Extraction and Enzymatic Hydrolysis of *Spirulina* Protein as Antioxidant

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Abstract

Spirulina sp. is a microalgae species that is consumed as a dietary supplement due to its high nutrition content, especially protein and its bioactivity as a radical scavenger. Extraction and hydrolysis processes are expected to enhance *Spirulina*'s functional characteristic and its bioactivity to inhibit free radicals 1,1-diphenyl-2-picryl-hydrazyl (DPPH). The aim of this study was to compare the effect of two different extraction methods on protein concentration, degree of hydrolysis, DPPH free radical inhibition, and amino acid profile of *Spirulina* sp. hydrolysate. *Spirulina* sp. were extracted using two different methods, physical and chemical methods. The crude protein extracts were analysed using Biuret method and hydrolysed using papain enzyme. The protein hydrolysate was finally run through some analysis such as hydrolysis degree, amino acid profile, and DPPH scavenging activity. The two different extraction methods effected on protein concentration and degree of hydrolysis significantly ($p < 0,05$). Protein concentration of *Spirulina* sp. that were extracted using physical method is 67,4 ± 14,35 mg/mL, while *Spirulina* sp. that were extracted using chemical method is 53,41 ± 3,90 mg/mL. Hydrolysis degree of protein hydrolysate extracted using physical method is 77,141 ± 11,945% while *Spirulina* sp. that were extracted using chemical method is 35,081 ± 15,884%. Both *Spirulina* sp. extract and hydrolysate %inhibition decreased after storage. Hydrolysate extracted using physical method contains 8.711,56 mg/kg of amino acid, while hydrolysate extracted using chemical method contains 3.391,56 mg/kg.

Keywords: DPPH radical inhibition, extraction, protein hydrolysate, *Spirulina* sp.

1. Introduction

Spirulina sp. is blue-green microalga that classified as *Cyanobacteria.* This microalga is known for its high content of protein that ranges from 64 to 73%. Spirulina sp. proximate compositions include 12–17% of carbohydrate, 10,3–11,6% of nitrogen, 5–7% of fat, and 0,9% of phosphor (Martin, Echevarrieta, & Otero, 2019). Food and Drug Admission (FDA) has approved *Spirulina* sp. dried biomass consumption as dietary supplement with daily intake of 3–4,5 g (FDA 2013). This microalga also has been known for its bioactivity, such as anticancer (Sirait, Setyaningsih & Tarman, 2019), antidiabetic, antiinflammation (Prabakaran, Manikannan, & Moovendhan, 2020), and anti-malaria (Wulandari et al. 2018). One of the most potential bioactivity of *Spirulina* sp. that had been tested *in vitro* as well as *in vivo* is antioxidant (Yu et al., 2016). *Spirulina*'s bioactivities are allegedly related to its high protein content.

Despite its high protein content, *Spirulina*'s protein has a lower biological value than egg's protein, which is 68% and 94,7%. Therefore, protein extraction is needed to increase *Spirulina* sp. protein availability that is quite low due to its hard-to-digest cell wall that is made of cellulose and muramic acid (Vernes et al. 2019). Carizzo, Conte, Sommella, Damato, Ambrosio, et al., (2018) also stated that protein extraction process can also improve the stability of bioactive peptides resulting from the process of hydrolysis inside human gastro intestinal tract, so that it can be utilized more optimally by human's body. Extraction methods that can be used to extract proteins from microalgae i.e., by physical and chemical methods. Chemical methods are carried out by utilizing protein solubility at its isoelectric point (Lisboa, Pereira, & Costa, 2017). Protein

extraction using the physical method can be done by combining freeze-thaw and sonication methods (Vernes et al. 2019). The effectiveness of protein extraction methods physically and chemically is not yet known.

Spirulina sp. protein can be hydrolysed to produce protein hydrolysate containing peptides and amino acids. The hydrolysis process increases digestibility of Spirulina sp. from 74.1% to 100%. It also increases Spirulina sp. inhibition activity of free radicals from 25.35% to 73.25% (Lisboa, Pereira, & Costa, 2017). The hydrolysis process can be done using dry biomass or crude protein of Spirulina. The effect of using different materials during hydrolysis process is not yet known.

