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Characterization of Protein Concentrate from Spirulina Platensis Microalgae Using Microwave and Alkaline-Acid Extraction Methods

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ABSTRACT

Background*: Spirulina platensis* is a microalgae type of *Cyanobacteria* in the form of filaments that resemble spirals (helix) ranging in size from 3-30 µm. *Spirulina platensis* is rich in nutritional sources, one of which is protein. However, the utilization of protein is limited by the rigidity of the cell wall of spirulina. The study aimed to compare protein extraction using various methods. **Methods**: Extraction process was carried out to obtain *Spirulina platensis* protein concentrate. Three methods were evaluated: physical extraction using a conventional microwave, alkali-acid extraction, and a combination of the two, namely, microwave and alkali-acid extraction. The protein extracts were dried and analyzed for its yield, protein content as well as its functional properties. **Results**: The spirulina powder contained 53.00±1.13% protein (dry basis). The alkaline-acid extraction produced protein concentrate with the highest protein content (67.04±3.85% dry basis), slightly higher than the combined methods (65.54±1.92% dry basis) and significantly higher than the microwave extraction (55.44±5.29% dry basis). However, the alkaline-acid extraction produced the lowest yield of protein concentrate powder (13.90±0.28%) as compared to the other methods. The protein concentrate had a moisture content ranging from 7.68-10.81%, water holding capacity (WHC) ranging from 0.95-1.64 g H_2O/g protein and foaming capacity (FC) ranging from 102-168%. **Conclusions**: Alkaline-acid extraction produced dried protein concentrate of spirulina with the highest protein content, the highest WHC and the lowest FC. The extraction methods/techniques not only affect the protein yield and content, but also its functionality.

Introduction

Protein is a very vital component of human nutrition; it is one of nutrients that will be in short supply in the future. Therefore, alternative protein sources and production methods are required to fulfil the demand for protein (Mahmood and Sibi, 2019). A study reported that microalgae synthesize proteins containing several important amino acids of high nutritional value. Spirulina is one of the richest protein sources from microbial origin (Benelhadj *et al.*, 2023). Spirulina

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is multicellular and filamentous cyanobacteria that grows in water, can be harvested and processed easily. It has very high content of macro and micronutrients, essential amino acids, proteins, lipids, vitamins, minerals and antioxidants (Soni *et al.*, 2017).

Numerous studies have reported the protein content of spirulina ranging from 46-70% dry basis (Bashir *et al.*, 2016, Becker, 2007, Benelhadj *et al.*, 2023, Dalla Costa *et al.*, 2022, López-Rodríguez *et al.*, 2021, Sela *et al.*, 2021). Thus, it is a highly potential source of proteins. However, the proteins were enclosed by cell walls containing water soluble polysacharide (Wang *et al.*, 2018), peptidoglicans and lipopolysacharides (Coelho *et al.*, 2020). Extracting a specific component from microalgae is often prevented by the intrinsic rigidity of its cell wall. Therefore, a process that facilitates cell disruption is required to permit complete access to internal components and facilitate the extraction process (Safi *et al.*, 2014).

Several techniques or methods used to disrupt or break down the cell walls of microalgal cells include mechanical methods, physical methods, chemical methods, enzymatic methods, and combinations thereof (Parimi *et al.*, 2015, Safi *et al.*, 2014, Weber *et al.*, 2022, Zheng *et al.*, 2011). In the case of protein extraction, besides disrupting cell walls, pH modification to increase its solubility then continued with precipitation is necessary (Bashir *et al.*, 2016, Benelhadj *et al.*, 2023). Previous study using a combination of high pressure homogenization and alkaline-acid extraction produced protein isolate with 75% protein content (Mahmood and Sibi, 2019), whereas the same method produced protein isolate with 80.6% protein content (Parimi *et al.*, 2015). Another study, produced freeze dried protein extract containing 74% protein content (Bashir *et al.*, 2016), by using a combination of microwave and alkaline-acid extraction. A combination of sonication, agitation and alkaline extraction produced protein extract containing 38% of protein (Lupatini *et al.*, 2017). A microwave assisted extraction (MAE) that used in chlorella extraction produced 69% protein dry weight in the protein extract (Margenat *et al.*, 2023). Another study using ultrasound assisted extraction with ethanol and methanol as a solvent, produced protein recovery of 20-40% (Sela *et al.*, 2021). Alternatively, a conventional microwave extraction may be used for practical reasons. Both this method alone and its combination with alkali-acid extraction can potentially produce protein extract, but their effectiveness relative to other methods remains unknown.

