Optimization of chlorophyll extraction solvent of bulung sangu (Gracilaria sp.) seaweed

M M V Sasadara1*, N M D M W Nayaka1, P E S K Yuda1, N L K A A Dewi1, E Cahyaningsih1, I G P Wirawan2, D S Ilalahi2

1Department of Natural Medicine, Faculty of Pharmacy, Universitas Mahasarakmat Denpasar, Bali
2Department of Biotechnology, Faculty of Agriculture, Udayana University, Bali

*Corresponding author email: mariasasadara@unmas.ac.id

Abstract. Algae are a photosynthetic organism, affordable and naturally rich in nutrients and a valuable source of bioactive substances such as natural pigments. Bulung sangu (Gracilaria sp.) is red macroalgae that wildly grows and distributes in Bali. The aim of this work was to optimize the solvent to extract the chlorophyll content of Bulung sangu. The pigment extraction was carried out using different solvents (100% methanol, 100% ethanol, and 90% acetone). The chlorophyll contents including chlorophyll a,b,c,d and total chlorophyll were measured using spectrophotometry UV-VIS and expressed in µg/g of algae. The results showed that chlorophyll c could not be extracted using all used solvent, while chlorophyll b can only be extracted using acetone. Acetone produced the highest concentration of chlorophyll a (717.52 ± 9.71 µg/g), chlorophyll b (7.23 ± 0.24 µg/g), chlorophyll d (21.93 ± 1.07 µg/g), and chlorophyll total (746.67 ± 8.99 µg/g) compared to other solvent, that were significantly different (p<0.05). The second solvent to produce the highest concentration of chlorophyll a, d, and total chlorophyll was methanol which produced 578.77 ± 9.74 µg/g, 5.50 ± 0.12 µg/g and 584.27 ± 9.62 µg/g of chlorophyll content, respectively, followed by ethanol which produced 520.98 ± 2.52 µg/g of chlorophyll a, 3.56 ± 0.25 µg/g for chlorophyll d, and 524.54 ± 2.30 µg/g for total chlorophyll. Acetone is considered the most effective solvent to extract the chlorophyll content of Bulung sangu.

1. Introduction
Macroalgae or seaweed are unicellular or multicellular photosynthetic organisms similar to plants, which live by attaching to rocks or other rigid substrates in the coastal area [1]. Currently, natural products derived from algae, both macroalgae, and microalgae, are becoming the focus of various pharmaceutical, food, and biotechnology industries [2]. Macroalgae is a source of critical bioactive metabolites that have been widely explored and used in drug development and treatment of various health problems. Macroalgae are rich in vitamins, minerals, protein, polysaccharides, steroids, and fiber. Macroalgae also contain various types of carotenoid and chlorophyll pigments [3].

Chlorophyll is one of the important bioactive compounds produced by macroalgae and microalgae in high quantities. Chlorophyll is the primary pigment that plays a role in photosynthesis by absorbing the energy of sunlight to synthesize oxygen and carbohydrates needed as nutrients. There are four types of chlorophyll: chlorophyll a, chlorophyll b, chlorophyll c, and chlorophyll d. Chlorophyll is essential for algae growth and survival [4]. The presence of chlorophyll a in algae is complemented by supporting pigments, namely chlorophyll b, c, or d and carotenoids, which protect chlorophyll a from photo-oxidation [5].
Chlorophyll is crucial for the growth of algae and has been used in various fields of life, especially health. Chlorophyll showed bioactive properties as antimutagenic, chemopreventive, antioxidant, and anti-inflammatory. Chlorophyll also rebalance the gut microbiota and used in food safety aspect for inactivating of microorganism [6] Chlorophylls are greenish, non-polar pigments present in all autotrophic algae that include porphyrin or hydro porphyrin ring centrally linked to a magnesium atom and allow light to be converted into biological energy [7].