2. **Material and Methods**

The ingredients used in this study include *Spirulina* sp. powder obtained from PT. Nanotech Herbal Indonesia, NaOH (Merck, Germany), HCl (Merck, Germany), papain (Sigma Aldrich), bovine serum albumin (Sigma Aldrich), ascorbic acid (Merck, Germany), methanol (Smartlab), copper(II)sulphate pentahydrate (Sigma Aldrich), potassium sodium tartrate (Sigma Aldrich), potassium iodide (Merck, Germany), dan 1,1-diphenyl-2-picrylhydrazyl (DPPH) (Sigma Aldrich).

This research consists of two main stages, namely protein extraction using two different methods and enzymatic hydrolysis using papain. Spirulina sp. powder was analysed for its physical and chemical characterization. Physical characterizations were done by observing *Spirulina* sp. appearance, smell, and texture, and also measuring *Spirulina* sp. size particle under microscope then analysed using ImageJ software. On the other hand, chemical characterizations were done by measuring water and protein content of *Spirulina* sp. powder based on AOAC (2015). Protein extraction using physical method refers to Wang & Zhang (2017) with some modifications. Spirulina sp. powder as much as 3% (w/v) was sonicated with the frequency of 40 kHz for 30 minutes, then continued with 4 cycles freeze-thaw consist 12 hours of freezing in -18°C, and 12 hours of thawing at room temperature. The process of protein extraction using chemical method refers to Lisbon et al. (2016) and Parimi et al. (2015) with some modifications. Spirulina sp. was dissolved in NaOH 1% pH 11 with a concentration of 3% (w/v), then precipitated using HCl 1 M pH 4. Crude protein extracts that were obtained were analyzed using biuret method refers to Gournall et al. (1949). The protein hydrolysis process refers to Barkia et al. (2018) with some modifications. Crude protein extracts were hydrolyzed using papain enzyme 6% (w/w) in temperature 60°C and pH 6 for 4 hours. Obtained hydrolysates were run some analysis such as degree of hydrolysis (Hoyle & Meritt 1994), amino acid profile (AOAC 2005), and inhibition of DPPH free radical activity (Blois 1958).

3. **Results and Discussion**

Characterization of *Spirulina* sp. Powder

Spirulina sp. powder has a dark green appearance, smooth powdery texture, and fishy aroma. The color of Spirulina sp. powder is caused by bio pigment content, phycocyanin, and chlorophyll. The smell of Spirulina sp. comes from mineral salts contained in the cultivation medium and volatile compounds in Spirulina sp. (Ekantari, Marsono, Pranoto, & Harmayani, 2017). Spirulina sp. powder has an average diameter size of 64,206 μm. The particle size of Spirulina sp. powder affects the extraction process. Ardyanti, Suhendra, & Puta (2020) stated that enhancement of surface area made the interaction of the solvent with the material easier, therefore the extraction process would work optimally. Moreover, the process of reducing particle size can also damage the cell wall. The damage of the cell wall can cause the targeted compounds inside the raw material to be more easily accessible to the solvent during the extraction process.

Spirulina sp. powder has moisture content of 1,11% and protein content of 57.88%. *Spirulina* sp. powder moisture content was influenced by the drying method. Bennamoun, Afzal, & Leonard (2015) state that the effective temperature for drying out *Spirulina* sp. biomass without causing

protein denaturation is 55–60 °C. On the other hand, Michael, Kyewalyanga, & Lugomela (2019) stated *Spirulina* sp. protein content was influenced by the nutrient content in cultivation media, especially nitrogen. Nitrogen is the most important element in cell division and growth, as well as the formation of amino acids, amide, nucleotides, and nucleoproteins in cells. *Spirulina fusiformis* cultivated in Zarrouk media contains 65% of protein, while *Spirulina* sp. cultivated in Walne media contains 50.05% of protein. Zarrouk's media contains $NaHCO_3$, $NaNO_3$, K_2HPO_4, K_2SO_4, NaCl, $MgSO_4.7H_2O$, $CaCl_2$, $FeSO_4.7H_2O$, EDTA, and micronutrients. On the other hand, Walne provides two sources of nitrogen, ammonium (NH_4^+) and nitrate (NO_3^-) (Sirait et al. 2019). Another factor that affects the protein content of *Spirulina* sp. is environmental conditions of cultivation media, such as pH, salinity, temperature, and light intensity. *Spirulina* sp. grows optimally on culture media that have salinity ranging from 15–18 ppt, temperature 35 °C, light intensity 2.000–3.500 lux, and pH ranges from 8–11 (Ekantari, et al. 2017).