Apart from nutritional values, proteins play a crucial role in shaping the texture of food products during processing. Due to their unique characteristics and structural properties, different proteins exhibit distinct functional performances, making them suitable for specific food applications (Zhang *et al.*, 2021). It is well-established that changes in the three-dimensional conformation of proteins are associated with a modification of the functional properties (Shevkani *et al.*, 2015). Several functional properties of proteins included water holding capacity (WHC) and foaming capacity (FC).

WHC refers to the ability to avoid water releasing from the protein's three-dimensional structure (Zayas, 1997) also considered as the ability of proteins to hold water when subjected to an external force (pressure, centrifugation, or heating) (Zayas, 1997). Others considered it measures the ability of proteins to absorb water against gravity (Mahmood and Sibi, 2019). The WHC of spirulina protein isolate produced by a combination of microwave and alkaline-acid extraction was 2.96 ml/g protein (Bashir *et al.*, 2016). A combination of high-pressure homogenization and alkaline-acid extraction produced protein isolate with the WHC ranging from 0.902-1.34 g H_2O/g protein (Mahmood and Sibi, 2019).

Some proteins are excellent foaming agents. Proteins in nature have a wide range of FC. The solubility, surface flexibility and hydrophobicity of proteins are the main determinants of an efficient foam formation (Zhang *et al.*, 2021). A combination of high-pressure homogenization and alkaline-acid extraction produced protein isolate of spirulina with FC ranging from 19-41% (Mahmood and Sibi, 2019). The study also showed that the higher the pH the lower the FC.

The functional properties of protein are affected by

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the characteristics of each protein thus are directly determined by how the proteins are isolated/extracted from their natural sources. Therefore, apart from evaluating the quantity of protein extracted, evaluating the functional properties of the proteins such as WHC and FC were important, as both values determined the application of protein concentrate in the food system.

The aims of the present study were: (1) to compare three different methods of protein extraction namely: physical (conventional microwave) extraction, chemical (alkaline-acid extraction) and a combination of microwave and alkaline-acid extraction; (2) to evaluate the functional properties of the dried protein extract produced by various methods.

Materials and Methods

Materials

Spirulina powder was purchased from a marketplace in Indonesia in the November 2022. The product had a dark green appearance and was packed in 100 g containers for characterization, the spirulina powder was examined under a microscope at 1000 x magnification.

Conventional microwave extraction

Spirulina powder was suspended in pure water with a ratio of 1:10, the suspension was then homogenized. Afterwards, the spirulina suspension was heated up in a conventional microwave (Samsung M745, South Korea) with a power of 400 Watts for 1 minute. The solution was centrifuged at 5000 rpm for 15 minutes. Supernatant was separated and dried in a cabinet drier at 60 ºC for 12 hours. The dried protein concentrate was stored in airtight packaging at room temperature until analysis.

Alcali-acid extraction

Spirulina was suspended in pure water with a ratio of 1:10, and the suspension was then homogenized. Afterwards, 1 M NaOH was added until alkaline pH of 11 was reached to increase protein solubilization. Subsequently the solution was centrifuged at 5000 rpm for 25 minutes. The supernatant was collected and added with 1 M HCL until an acidic pH of 4 to precipitate the protein. The precipitation of protein in acidic pH was facilitated by centrifugation at 5000 rpm for 25 minutes. The pellet was collected and dried at 60 ºC for 12 hours. The dried protein concentrate was stored in airtight packaging at room temperature until analysis.

Combination of microwave and alkaline-acid extraction

Spirulina powder was suspended in pure water with a ratio of 1:10, the suspension was then homogenized. Afterwards, the spirulina suspension was heated up in a conventional microwave (Samsung M745, South Korea) with a power of 400 Watts for 1 minute. Afterwards, 1 M NaOH was added until alkaline pH of 11 was reached to increase protein solubilization. Subsequently the solution was centrifuged at 5000 rpm for 25 minutes. The supernatant was collected and added with 1 M HCL until an acidic pH of 4 to precipitate the protein. The precipitation of protein in acidic pH was facilitated by centrifugation at 5000 rpm for 25 minutes. The pellet was collected and dried at 60 ºC for 12 hours. The dried protein concentrate was stored in airtight packaging at room temperature until analysis.