One of the widely used macroalgae is red algae (Rhodophyta). Rhodophyta contains agar, carrageenan and consists of several pigments such as phycoerythrin, phycobilin, phycocyanin, xanthophyll, chlorophyll, and -carotene [8]. Red algae production is estimated to be more than 3.8 million tons/year. China and Indonesia are the most producing countries for Gracilaria, a Rhodophyta genus [9].

Various studies have shown that red algae (Rhodophyta) contain high chlorophyll-a levels. Bulung sangu (Gracilaria sp.) is one of the most widespread seaweeds in Bali and is popularly consumed as a vegetable. Like most other Rhodophyta, Bulung sangu is a potential phytochemical source with various bioactivities. Bulung sangu ethanol extract contains a high level of n-hexadecanoic acid, and showed potential antioxidant and anti-inflammatory activity [10]. Bulung sangu is also potential in regulating blood cholesterol level. Phytochemical identification using thin-layer chromatography showed the presence of several types of carotenoid pigment, along with chlorophyll [11].

Various studies have shown the presence of chlorophyll in Gracilaria algae. Gracilaria verrucosa growing on the coast of Gunung Kidul showed 7.132 mg/g of chlorophyll a and 8.335 mg/g of chlorophyll b [12]. In addition, Gracilaria lemaneiformis obtained from Chinese waters also showed chlorophyll reaching 0.036 g/g [13]. The research was also conducted on chlorophyll in the brown algae Padina australis Hauck with the results obtained as much as 0.381 g/g in Blangko Waters and 0.143 g/g in Tongkaina Waters [14].

Several techniques can be used to isolate chlorophyll in photosynthetic organisms, such as maceration extraction with organic solvents, supercritical fluid extraction, subcritical water extraction, and ultrasound-assisted extraction [15]. The success of the isolation process and the amount of extract produced depend on the extraction method and solvent used. Chlorophyll can be extracted using organic solvocents with different polarity levels. Several studies have shown the use of various solvents to extract chlorophyll, such as ethanol, methanol, and acetone [4]. Under the same conditions of extraction time and temperature, solvent and sample composition are the critical parameters affecting the extraction results [16]. In addition to selecting the suitable extraction method, the solvent is critical to obtain maximum extraction results with minimal changes in functional properties [17].

Bulung sangu are one of the potential sources of chlorophyll. This research was conducted to optimize the solvent used in the chlorophyll extraction process in Bulung sangu (Gracilaria sp.). The optimization process was carried out on the three most common organic solvents used in chlorophyll extraction: ethanol, methanol, and acetone.

2. **Material and Methods**

Bulung sangu (Gracilaria sp.) macroalgae used in this study were collected at Serangan, Bali. Fresh and red Bulung sangu was cleaned from impurities and washed under running water. Clean Bulung sangu was then chopped to reduce the size. The pigment extraction method was adapted from Osorio et al. (2020) [7]. 100% ethanol (Merch, p.a), 100% methanol (Merch, p.a) and 90% acetone (Merch, p.a) were used as the extraction solvent. 25mg of wet sample was then pounded with 10ml of each solvent followed by filtration and vortex treatment for 1 minute. The sample was then centrifuged with 2500 rpm of speed for 10 minutes. The supernatant was collected for another centrifuge treatment at 5000 rpm for 5 minutes. The supernatants were then collected for the chlorophyll measurement. The absorbances were then measured using Spectrophotometry UV-VIS (UV-1800 Shimadzu double beam) in five wavelengths 632, 652, 665, 695, and 750 nm. Chlorophyll a, b, c, d, and total Chlorophyll were calculated using the equation according to Osório et al., 2020 [7]. The chlorophyll contents
concentrations were obtained after substitution of the absorbance of each wavelength in the equation. The results were expressed in µg/g. The results were then statistically analyzed using one-way ANOVA and Tukey’s HSD post hoc with a 95% of confidence level. A significant value of <0.05 was considered statistically different. Statistical analysis was performed using IBM SPSS version 25 (IBM Corp., USA). Data are expressed as mean ± standard deviation (n = 3).