Protein Extract of *Spirulina* sp.

Extraction is the process of separating a desired substance from a mixture using a specific solvent (Zhang, Lin, & Ye 2018). *Spirulina* sp. crude protein extracts were measured protein concentration using Biuret method so that the yield was obtained. The result of protein concentration and protein yield of *Spirulina* sp. crude extract can be seen in Table 1.

Tabel 1 Protein concentration and yield of *Spirulina* sp. protein extract

Sample	Extraction method	Protein concentration (mg/mL)	Protein yield (%)
Spirulina sp. powder	Physical method (*freeze thaw* and sonication)	67.34 ± 14.35^a	$22.45 \pm 3,.3^a$
	Chemical method (isoelectric pH)	53.41 ± 3.9^b	17.80 ± 1.21^b

Note: Numbers in the same column followed by the same letter indicate no significant difference at the test level of 5% (T-test independent sampel).

The results of *Spirulina* sp. protein yields and concentrations showed that two different methods of protein extraction affect *Spirulina* sp. protein concentration and yield significantly ($p > 0.05$). Combination of freeze-thaw and sonication methods resulted higher protein concentrations and yields than the isoelectric method. During the freeze-thaw method, *Spirulina* sp. would form crystals due to freezing conditions and the cells were stretched during the thawing process. Those phenomena occurred repeatedly each cycle and caused *Spirulina* sp. cell walls to be damaged (Lee, Show, & Ling, 2017). The Sonication method utilizes ultrasonic waves that can cause cavitation in *Spirulina* sp. cells so that the cell wall is degraded. This process causes proteins inside the cell to become accessible to be extracted by the solvent (Chia, Wayne, Manickam, Show, & Nguyen, 2020). The *Spirulina* sp. that was extracted using chemical method obtained less yield than physical method. Lisboa *et al.* (2017) stated that *Spirulina* sp. protein extracted by chemical methods obtained 53,8% of crude protein extract. Low concentration value and protein yield in *Spirulina* sp. extracted by chemical methods could be caused by different extraction conditions, such as solvent concentration and soaking time of *Spirulina* sp. powder in alkali. Devi, Suptijah, & Nurilmala, (2017) stated that the concentration of the alkaline solution and the length of immersion of the sample in the alkaline solution during the protein extraction process affect the resulting yield. The use of alkaline solutions with concentrations that are too low is less effective in dissolving proteins, while the concentration of solutions that are too high can cause some

proteins to malfunction. Another factor that caused low yields resulting from the extraction process by chemical methods was the formation of protein clusters and denaturation of some proteins due to interaction with alkali. This interaction could be caused by damage or changes in the surface of proteins resulting from interaction with alkaline. Protein cluster formation was caused by protein aggregation that could reduce protein solubility in the solvent (Lee et al. 2017).

Hydrolysis Degree of *Spirulina* sp. Hydrolysate

Hydrolysate of *Spirulina* sp. protein extracts and hydrolysate of *Spirulina* sp. powder were analyzed using SN-TCA methods to acknowledge the degree of hydrolysis. The degree of hydrolysis is the value of peptide bonds that are broken down and catalyzed by proteolytic enzymes during the hydrolysis process expressed in percent units. Analysis of hydrolysis degree was done using trichloro acid precipitation. The degree of hydrolysis was expressed by calculating the ratio between the total protein before and after hydrolysates dissolved in TCA (Sossella et al. 2020). The hydrolysis degree of *Spirulina* sp. protein extract can be seen in Table 2.

Table 2 Hydrolysis degree of *Spirulina* sp. hydrolysate

Sample	Degree of Hydrolysis (%)
Protein hydrolysate extracted using physical methods	77.14 ± 11.95^{b}
Protein hydrolysate extracted using chemical methods	42.71 ± 10.69^{a}
Hydrolysate of *Spirulina* Powder (control)	35.08 ± 15.88^{a}

Note: Numbers in the same column followed by the same letter indicate no significant difference at the test level of 5% (DMRT multiple hose test).

