

Extraction of antioxidants from *Chlorella sp.* using subcritical water treatment

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Abstract. *Chlorella sp.* microalgae is one of the main source of natural bioactive compounds used in the food and pharmaceutical industries. Subcritical water extraction is the technique that offers an efficient, non-toxic, and environmental-friendly method to obtain natural ingredients. In this work, the extracts of *Chlorella sp.* microalgae was evaluated in terms of: chemical composition, extraction (polysaccharides) yield and antioxidant activity, using subcritical water extraction. Extractions were performed at temperatures ranging from 100°C to 300°C. The results show that by using subcritical water, the highest yield of polysaccharides is 23.6% that obtained at 150°C. Analysis on the polysaccharides yield show that the contents were highly influenced by the extraction temperature. The individual antioxidant activity were evaluated by in vitro assay using a free radical method. In general, the antioxidant activity of the extracts obtained at different water temperatures was high, with values of 31.08-54.29%. The results indicated that extraction by subcritical water was effective and *Chlorella sp.* can be a useful source of natural antioxidants.

1. Introduction

Microalgae are particularly attractive as natural sources for bioactive compounds since they have the potential to produce the compounds in culture which enables the production of structurally complex molecules [1-4]. The capability of microalgae to synthesize variety of bioactive chemicals is due to its multitude of physiological, biochemical and molecular strategies to cope with stress. As photosynthetic organisms, microalgae contain chlorophyll that can be used for food and cosmetic purposes [5]. They can also be used in pharmaceutical industries, as some species of microalgae produce bioactive compounds such as antioxidants, anticancer, anti-inflammatory, antiviral, antibiotics and antitoxins [6]. Besides, microalgae are used as nutrient supplements for human consumption as they are high in protein, vitamins and polysaccharides contents [7]. They are also have been described to secrete a wide range of compounds that could be potentially employed as functional ingredients including polyphenols, carotenoids and other antioxidant pigments [8].

Chlorella sp. is a type of microalgae which can normally be found in freshwater. It is unicellular photosynthetic algae which contains green photosynthetic pigments chlorophyll in its chloroplast and also contains lutein and other primary carotenoids such as carotene [9]. Recently, *Chlorella sp.* is known and commercialized because of its nutrients and benefits. This kind of microalgae has the capabilities



to produce secondary metabolites including polysaccharides. Polysaccharides extracted from algae can be considered as source of dietetic fiber which has identical functionality as prebiotics since they are indigestible in the human digestive system [10].

Extraction processes for the separation of bioactive compounds have been developed to obtain highly purified products and rendering them useful in a wide range of applications. These technologies could provide an innovative approach to increase the production of the desired compounds including extraction of bioactive compounds from microalgae. Many extraction techniques have been developed in extracting the bioactive components. The current techniques mostly involve solvents and heating [11] such as soxhlet, microwave, ultrasonic and supercritical extraction technique.

Subcritical water extraction (SWE) is one of the potential technique that can be applied to enhance the selectivity and the yield of desirable bioactive compounds. This extraction using hot water under pressure, has recently emerged as a useful tool to replace the traditional extraction methods. SWE is an environmental-friendly technique that can provide higher extraction yields from solid samples. SWE is carried out using hot water (from 100 to 374°C) under high pressure (usually from 10 to 60 bar) to maintain water in the liquid state. In general, the use of SWE provides a number of advantages over traditional extraction techniques. These are, mainly low extraction times, higher quality of the extracts, lower costs of the extracting agent, and an environmentally compatible technique [12].

In the last decade, subcritical water has gained much interest from researchers as a green method for extracting compounds from various materials. The goal of the present investigation was to evaluate the extracts from the *Chlorella sp.* microalgae using subcritical water as method of extraction at different temperature conditions. For this purpose, the extracts were evaluated based on extraction yield and polysaccharides contents. A study on the effect of the temperature on the extraction efficiency of the antioxidant compounds was also performed.

2. Materials and Method

2.1. Samples and chemicals

Chlorella sp. bluegreen algae (derived from *Chlorella vulgaris*) was purchased from PureBulk, USA. Ethanol, methanol, Folin–ciocalteu reagent, sodium carbonate, gallic acid, diphenyl-1-Picryl-Hydrazyl-Hydrate (DPPH) was purchased from Sigma Aldrich, Malaysia. All chemicals were of analytical grade and used as received, without further purification.

