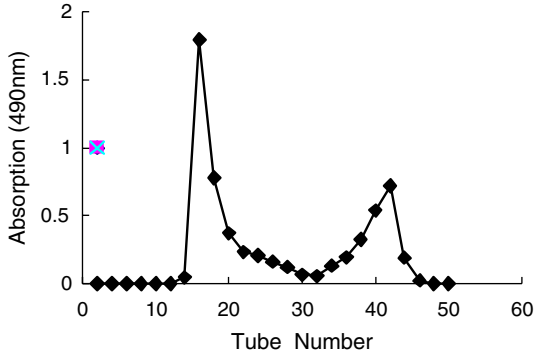

Fig. 1. The purification of crude *Chlorella pyrenoidosa* polysaccharides by Sepharose 4B column chromatography.

Table 2
Polysaccharide yields under the conditions indicated using $L_{16}(4^5)$ orthogonal design

Run	Temperature of incubation	Intensity of ultrasound	Duration of ultrasound	Time of incubation	Concentration of ethanol	Polysaccharide yield (g kg^{-1})
1	I	I	I	I	I	0.94
2	I	II	II	II	II	8.06
3	I	III	III	III	III	19.5
4	I	IV	IV	IV	IV	18.3
5	II	I	II	III	IV	16.2
6	II	II	I	IV	III	14.2
7	II	III	IV	I	II	8.43
8	II	IV	III	II	I	15.2
9	III	I	III	IV	II	34.0
10	III	II	IV	III	I	22.9
11	III	III	I	II	IV	16.4
12	III	IV	II	I	III	12.3
13	IV	I	IV	II	III	44.8
14	IV	II	III	I	IV	12.4
15	IV	III	II	IV	I	31.4
16	IV	IV	I	III	II	34.3
k_I[a]	11.7	14.4	16.4	8.51	17.6	
k_{II}[a]	13.5	18.9	17.0	21.1	21.2	
k_{III}[a]	21.4	20.0	20.3	23.2	22.7	
k_{IV}[a]	30.7	24.0	23.6	24.5	15.81	
R[b]	19.0	9.60	7.16	16.0	6.87	

[a] Average responses (k_I, k_{II}, k_{III}, k_{IV}) of each level about extraction yield.
[b] R value means range between four average responses of each level about extraction yield.

fractions of polysaccharides, with different molecular weights, were collected for further studies.

3.3. M_w of the polysaccharides

HPLC was applied to elucidate the molecular weights (M_w) of the polysaccharides (Fig. 2). Two independent peaks were identified and the average M_w values were 81,877 Da (S1) and 1749 Da (S2), respectively.

3.4. Chemical composition of the purified polysaccharides

The IR spectra of the S1 and S2 are shown in Fig. 3. All samples exhibited a broadly-stretched intense peak at around 3410 cm^{-1} characteristic of hydroxyl groups and a weak C–H band at around 2930 cm^{-1}. The relatively strong absorption peak at around1650 $cm^{-1}$and the weak one at around 1250 cm^{-1} also indicated the characteristic IR absorption of polysaccharide. The samples showed IR absorption at about 834 cm^{-1}, which was characteristic