The results showed two different extraction methods affect hydrolysis degree of *Spirulina* sp. hydrolysate significantly ($p > 0.05$). *Spirulina* sp. hydrolysate extracted by the physical method has the highest degree of hydrolysis, while the lowest was found in control. This indicates that protein extraction before the hydrolysis process increases the hydrolysis degree of hydrolysate due to the improvement of protease accessibility to protein molecules to initiate the hydrolysis process (Lisboa et al. 2017). Hydrolysate of *Spirulina* sp. extract using physical method produced the highest degree of hydrolysis. Lisboa et al. (2017) in their research compared the hydrolysis degree values of the three hydrolysates with different substrates, namely protein concentrates, protein isolates, and *Spirulina* sp. biomass. Hydrolysate of protein concentrate produced the highest degree of hydrolysis at 69.2%. Hydrolysate from *spirulina* sp. biomass obtained the lowest hydrolysis degree value at 58.1%. Extraction of proteins before hydrolysis can increase the degree of hydrolysis of the resulting hydrolysis. The protein extraction process can increase the accessibility of protease enzymes to protein molecules to initiate the process of hydrolysis. Haryati *et al.* (2018) also stated that high protein content on hydrolyzed substrates can increase the degree of hydrolysis of hydrolysis produced.

Protein hydrolysate extracted by the chemical method has a lower degree of hydrolysis than hydrolysate extracted by physical methods. Gao et al. (2020) stated that the extraction method using alkaline solution can increase total sulfhydryl groups and disulfide bonds in protein isolates. The active side of the papain enzyme works by breaking off proteins containing cysteine residue through a deprotonation reaction. Disulfide bonds are protein structures that are very hydrophobic and difficult to break off by the enzyme papain. Disulfide bonds and sulfhydryl groups can decrease the accessibility of the papain active side in attacking its target, cysteine residues.

DPPH Inhibition Activity of *Spirulina* sp. Protein Extract and Hydrolysis

Antioxidant activity can be expressed in percent inhibition which is a value that states the ability of antioxidant compounds to inhibit free radicals (Esfandi et al. 2019). Antioxidant analysis was done twice, after the hydrolysis process (day-0) and after hydrolysates were stored in freezer

for 210 days (day-210). Inhibition of DPPH free radical of *Spirulina* sp. protein extract and hydrolysate on day-0 and day-210 can be seen in Table 3.

Table 3 DPPH inhibition activity of *Spirulina* sp. extract and hydrolysate

Sample	Extraction method	DPPH inhibition activity (%)	
		Day-0	Day-210
Protein extract	Physical	40.51 ± 9.33	20.58 ± 0.82
	Chemical	32.88 ± 10.12	16.08 ± 1.38
Protein hydrolysate	Physical	50.77 ± 4.32	10.52 ± 4.84
	Chemical	35.10 ± 11.22	5.09 ± 0.47

Antioxidant activity has slightly increase after protein extract hydrolyzated with papain. Hydrolyzed protein extracted by the physical method shown the highest activity of DPPH free radical inhibition. Lisboa et al. (2017) stated that *Spirulina* sp. protein hydrolysates had scavenging activity 8.5 times higher than *Spirulina* sp. biomass because enzymatic hydrolysis process produced peptides and various amino acid sequences. Those peptides and amino acids sequences obtained are potential to have antioxidant capacity. Prastika, Laksmawati, Ratnayani, & Puspawati, (2018) also stated that the process of hydrolysis using enzymes could increase the ability of peptides to donate electrons to free radical compounds.

Spirulina sp. protein extract using physical method had lower DPPH inhibition activity on Day-0 than its hydrolysate. Aftari *et al.* (2017) stated that extraction time using the freeze-thaw method can affect antioxidant activity of *Spirulina.* Extraction process using freeze-thaw method for more than 4 hours in one cycle could decrease the percentage of inhibition value. The increasingly long extraction time caused the solvent to extract other components in the cell, thereby lowering the purity of the extracted protein. It also has an impact on decreasing the percent value of the inhibition in the resulting extract.

Both protein hydrolysate and extract of *Spirulina* sp. using chemical method had lower DPPH inhibition activity than protein hydrolysate and extract using physical method. This could be due to different extraction methods could produce protein and peptides with different antioxidant mechanism. Proteins and peptides have different antioxidant mechanisms, namely radical scavengers, chelating agents, reducing agents, and lipid peroxidation inhibitors. The mechanism of antioxidant activity can be determined through the physicochemical properties of amino acids. Amino acids that have aromatic rings such as tyrosine, tryptophan, and phenylalanine have an antioxidant mechanism of electron transfer. Hydrophobic peptides and proteins more optimally reduce oxidative reactions to lipids. (Esfandi, Walters, & Tsopmo, 2019).