2.2. Proximate analysis

The proximate compositions of *Chlorella sp.* was determined according to the Official Analytical Chemists Association (AOAC) method [13]. Moisture was determined by drying a sample in an air oven for 5 h at 105°C. The Kjeldahl method was used to determine the crude protein content using Tecator Kjeltec protein analyzer (FOSS, Hillerod, Denmark). The fat content was determined using the soxhlet method. Ash was determined by heating the samples in a furnace at 550°C for 8–12 h.

2.3. Subcritical water extraction

Extractions were performed with a batch subcritical fluid extraction system (Figure 1) at extraction temperature between 100 to 300 °C. Previous to each extraction, the molten salt bath heat-up was carried out for a few minutes. Likewise, all extractions were performed in stainless steel batch reactor (SUS316, 7.5mm x 150.4mm) cells, containing 0.5 g of sample.

The extraction procedure was as follows: (i) sample was loaded into reactor cell; (ii) cell was filled with 5 mL of distilled water; (iii) the air was drawn out by purging argon gas into the reactor cell; (iv) the reactor was then placed into the preheated molten salt bath to initiate the reaction; (v) after reaching 10 minutes, the reactor cell was taken out and quickly quenched in flowing tap water at room temperature to terminate the reaction. After that, the reactor content were centrifuged at 5000 rpm, for 10 minutes. Then, filtration process was took place using Whatman No.1 filter paper and filter funnel into a conical flask. The supernatant and residue were collected for further analysis.

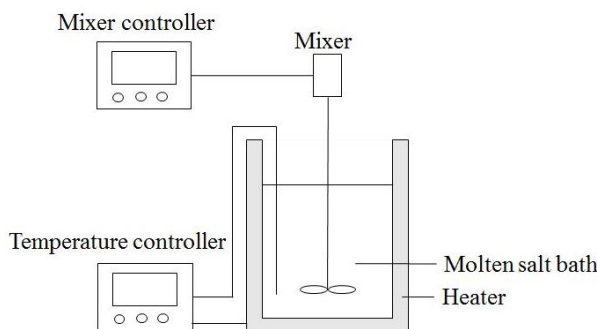


Figure 1. Subcritical fluid extraction system

The residue were dried at 60°C in drying oven for overnight and the weight was determined. The crude extracts yield was calculated by following equation:

$$\text{Crude extract yield (\%)} = \frac{W_{\text{microalgae}} - W_{\text{residue}}}{W_{\text{microalgae}}} \times 100\% \quad (1)$$

Where W_{residue} is the mass of residue (g) and $W_{\text{microalgae}}$ is the total mass of microalgae (g)

2.4. Polysaccharides yield determination

The supernatant was treated by using three volumes of absolute ethanol (v/v) and was then precipitated for incubation overnight at 4°C. The precipitate was centrifuged at 4000 rpm for 5 min and was washed three times using acetone. Afterward, the precipitate was freeze-dried using a vacuum freeze dryer (SCANVAC Freeze Dryer) at -108 °C for 72 hrs and the weight was determined. The yield of polysaccharides was calculated using the following equation:

$$\text{Polysaccharides yield (\%)} = \frac{W_{\text{polysaccharides}}}{W_{\text{microalgae}}} \times 100\% \quad (2)$$

Where $W_{\text{polysaccharides}}$ is the mass of polysaccharides (g) and $W_{\text{microalgae}}$ is the total mass of microalgae (g)

2.5. Total phenolic compound

The total phenolic content of the extract was determined by modified Folin–Ciocalteu method. Briefly, 200 µL of extract were made up to 3 mL with distilled water, mixed thoroughly with 0.5 mL of Folin–Ciocalteu reagent for 3 min, followed by the addition of 2 mL of 20% (w/v) sodium carbonate. The mixture was allowed to stand for a further 60 min in the dark, and absorbance was measured at 765 nm by UV- visible spectrophotometer. The total phenolic content was calculated from the gallic acid calibration curve.