Chemical analysis

Chemical analysis of spirulina powder and dried protein concentrate were performed using AOAC methods (Association of Official Analytical Chemists (AOAC), 2005). Chemical analysis included moisture and protein content. The total nitrogen content of the spirulina and dried protein concentrates were measured in accordance with the Kjeldahl method which includes destruction of the samples using sulphuric acid at high temperature and added with Kjehdahl catalist, until nitrogen was released as ammonium sulphate. Furthermore, the ammonium sulphate was distillated with an excess base to form ammonia gas. Finally, the quantity of total nitrogen present in the sample was determined through titration of the product from the previous step. A conversion coefficient of 6.25 was used for nitrogen to be converted to protein.

Functional properties of protein concentrate

The WHC and FC were measured to analyze the functional properties of protein concentrate.

WHC: One g of dried protein concentrate (W0) was mixed with water, and the change in weight was

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measured before (W1) and after water absorption (W2). The change in weight indicated the water being absorbed was divided by the weight of dried protein sample (Zhang *et al.*, 2021).

$$WHC = \frac{W2 - W1}{W0}$$ **Equation 1**

FC: One g of dried protein concentrate was mixed with water and whipped to form foam. The volume before (V1) and after whipping (V2) was measured. The change of volume of foam divided by volume of foam after whipping multiplied by 100% (Onwuka and Onwuka, 2005).

$$FC\ (\%) = \frac{V2 - V1}{V2} x\ 100$$ **Equation 2**

Statistical analysis

Data was analyzed using ANOVA at 95% confidence level. When significant differences were detected, Tukey's test was conducted for post-hoc analysis using Minitab 2016 software.

Results

Characteristics of spirulina powder

In this study, the material used to produce protein concentrate was a commercial spirulina powder available in the marketplace. Spirulina had undergone several processing steps such as drying, grinding, sieving and packaging. Spirulina powder was examined under the microscope with 1000 x magnification and is presented in **Figure 1**.

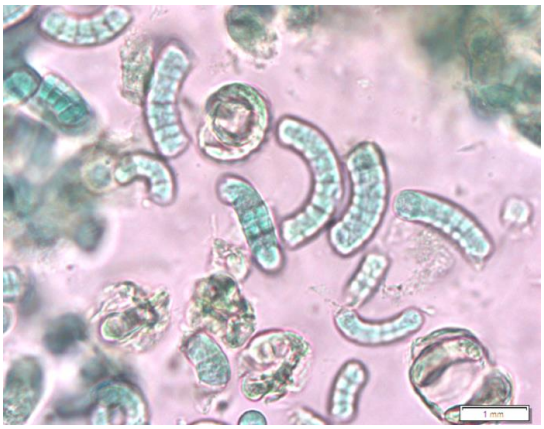

Figure 1. Microscopic image of spirulina powder (1000x).

The spirulina powder had a dark green appearance with the Lightness (L*) of 39.40±0.06, indicating low lightness (**Table 1**). The redness (a) of the powder was -4.50±0.10, the negative value indicated the opposite color (green). Whereas the yellowness (b) was 7.80±0.06, indicating yellow than blue.

Spirulina powder contained protein of 53.08±1.13% dry basis (**Table 1**), thus can be regarded as a high protein source amongst other vegetable protein sources in nature. Whereas the moisture content was 10.46±0.38%, typical moisture content for powdery products.

Table 1. Protein content and appearance of commercial spirulina powder.

Components	Values
Protein (% db)	53.08 ± 1.13[a]
Moisture (%)	10.46 ± 0.38
Lightness (L*)	39.40 ± 0.06
Redness (a)	- 4.50 ± 0.10
Yellowness (b)	7.80 ± 0.06

[a] *Means±standard deviation;* ***db****: Dry basis.*

Protein content and yield of dried protein concentrates of spirulina from various methods

The protein content and yield of dried protein concentrates from various methods is presented in **Table 2**. Conventional microwave extraction produced the highest yield (28.20±2.97%) but the lowest protein content (55.44±5.29% dry basis). On the other hand, Alkaline-acid extraction produced the lowest yield (13.90±0.28%) but the highest protein content (67.04±3.85%). Interestingly, a combined microwave and alkaline-acid extraction did not produce the highest yield and protein content among the three methods. The protein content (65.54±1.92% on a dry basis) did not significantly differ from the alkaline-acid extraction only. Moisture content of the dried protein concentrates was within the range of 7.68-10.81%, with the dried protein concentrate produced by alkaline-acid extraction being the highest.