The inhibition value of all samples decreased after being stored with solution form in the freezer for 210 days. *Spirulina* sp. protein extract extracted using the physical method has the highest DPPH inhibition activity after 210 days of storage. The decrease is due to changes in the molecular structure of proteins that can affect the biological activity and functional properties of proteins. Types of changes in protein molecular structure during storage in low temperature include dissociation and subunit changes in protein oligomers, aggregation, and changes in protein conformation. Aggregation of peptides and proteins in a solvent could be caused by several factors, namely the phenomenon of unfolding, chemical bonds, and degradation due to other chemical compounds. Aggregation in protein hydrolysate were characterized by the presence of deposits caused by the unfolding proteins causing proteins to become insoluble. Aggregation of proteins and peptides is irreversible. This phenomenon could cause bioactive peptides that were contained in hydrolysates to be partially or completely degraded. Thus, affecting the biological activity of bioactive peptides. The degree of protein damage caused by storage at low temperatures is influenced by storage temperature, storage length, freezing and thawing conditions, protein types, as well as the complexity of protein structures (Fennema, 1982).

Amino Acids Profile of *Spirulina* sp. Hydrolysates

Both protein hydrolysates are analyzed their amino acid content using HPLC. The analysis was aimed to compare the amino acid profiles of the two hydrolysates. Amino acid profiles of *Spirulina* sp. protein hydrolysate can be seen in Figure 1.

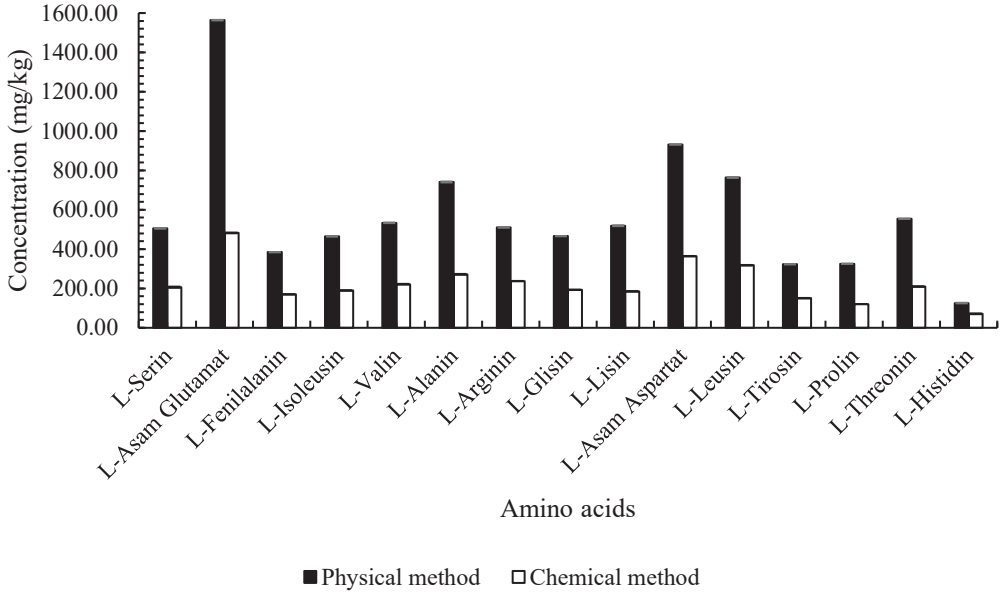

Figure1 Amino acid profiles of *Spirulina* sp. hydrolysate extracted using physical and chemical method

The results showed both *Spirulina* sp. protein hydrolysates contain 15 types of amino acids. Hydrolysate of protein extract extracted by the physical method has a higher amino acid content, which is 8,711.56 mg/kg, while hydrolysate of protein extract extracted by chemical method contains 3,391.56 mg/kg of amino acids. The most dominant amino acids in both hydrolysates are glutamic acid, aspartic acid, and leucine. The amino acid content in hydrolysate of protein extracts extracted by the physical method is higher than hydrolysate extracted by chemical methods. The composition of amino acids was influenced by the process of hydrolysis which breaks down proteins into peptides and amino acids. Sequences and amino acid structures in a peptide also greatly affect its antioxidant activity. Amino acid fractions containing glutamic acid and leucine have high antioxidant activity. Glutamic acid plays a role in preventing oxidation reactions, while leucine has high scavenging activity (Shazly et al. 2017).