3. Results and Discussion
Chlorophyll content obtained by extraction with three different solvents is shown in table 1. Acetone is the most efficient solvent for extracting chlorophyll by producing a higher chlorophyll content than the other solvents. Among all determined chlorophyll contents, acetone can extract chlorophyll a, b, and d, but not chlorophyll c. Chlorophyll c was not detected using all solvents, indicating a possible absence of this pigment in Bulung sangu. Meanwhile, chlorophyll b, which was detected in acetone extract, was not detected in ethanol and methanol extracts.

Table 1. The Contents of chlorophylls extracted red seaweed Bulung sangu (Gracilaria sp.) using different solvents.

|                | Methanol      | Ethanol       | Acetone       |
|----------------|---------------|---------------|---------------|
| Chlorophyll a  | 578.77 ± 9.73^a | 520.98 ± 2.52^b | 717.52 ± 9.71^c |
| Chlorophyll b  | n.d           | n.d           | 7.23 ± 0.24    |
| Chlorophyll c  | n.d           | n.d           | n.d           |
| Chlorophyll d  | 5.50 ± 0.12^a  | 3.56 ± 0.25^b  | 21.93 ± 1.07^c |
| Total Chlorophyll | 584.27 ± 9.62^a | 524.53 ± 2.31^b | 746.67 ± 8.99^c |

n.d. : not detected

Data are presented as mean ± standard deviation, and expressed in µg/g of algae. Within each line, different letters represent significant differences between results obtained with different extraction solvents, at p < 0.05.

Acetone is generally the solvent of choice for extracting chlorophyll because it produces different absorption peaks. However, not all chlorophyll is well extracted with acetone. The use of acetone showed poor chlorophyll extraction in some algae, such as green and blue-green algae [7,18–20]. Although in this study acetone showed statistically different results with the use of the other two solvents, it was different from the study by Osorio et al. which showed that chlorophyll extraction in red algae Porphyra spp. not influenced by the use of solvents, either acetone, methanol, ethanol, or N,N-Dimethylformamide (DMF) [7].

Chlorophyll is the primary pigment used by plants to capture light energy. The chlorophyll molecule consists of a porphyrin head and a long lipid-soluble hydrocarbon tail. The porphyrin head of chlorophyll consists of four nitrogen-containing pyrrole rings that form a ring surrounding magnesium ions. There are four types of chlorophyll: chlorophyll a, b, c, and d. Chlorophyll a and b are found in higher plants and algae, but chlorophyll a can also be found in cyanobacteria. Chlorophyll c is found in diatoms, dinoflagellates, and brown algae, while chlorophyll d in red algae [21]. Several literatures mention the presence of another type of chlorophyll, namely chlorophyll f that is found in stromatolites, a hard rock structure made by cyanobacteria [22]. Other literature also mentioned another type of chlorophyll, namely chlorophyll e, which is rare and only found in golden algae [23]. All types of chlorophyll, other than chlorophyll a, are considered accessory chlorophyll molecules [22].

In this study, chlorophyll a showed the highest concentration compared to other chlorophylls. According to Dasgupta (2015) red algae contain chlorophyll a and d, but not chlorophyll b and c [24]. Chlorophyll a is a green pigment found in all land plants and algae to carry out photosynthesis. Chlorophyll a is responsible for absorbing light in the red-orange and blue-violet light spectrum. While chlorophyll b only absorbs light with a blue-violet wavelength. Chlorophyll b is an auxiliary pigment in
the photosynthesis process and is found in plants and several types of algae, mainly green algae [23]. Chlorophyll c was not detected in Bulung sangu, but a small amount of chlorophyll b was detected in the acetone extract. Nevertheless, the value of chlorophyll b cannot be considered because the quadratic equations used in acetone, although more reliable than the equation for methanol, can produce small amounts of chlorophyll b values that are not present in the pigment [18,25]. In addition, the use of acetone and alcohol may also have an effect, especially in causing chlorophyll degradation due to the high acidic nature of the two solvents. When degradation occurs at low pH conditions, chlorophyll can lose magnesium ions, which will produce pheophytin, or may uptake oxygen, producing allomerization products [7,26]. The degradation product has a different spectrum from chlorophyll so that it can interfere with the chlorophyll determination process, which results in the formation of an enlarged chlorophyll a peak so that other chlorophyll peaks such as chlorophyll b, c, and d become lower and wider [18,25]. However, even if this happens, in this study the presence of chlorophyll b was not detected in the methanol extract.