Functional property of protein concentrates of spirulina from various methods

The functional properties of dried protein concentrates included water holding capacity and foaming capacity are presented in **Table 2**.

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Table 2. Components and functional properties from various methods.

Variables	Extraction methods		
	Microwave	Alkaline-acid	Combined microwave and alkaline-acid
Components			
Protein (% db)	55.44 ± 5.29*[b]	67.04 ± 3.85[a]	65.54 ± 1.92[a]
Moisture (%)	7.68 ± 0.83[b]	10.81 ± 1.03[a]	8.59 ± 0.81[b]
Yield (%)	28.20 ± 2.97[a]	13.90 ± 0.28[c]	15.90 ± 2.98[b]
Functional properties			
WHC (g H_2O/g protein)	0.95 ± 0.12*[b]	1.64 ± 0.39[a]	1.63 ± 0.27[a]
FC (%)	163.70 ± 37.20[a]	102.00 ± 12.13[b]	116.30 ± 25.80[b]

* *Means±standard deviation;* ***db****: Dry basis;* ***WHC****: Water holding capacity;* ***FC****: Foaming capacity; Values with different superscript letters within the same row are significantly different (P<0.05) using ANOVA and Tukey's post-hoc test..*

Water holding capacity is an important functional property for protein. **Table 2** showed that microwave extraction produced dried protein concentrate with the lowest water holding capacity, whereas alkaline-acid extraction produced dried protein concentrate with similar water holding capacity to that of the combined method. On the contrary, foaming capacity was the highest in dried protein concentrate produced by microwave extraction, but the property was similar in the other two methods.

Discussion

Spirulina has undergone several processing steps such as drying, grinding, sieving and packaging (Demarco *et al.*, 2022, Fábio de Farias *et al.*, 2019). Spirulina is characterized by its regularly coiled trichomes (Wang and Zhao, 2005), but straight or nearly straight variants were also observed (Dalla Costa *et al.*, 2022). Although, previous studies reported that the conversion from helical laments to irregularly curved or even linear shaped was considered as permanent degeneration that cannot be reversed and produces abnormal morphologies due to some stressful condition, recent study found that the linear filament could be reverted to the original morphological trait (Wang and Zhao, 2005). Spirulina powder appeared more fragmented and damaged under the microscope (**Figure 1**) which may be caused by handling, drying and powdering after harvesting (Dalla Costa *et al.*, 2022, Demarco *et al.*, 2022). The dark green color of the spirulina cells appeared under the microscope indicating that the cells produced chlorophyl in large amount (Diaconu *et al.*, 2023).

The color measurement of the Spirulina powders also presented as redness (a) which was -4.5, the negative value indicates the opposite color of red, which is green. In a study by (Diaconu *et al.*, 2023), the change in color of spirulina was reported as a sign of the aquatic ecosystem that has been polluted by heavy metals that can affect microalgae growth and destroy the chlorophyl. However, chlorophyl may undergo degradation during drying process of spirulina with high temperature, the product was named pheophytins (Dalla Costa *et al.*, 2022). This may also affect the change in color from the color of fresh spirulina biomass towards the color of the spirulina powder. The dark green color was also supported by the value of lightness (L*) which was 39, the value of lightness was between 0 (black) -100 (white), with a value in the middle (50) was gray. Therefore, a value below 50 indicates a darker color. In the case of spirulina powder, it was dark green. The yellowness (b) was positive, indicating the presence of yellow pigment although not predominant, given a small value of yellowness was 7.8. The study of (Dalla Costa *et al.*, 2022) reported the presence of carotenoids such as ß-carotene, zeaxanthin and xanthophyls in spirulina powder.