2.6. Antioxidant analysis (DPPH)

DPPH (2,2-diphenyl-1-picryl-hydrazyl-hydrate) free radical scavenging assay [14] was performed to determine the antioxidant activity. 0.1 mM solution of DPPH in methanol was prepared and 1 ml of this solution was added to 3mL extracts and incubated in the dark place for 30 minutes. After that, the absorbance was measured using UV-visible spectrophotometer at 517 nm. 4mL of distilled water was used as blank. While, 3mL of methanol solution and 1mL of DPPH solutions were mixed together which act as control. The changes in color of the mixture were observed. Then, the absorbance was measured. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The scavenging activity of DPPH radical in percentage calculated using following equation:

$$\text{Antioxidant activity (\%)} = \frac{A_0 - A_1}{A_0} \times 100\% \quad (3)$$

Where A_0 is the absorbance of the control sample and A_1 is the absorbance of the extracts.

3. Result and Discussion

3.1. Chemical composition of *Chlorella sp.*

The chemical composition of natural ingredients is very important not only from the perspective of human nutritional health, but also for assessment of the potential development and application of materials in food and pharmaceutical system. Proximate analysis were conducted on *Chlorella sp.* (Table 1) and the results showed that the microalgae contained $37.05 \pm 1.38\%$ carbohydrate, $52.25 \pm 0.19\%$ crude protein, $3.73 \pm 1.21\%$ moisture, $2.51 \pm 0.14\%$ crude oil, and $4.45 \pm 0.06\%$ ash.

Table 1. Composition percentage of *Chlorella sp.*

Component	Content (%)
Moisture content	3.73 ± 1.21
Carbohydrate	37.05 ± 1.38
Crude protein	52.25 ± 0.19
Crude oil	2.51 ± 0.14
Ash	4.45 ± 0.06

The analysis shows that *Chlorella sp.* was high in carbohydrate content (37.05%). Previous studies have shown that the carbohydrate content of few microalgae varies from 18.5 to 45.8% [15]. *Chlorella sp.* was within this range, indicating that it possessed considerable polysaccharides content.

3.2. Extraction yield

SWE of the *Chlorella sp.* was carried out at 100-300 °C for 10 min. Figure 2 shows the crude extraction yield of microalgae with different temperatures. It shows that extraction temperature of 200°C gave a maximum amount of crude yield. The crude yield at 100°C was 25.59% and it reached 50.10% yield at 200°C. The crude yield of the extracts start to decrease at 250°C until it reach 300°C with 16.07% of yield extracted.

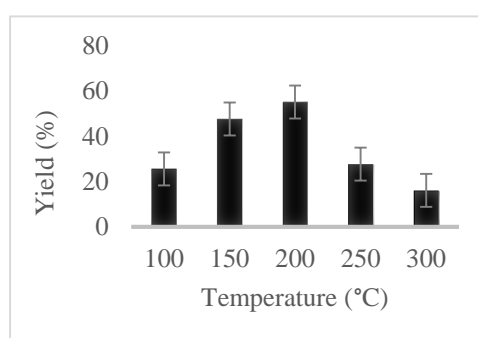


Figure 2. Yield of *Chlorella sp.* crude extracts at different temperature

Figure 3 shows the effects of extraction temperature on the polysaccharides yield. As described in Figure 3, the effect of temperature on polysaccharides yield was significant. The polysaccharides yield increased with the rise of extraction temperature from 100°C to 150°C and decreased from 150°C to

300°C. The minimum and maximum polysaccharides yield were 5.1% at 300°C and 23.6% at 150°C, respectively.

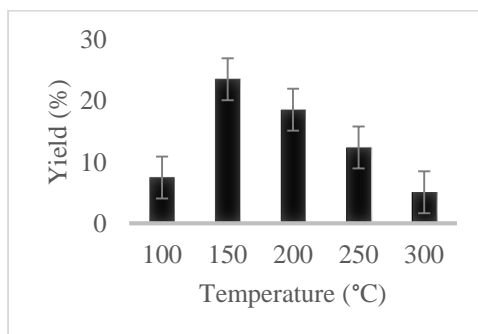


Figure 3. Yield of *Chlorella sp.* polysaccharides extracted at different temperature

When temperature increased from 100 to 150°C, the dielectric constant of subcritical water significantly decreases. At such high temperature, polysaccharides could dissolve in subcritical water as much as they dissolve in the organic solvents. However, polysaccharides yield decreased at 200°C and above possibly due to further degradation of polysaccharides and some compounds and decreasing of dielectric constant provided less effective contribution to the polysaccharides recovery. In general, it has been observed that amorphous polysaccharides to water soluble products increases rapidly as the temperature increase from 100 to 150°C and it decrease when the temperature reaches 200°C. In addition, other studies reported that polysaccharides like celluloses remain stable below 200°C [16]. Polysaccharides in microalgal cell wall were complex and diversified. Moreover, the chemical structures of the polysaccharides were vary for different species. It was difficult to utilize an ambient temperature for extraction of polysaccharides due to complexity and inherent tensile strength of the cell wall. Therefore, additional detailed study is required to explain the characteristics of the extracted polysaccharides and the effects on every point of temperature within subcritical phase.