4. CONCLUSION

A combination of freeze-thaw and sonication methods was able to extract *Spirulina* sp. proteins higher than alkali dissolution and precipitation at the isoelectric point, which is indicated by the resulting protein concentration of 67,4 ± 14,5 mg / mL and 53,41 ± 3,90 mg/mL. The highest of Degree of enzymatic hydrolysis of protein extract from Hydrolysate of protein extract extracted by freeze-thaw and sonication methods also has higher hydrolysis degree values and amino acid composition, which is 77,14 ± 11,95% and 8.711,56 mg/kg. Different extraction methods do not affect the *Spirulina* sp. DPPH free radical activity significantly.

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REFERENCES

[AOAC] Association of Official Analytical Chemist. 2005. *Official Method of Analysis of The Association of Official Analytical of Chemist.* Arlington (US): The Association of Official Analytical Chemist, Inc.
Aftari, R. V., Rezaei, K., Bandani, A. R., & Mortazavi, A. (2017). Antioxidant activity optimization of *Spirulina platensis* C-phycocyanin obtained by freeze-thaw, microwave-assisted, and ultrasound-assisted extraction method. *Quality Assurance and Safety of Crops & Foods. 9*(1): 1-9. doi: https://doi.org/10.3920/QAS2015.0708.
Ardyanti, N. K. N. T., Suhendra, L., & Puta, G. G. P. (2020). Pengukuran ukuran partikel dan lama maserasi terhadap karakteristik ekstrak *virgin coconut oil* wortel (*Daucus carota* L.) sebagai pewarna alami. *Jurnal Rekayasa dan Manajemen Agroindustri, 8*(3): 423-434.
Barkia, I., Al-Haj, L., Abdul, H. A., Zakaria, M., Saari, N., & Zadjali, F. (2018). Indigenous marine diatoms as novel sources of bioactive peptides with antihypertensive and antioxidant properties. *International Journal of Food Science & Technology,* 1-9. doi:10.1111/ijfs.14006.
Bennamoun, L., Afzal, M. T., & Leonard, A. (2015). Drying of alga as a source of bioenergy feedstock and food supplement – A review. *Renewable and Sustainable Energy Reviews, 50:* 1203-1212. doi: https://doi.org/10.1016/j.rser.2015.04.196.
Blois, M. S. (1958). Antioxidant Determinations by the use of a stable free radical. *Nature, 181,* 1199-1200.
Carrizo, A., Conte, G. M., Sommella, E., Damato, A., Ambrosio, M., Sala, M., Scala, M. C., Aquino, R. P., Lucia, M. D., Madonna, M., et al. (2018). Novel potent decameric peptide of *Spirulina platensis* reduces blood pressure levels through a PI3K/AKT/enos-dependent mechanism. *Nonpharmacological Treatment, 73:* 449-457. doi: 10.1161/HYPERTENSIONAHA.118.11801.
Chia, S. R., Wayne, C. K., Manickam, S., Show, P., & Nguyen, H. P. (2020). Sonoprocessing-assisted solvent extraction for the recovery of pigment-protein complex from Spirulina platensis. *Chemical Engineering Journal, 398.* doi: 10.1016/j.cej.2020.125613.
Devi, H. L. N. A., Suptijah, P., Nurilmala, M. (2017). Efektifitas alkali dan asam terhadap mutu kolagen dari kulit ikan patin. *JPHPI, 20*(2), 255-265. doi: http://dx.doi.org/10.17844/jphpi.v20i2.17906.
Ekantari N., Marsono, Y., Pranoto, Y., & Harmayani, E. (2017). Pengaruh media budidaya menggunakan air laut dan air tawar terhadap sifat kimia dan fungsional biomassa kering *Spirulina* sp.. *AGRITECH, 37*(2), 173-182. doi:10.22146/agritech.10843.
Esfandi, R., Walters, M. E., & Tsopmo, A. (2019). Antioxidant properties and potential mechanism of hydrolysed proteins and peptides from cereals. *Heliyon, 5*(4): 1-26. doi: doi.org/10.1016/j.heliyon.2019.e01538.
Fennema, O. (1982). Behavior of Proteins at Low Temperatures. *Food Protein Deterioration,* 109–133. doi:10.1021/bk-1982-0206.ch006.
Gao, H., Korn, J., Ferretti, S., Monahan, J. E., Wang, Y., Singh, M., et al. (2015). High-throughput screening using patient-derived tumor xenografts to predict clinical trial drug response. *Nat. Med, 21***,** 1318–1325. doi: https://doi.org/10.1038/nm.3954.
Lee, S., Show, P., & Ling, T. (2017). Single-step disruption and protein recovery from *Chlorella vulgaris* using ultrasonication and ionic liquid buffer aqueous solutions as extractive solvents. *Biochemical Engineering Journal, 124*, 26-35. doi:10.1016/j.bej.2017.04.009.
Lisboa, C., Pereira, A., & Costa, J. (2017). Microalgal protein hydrolysis in different bioreactor types. *African Journal of Microbiology Research*, *10*(3), 79–86. doi: 10.5897/AJMR2015.7760.
Martin, C. M. V., Echevarrieta, L., & Otero, C. (2019). Advantageous preparation of digested proteic extracts from *Spirulina* sp. biomass. *Catalyst, 145*(9), 1-15. doi:10.3390/catal9020145.
Michael, A., Kyewalyanga, M. S., & Lugomela, C. V. (2019). Biomass and nutritive value of *Spirulina* (*Arthrospira fusiformis*) cultivated in a cost-effective medium. *AnnMicrobiol*, *69,* 1387–1395. doi: org/10.1007/s13213-019-01520-4.