Chlorophyll is one of the most valuable bioactive compounds that can be extracted from algae. Chlorophyll can be extracted from wet or dry materials. Chlorophyll is used as a coloring agent because of its ability to selectively absorb light at specific wavelengths and produce a green color. This makes chlorophyll widely used in industry, especially the food industry. Chlorophyll is also widely used in pharmaceutical products. Chlorophyll accelerates wound healing, stimulates tissue development, and prevents the growth of bacteria [4]. Chlorophyll showed bioactive properties as antimutagenic, chemopreventive, antioxidant, and anti-inflammatory. Chlorophyll also rebalances the gut microbiota and is used in the food safety aspects for inactivating microorganisms [6]. Chlorophyll has a structure similar to hemoglobin, so it is predicted to stimulate tissue development by accelerating the exchange of carbon dioxide and oxygen. This activity, coupled with antioxidant properties, especially those derived from chlorophyll, makes chlorophyll widely used in various pharmaceutical products [4].

4. Conclusion
Bulung sangu (Gracilaria sp.) can be a potential source of chlorophyll with acetone considered the most optimum solvent to extract the chlorophyll content. There is no presence of chlorophyll c detected using all solvents, while chlorophyll b can only be extracted using acetone. This research shows other potential utilization of Bulung sangu, in which it can be a good source of chlorophyll.

References
[1] Guiry MD. What are algae? 2019:3–5. http://www.seaweed.ie/algae/seaweeds.php.
[2] Khan MI, Shin JH, Kim JD. The promising future of microalgae: Current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. Microb Cell Fact 2018;17:1–21. https://doi.org/10.1186/s12934-018-0879-x.
[3] de Almeida CLF, Falcão H de S, Lima GR d. M, Montenegro C de A, Lira NS, de Athayde-Filho PF, et al. Bioactivities from marine algae of the genus Gracilaria. Int J Mol Sci 2011;12:4550–73. https://doi.org/10.3390/ijms12074550.
[4] Hosikian A, Lim S, Halim R, Danquah MK. Chlorophyll extraction from microalgae: A review on the process engineering aspects. Int J Chem Eng 2010;2010. https://doi.org/10.1155/2010/391632.
[5] Hong JE, Lim JH, Kim TY, Jang HY, Oh H Bin, Chung BG, et al. Photo-oxidative protection of chlorophyll a in c-phycocyanin aqueous medium. Antioxidants 2020;9:1–13. https://doi.org/10.3390/antiox9121235.
[6] Queiroz Zepka L, Jacob-Lopes E, Roca M. Catabolism and bioactive properties of chlorophylls. Curr Opin Food Sci 2019;26:94–100. https://doi.org/10.1016/j.cofo.2019.04.004.
[7] Osório C, Machado S, Peixoto J, Bessa S, Pimentel FB, Alves RC, et al. Pigments content (Chlorophylls, fucoxanthin and phycobiliproteins) of different commercial dried algae. Separations 2020;7:1–14. https://doi.org/10.3390/separations7020033.
Pereira L. Seaweeds as source of bioactive substances and skin care therapy-Cosmeceuticals, algotherapy, and thalasstontherapy. vol. 5. 2018. https://doi.org/10.3390/cosmetics5040068.