3.3. Antioxidant analysis

Once the different extracts were obtained, the next step was their functional characterization in terms of antioxidant activity. As mentioned, free radical method (DPPH) was used to measure the antioxidant activity of the extracts. Figure 4 shows the antioxidant activities of the extracts obtained, at different subcritical water temperatures.

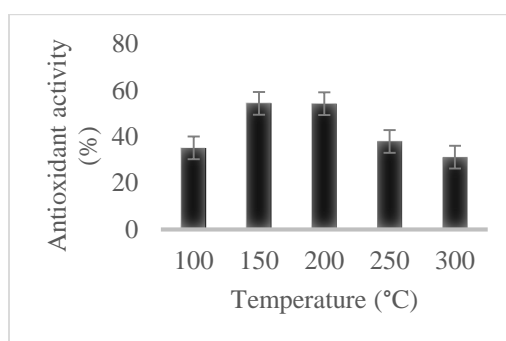


Figure 4. Antioxidant activity of the extracts at different temperature

As can be seen the highest antioxidant activities for *Chlorella sp.* extracts was obtained at 150°C with 54.29% inhibition. When the temperature increased from 100 to 150°C, the antioxidant activity was also increased from 35.06% to 54.29%. The antioxidant activity of the extracts were decreased when the extraction temperature reach 250°C and above with 31.08% inhibition at 300°C. This may due to the decomposition of most of the component in the extracts at very high temperature. Comparatively, for the extracts obtained at 200°C, the antioxidant activities (54.11%) found were almost the same as those observed at 150°C.

Figure 5 shows the total phenolic content of the extracts at different temperature. As the temperature increases from 100 to 150°C, the total of phenolic compound also increases. At 150°C, the highest recovery of phenolic compound was obtained with the value of 57.1mg/g. Overall, the results show that extraction yield increases as the temperature increases as a result of increased solubility of phenolic compounds in water. However, it drop when it reached 200°C and it might due to the degradation of phenolic compound. In addition, previous studies have reported that, degradation of phenolic compounds was observed above 180°C [17].

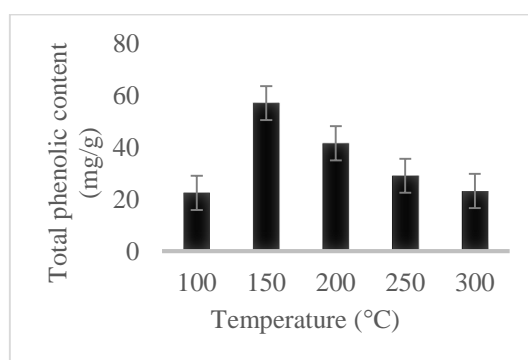


Figure 5. Total phenolic content of the extracts at different temperature

This suggested that other compounds should have a positive influence on the total antioxidant activity of the extracts at 200°C. The presence of phenolic compounds alone, cannot explain the antioxidant capacity observed. Some studies have reported that Maillard reaction occurred during the extraction at high temperature which resulted in the formation of new antioxidant compounds [18]. Chemical composition of this microalgae with the presence of carbohydrates together with amino acids could generate some compounds during the extraction at high temperature. These compounds could have an effective influence on the total antioxidant activity of the extract produced.

4. Conclusion

This study showed that extraction by subcritical water, produces polysaccharides-rich extracts from *Chlorella sp.* The recommended operating condition for subcritical water extraction to obtain the highest concentration of polysaccharides is 150°C with yield 23.6%. Antioxidant was efficiently extracted from *Chlorella sp.* with environmentally, non-toxic subcritical water. The extracts, which are free from organic solvent residue and exhibit high free radical scavenging activities should be ready for used as antioxidants for food and pharmaceutical products.

Acknowledgement

The authors gratefully acknowledge Universiti Putra Malaysia and financial support from the Fundamental Research Grant Scheme (FRGS) provided by the Ministry of Higher Education Malaysia.

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