Protein content of spirulina powder used in the present study was 53.08±1.13% dry basis. There were variations in the protein content of spirulina powder reported by numerous studies. Previous

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study (Becker, 2007) reported that spirulina contained 46-63% protein on dry basis, whereas others reported spirulina contained 60-70% protein dry basis (Bashir *et al.*, 2016, Benelhadj *et al.*, 2023, López-Rodríguez *et al.*, 2021). The protein content of spirulina powder in our study was slightly higher than that reported by (Sela *et al.*, 2021) which was 49.51%, despite this study was also conducted in Indonesia, thus the environment for cultivation may have similar geographical and climate to spirulina that we used. Nevertheless, Dalla Costa *et al.* also found variations in the protein content of spirulina powder marketed in Italy ranging from 54.8-60.56% dry basis (Dalla Costa *et al.*, 2022). The amount of protein in spirulina can vary widely by modifying the medium chemical composition (Diaconu *et al.*, 2023). In addition, proteins were synthesized and accumulated in the cell biomass of spirulina over a cultivation period, a change in the levels of proteins involved in central metabolic processes may take place as these cyanobacteria respond to stress (Giner-Lamia *et al.*, 2016).

Alkaline-acid extraction method produced dried protein concentrate with the highest protein content of 67.04±3.85% dry basis, slightly higher but not significantly different from that produced by a combination of microwave and alkaline-acid extraction (65.54±1.92% dry basis). The results here were lower than that reported by other studies using alkaline-acid extraction (Bashir *et al.*, 2016, Mahmood and Sibi, 2019, Parimi *et al.*, 2015). Previous study reported protein content of around 75% of the protein extract, however, they pre-treated the spirulina suspension with high pressure homogenization prior to alkaline-acid extraction (Mahmood and Sibi, 2019). The method we applied was similar to a study in Pakistan (Bashir *et al.*, 2016), the difference was in the use of 2 M NaOH instead of 1 M NaOH for alkaline treatment that produced freeze dried protein extract containing 74% of protein. Thus, the higher the molarity of the alkaline solution the more protein can be extracted, because the solubility of the protein was the highest in alkaline condition. This results were also reported by another study (Parimi *et al.*, 2015) which optimized a wide range of alkaline pH, the solubilization time and the biomass concentration to produce protein extract of spirulina resulting in protein content of 80.6%. In their study, the spirulina suspension was pre-treated with high pressure homogenization. The study also reported that optimum solubilization pH was between 8-11, above pH 11 a decreased protein solubility was observed, possibly due to denaturation (Parimi *et al.*, 2015). On the contrary, the results we obtained were higher than that reported by another study (Lupatini *et al.*, 2017) which produced 75% yield containing 38% protein. The study employing alkaline extraction combined with sonication and agitation, the lower protein content may be attributed to not combining the alkaline extraction with the isoelectric/acid pH to facilitate the protein precipitation. Thus, from our study and others it can be concluded that treated with alkaline condition until pH 11 was the best condition for protein solubility, followed by precipitation at isoelectric (acid) pH until 4 was able to increase the recovery of the spirulina proteins. In addition, pre-treatment using high-pressure homogenisation was proven to facilitate cell disruption of the spirulina better than ultrasound and autoclaving (Parimi *et al.*, 2015, Safi *et al.*, 2014).

We used a conventional microwave to facilitate cell disruption for practical reasons, however, the method was only able to increase protein content from 53% (raw material) to 55% (dried protein extract) dry basis, indicating more impurities still present in the product. Microwave heating extraction involves electromagnetic radiation in the microwave frequency range in the sample to rotate and produce thermal energy in the solvent. Energy induces the vibration of polar molecules with a rapid increase in temperature and eventually increases the efficiency of extraction process (Rokicka *et al.*, 2021). However, the use of a conventional microwave method with a power of 400 watts for 1 minute in our study was not enough to maximize the extraction of the protein.

The microwave assisted extraction (MAE) was reported to produce better results. The MAE

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applied in chlorella extraction increased the protein content from 48% dry weight in the raw material to 69% dry weight in the protein extract (Margenat *et al.*, 2023). The conventional microwave method that we used was set at 400 Watts similar to that in the study of Margenat. However the time was set differently, 1 minute in our study and 10 minutes in this study (Margenat *et al.*, 2023). In addition, the suspension of chlorella in the study was conditioned at pH 9, thus more like our third method, a combination of conventional microwave and alkaline-acid extraction method. Nonetheless, MAE had several advantages as one of physical treatments for cell disintegration (Rokicka *et al.*, 2021) such as require a relatively low energy input, fast heating and short reaction times (Kapoore *et al.*, 2021). Among the cell disintegration using physical and mechanical techniques, high pressure homogenization was the most effective because it could produce 90% protein recovery in the product (Safi *et al.*, 2014).