Kim JK, Yarish C, Hwang EK, Park M, Kim Y. Seaweed aquaculture: Cultivation technologies, challenges and its ecosystem services. Algae 2017;32:1–13. https://doi.org/10.4490/algae.2017.32.3.3.

Sasadara MMV, Wirawan IGP, Jawi IM, Sritamin M, Dewi NNA, Adi AAAM. Anti-inflammatory effect of red macroalgae bulung sangu (Gracilaria sp.) extract in UVB-irradiated mice. Pakistan J Biol Sci 2021;24:80–9. https://doi.org/10.3923/pjbs.2021.80.89.

Julyasih KSM, Wirawan IGP. Potential effect of macro alga Caulerpa sp. and Gracilaria sp. extract lowering malondialdehyde level of wistar rats fed high cholesterol diet. Int J Biosci Biotechnol 2017;5:71–9.

Febrianto W, Djunaedi A, Suryono S, Santosa GW, Sunaryo S. Potensi Antioksidan Rumput Laut Gracilaria verrucosa Dari Pantai Gunung Kidul, Yogyakarta. J Kelaut Trop 2019;22:81. https://doi.org/10.14710/jkt.v22i1.4669.

Wu H, Jiang H, Liu C, Deng Y. Growth, pigment composition, chlorophyll fluorescence and antioxidant defenses in the red alga Gracilaria lemaneiformis (Gracilariaceae, Rhodophyta) under light stress. South African J Bot 2015;100:27–32. https://doi.org/10.1016/j.sajb.2015.05.017.

Kalalo JL, Mantiri D, Rimper J. Pigment analysis of Brown Algae Padina australis Hauck from Sulawesi sea. J Pesisir Dan Laut Trop 2014;2:8. https://doi.org/10.35800/jplt.2.1.2014.6352.

Turkmen N, Sari F, Velioglu YS. Effects of extraction solvents on concentration and antioxidant activity of black and black mate tea polyphenols determined by ferrous tartrate and Folin-Ciocalteu methods. Food Chem 2006;99:835–41. https://doi.org/10.1016/j.foodchem.2005.08.034.

Do QD, Tran-Nguyen PL, Huynh LH, Soetaredjo FE, Ismadji S, et al. Effect of extraction solvent on total phenol content, total flavonoid content, and antioxidant activity of Limnophila aromatica. J Food Drug Anal 2014;22:296–302. https://doi.org/10.1016/j.jfda.2013.11.001.

Monteiro M, Santos RA, Iglesias P, Couto A, Serra CR, Gouvinhas I, et al. Effect of extraction method and solvent system on the phenolic content and antioxidant activity of selected macro- and microalgae extracts. J Appl Phycol 2020;32:349–62. https://doi.org/10.1007/s10619-019-01927-1.

Connan S. Spectrophotometric Assays of Major Compounds Extracted from Algae. In: Stengel DB, Connan S, editors. Nat. Prod. From Mar. Algae Methods Protoc. Methods Mol. Biol., vol. 1308, New York, United States of America: Springer Science+Business Media New; 2015, p. 1–439. https://doi.org/10.1007/978-1-4939-2684-8.

Ritchie RJ. Consistent sets of spectrophotometric chlorophyll equations for acetone, methanol and ethanol solvents. Photosynth Res 2006;89:27–41. https://doi.org/10.1007/s11120-006-9065-9.
[25] Ritchie RJ. Universal chlorophyll equations for estimating chlorophylls a, b, c, and d and total chlorophylls in natural assemblages of photosynthetic organisms using acetone, methanol, or ethanol solvents. Photosynthetica 2008;46:115–26. https://doi.org/10.1007/s11099-008-0019-7.

[26] Brereton RG, Rahmani A, Liang Y-z, Kvalheim OM. Investigation of the allomerization reaction of chlorophyll a: use of diode array HPLC, mass spectrometry and chemometric factor analysis for the detection of early products. Photochem Photobiol 1994;59:99–110. https://doi.org/10.1111/j.1751-1097.1994.tb05007.x.