Prabakaran, G., Manikannan, K., & Moovendhan, M. (2020). Extraction and characterization of phycosyanin from *Spirulina platensis* and evaluation of its anticancer, antidiabetic, and antiinflamatory effect. *International Journal of Biological Macromolecules, 153:* 1-9. doi: 10.1016/j.ijbiomac.2020.03.009.

Prastika, H. H., Laksmawat,i A., Ratnayani, K., & Puspawati, N. M. (2019). Penggunaan enzim pepsin untuk produksi hidrolisat protein kacang gude (*Cajanus cajan* (L.) Millsp.) yang aktif antioksidan. *Indonesian E-Journal of Applied Chemistry, 7*(2), 180-188.

Shazly, A., Mu, H., Liu, Z., El-Aziz, M., Qin, F., Zhang, S., He, Z., & Chen, J. (2018). Release of antioxidant peptides from buffalo and bovine caseins: Influence of proteases on antioxidant capacities. *Food Chemistry*, *274.* doi: 10.1016/j.foodchem.2018.08.137.

Sirait, P. S., Setyaningsih, I., & Tarman, K. (2019). Anticancer activity of *Spirulina* cultivated in walne and organic media. *Jurnal Pengolahan Hasil Perikanan Indonesia, 22*(1), 50-59. doi: https://doi.org/10.17844/jphpi.v22i1.25876.

Vernes, L., Avert-vian, M., El Maatoui, M., Tao, Y., Bornard, I., & Chemat, F. (2019). Application of ultrasound for green extraction of proteins from spirulina. Mechanism, optimization, modeling, and industrial prospects. *Ultrasonics Sonochemistry, 54:* 48-60. doi: https://doi.org/10.1016/j.ultsonch.2019.02.016.

Wang, X., & Zhang, X. (2012). Optimal extraction and hydrolysis of *Chlorella pyrenoidosa* proteins. *Bioresource Technology, 126*, 307–313. doi:10.1016/j.biortech.2012.09.059.

Widhianingsih, Ridho, A., Hartati, R., & Harmoko. (2008). Kandungan nutrisi kandungan nutrisi *Spirulina* sp. yang dikultur pada media yang berbeda. *Ilmu Kelautan, 13*(3): 167-170. doi:10.14710/ik.ijms.13.3.167-170.

Wulandari, D., Setyaningsih, I., & Asih, P. (2016). Extraction and In Vitro Antimalarial Activity Phycocyanin from Spirulina platensis. *Jurnal Pengolahan Hasil Perikanan Indonesia, 19*. 17-25. doi: 10.17844/jphpi.2016.19.1.17.

Yu, J,, Hu, Y., Xue, M., Dun, Y., Li, S., Peng, N., Liang, Y., & Zhao, S. (2016). Purification and identification of antioxidant peptides from enzymatic hydrolysate of *Spirulina* sp.. *J. Microbial. Biotechnol, 26*(7): 1216-1223. doi:10.4014/jmb.1601.01033.

Zhang, Q. W., Lin, L. G., & Ye, W. C. (2018) Techniques for extraction and isolation of natural products: a comprehensive review. *Chinese Medicine, 20*(13). doi: doi.org/10.1186/s13020-018-0177-x.