It is necessary to measure WHC to evaluate the interactions between protein and water in food systems, thus, to be able to identify its suitable application. WHC was the lowest in dried protein concentrate produced by microwave extraction whereas those produced by alkaline-acid extraction and a combined method were comparable but higher than that of the microwave extraction. The WHC of our dried protein concentrate produced by alkaline-acid extraction or a combined method were 1.63-1.64 g H_2O/g protein thus, lower than the WHC of spirulina protein isolate produced by the same method in the study in Pakistan which was 2.96 ml H_2O/g protein (Bashir *et al.*, 2016) but comparable to those reported by (Mahmood and Sibi, 2019) ranging from 0.902-1.34 g H_2O/g protein. The WHC of protein is affected by protein intrinsic properties and by concentration, ionic strength, pH, temperature, and process conditions (Zhang *et al.*, 2021). The alkaline-acid extraction method obviously changed the pH and ionic strength of the protein and thus contributed to the WHC. This might explain the difference between the WHC of dried protein concentrate produced by the conventional microwave extraction and the alkaline-acid extraction. In relation to temperature, the protein concentrate was dried at 60 ºC for 12 hours. A study on Faba bean protein reported that the WHC did not change after dry heating at 75 ºC with the values of around 1.25 g H_2O/g protein, while the WHC increased by 86.4% upon heating up to 150 ºC (Bühler *et al.*, 2020).

Some proteins are excellent foaming agents, a foam is defined as a two-phase mixture in which the gaseous phase is surrounded by a continuous phase (liquid or solid) (Zhang *et al.*, 2021). The FC of dried protein concentrate produced by alkaline-acid extraction was the lowest among the three methods. This result agreed with those reported by (Mahmood and Sibi, 2019), where alkaline treatment in that study also produced protein isolate with the lowest FC. Another study reported that protein fractions in spirulina, consisted of albumin-like protein fraction, globulin-like protein fraction and prolamin-like protein fraction which demonstrated different WHC as well as FC (Benelhadj *et al.*, 2023). Globulin exhibited the highest FC (341%) followed by albumin (153%) and prolamin (118%). Thus, depending on the predominant protein fraction of spirulina being extracted using various methods will affect the foaming capacity.

The strength of the present study was the use of relatively simple methods to extract protein from spirulina which produced protein concentrate ranging from 55-67% dry basis. However, the comparison between three distinct methods did not provide more elaborate information of each method that becomes the limitation of this study. For example, the use of conventional microwave extraction with a fixed duration and power was not able to maximize the protein extraction. In the future, the use of conventional microwave extraction may have to be optimized by combining a range of duration and power to obtain the highest yield of protein. In addition, a combination of several methods to optimize protein extraction is an open area for further research. A combination of several methods may also produce protein with different functional properties which are also interesting to investigate.

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Apart from protein extraction, in the future, assessing the bioactivity of the protein concentrate is necessary, since this microalga is rich in bioactive compounds that may be extracted by the techniques applied. Nevertheless, assessing the safety aspect of spirulina is equally important before it is widely used for human consumption.

Conclusions

The utilization of *Spirulina platensis* as a protein sources was limited by the rigidity of its cell wall. Three extraction techniques were used to disrupt the spirulina cell wall, namely microwave, alkaline-acid and a combination of microwave and alkaline-acid extraction. The alkaline-acid extraction produced dried protein concentrate with the highest protein content (67.04% dry basis) but the lowest yield (13.9%). The water holding capacity of the dried protein concentrate was the highest in alkaline-acid extraction, and the lowest in conventional microwave. On the contrary, the foaming capacity was the highest in the conventional microwave extraction and the lowest in alkaline-acid extraction. The extraction techniques not only affected the yield and protein content but also the functionality of the protein concentrate.

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Authors' contributions

Siti Narsito W designed the study, interpreted the data and wrote the manuscript; Septian Dwi N and Raden Satrio Mukti Wibowo J conducted the research, collected and analyzed the data; Harijono H interpreted the data and reviewed the manuscript. All authors read the final of manuscript and approved it for publication.

Conflict of interest

The authors declare no conflicts of interest.

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