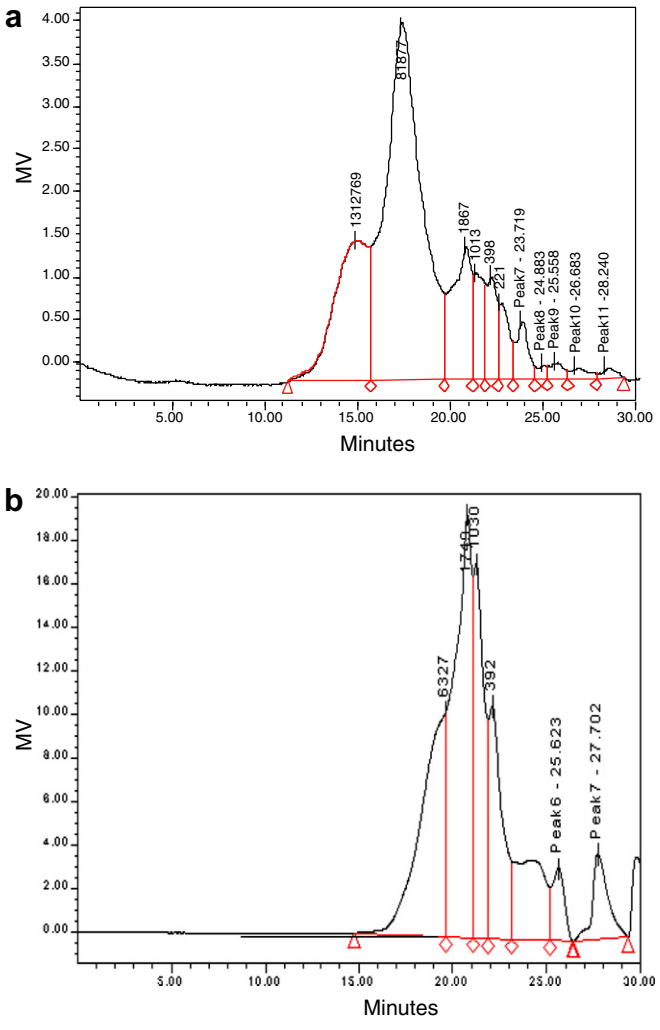

Fig. 2. Results of the molecular weights (M_ws) of the polysaccharide of S1 (a) and S2 (b).

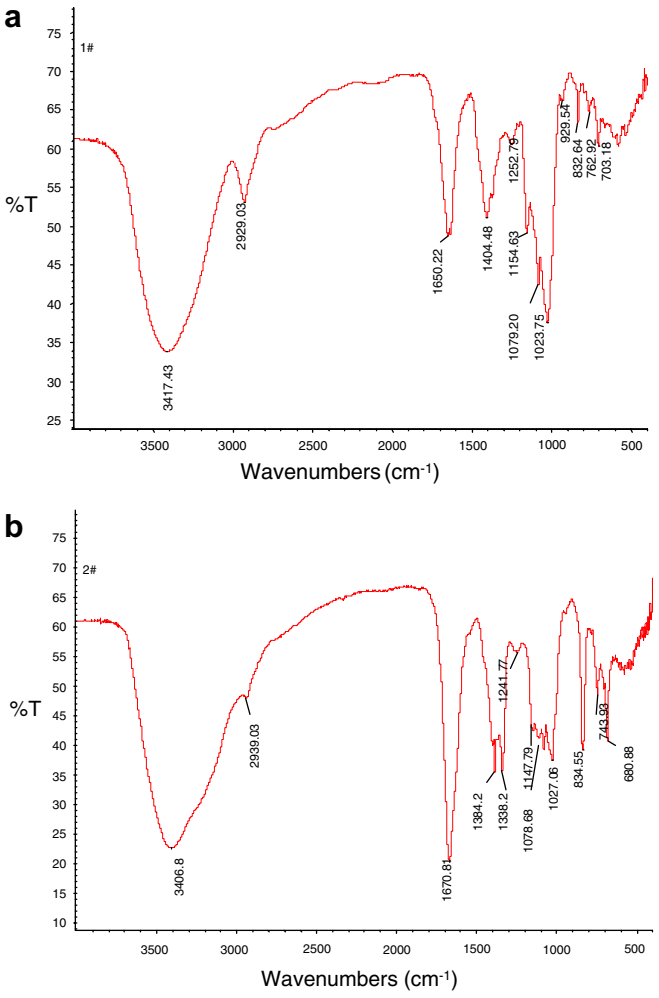

Fig. 3. FT-IR spectra of the polysaccharide of S1 (a) and S2 (c).

of α-pyranoses. A symmetrical stretching peak near 1384–1338 cm^{-1} in Fig. 3 A was an indication of the presence of carboxyl groups.

GC traces of the polysaccharide hydrolyzates, compared with standard saccharides (rhamnose, arabinose, xylose, mannose, glucose and galactose) showed the monosaccharide components of the samples. The results indicated that mannose (relative mass 78.0%) was the predominant monosaccharides of S1 with low levels of glucose (relative mass 13.2%). The predominant monosaccharides of S2 were mannose (relative mass 76.5%). In additional to mannose, there was glucose (relative mass 10.5%); an uncertain monosaccharide (relative mass 8.4%) was also present but at even lower level.

4. Conclusions

In this work, the extraction conditions of polysaccharide in *C. pyrenoidosa* were optimized as 400 W of ultrasound for 800 s, then followed by incubation in a water bath at 100 °C for 4 h in the presence of 80% ethanol. Two different polysaccharides were isolated and their chemical com-

positions were determined. Compared with the known methods of purification of seaweed polysaccharides, used extensively in the industry, the procedure is effective in polysaccharide extraction for its shorter time and higher yields and may be potential useful for mass production of seaweed polysaccharides. Further study should be carried out to elucidate bioactivity through animal experiments with the purpose of applying the polysaccharides in the food industry, which is already underway in our lab.

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