The quality of *Ulva lactuca* fatty acid microemulsion with ascorbic acid antioxidant during storage

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**Abstract.** This study aims to determine the effect of temperature and storage duration on *Ulva lactuca* fatty acid quality in microemulsion with ascorbic acid antioxidants. *U. lactuca* is a green algae obtained from coastal areas in Trenggole, Yogyakarta. The stages in this study were extraction of *U. lactuca* fatty acid, production of its microemulsion, and addition of ascorbic acid antioxidant. The extraction of fatty acid was carried out using hexane and ethanol solvents with a ratio of 1:19 (v/v) at 70°C for 3 hours. Furthermore, the microemulsion production was done by using three different types of surfactants, including Tween 80, Tween 20, and Span 80, at a ratio of 7.0:7.5:5.5 (v/v), respectively, with 80% water content and 200 ppm of *U. lactuca* fatty acid. This study consisted of three treatments of ascorbic acid antioxidants, namely 0, 100, and 200 ppm. The microemulsions with ascorbic acid were stored at 10, 30, and 50°C. The quality measurements were carried out between 4 to 20 days. The results showed the temperatures and storage time significantly affected turbidity, antioxidant activity, peroxide, and TBA value up to the 20th day of storage. The turbidity value increased more than 1% on the 20th day, while the peroxide and TBA values were still in the standard of food products. Therefore, the use of ascorbic acid as an antioxidant with a concentration of 200 ppm was appropriate to inhibit the rate of damage to the microemulsion.

1. Introduction
Seaweed is one of the most abundant biological resources in Indonesia. According [1], the country provides 34.47% of the world's seaweed production, around 9.30 million tons. One of the most abundant seaweeds in Gunungkidul Yogyakarta is the Chlorophyceae, *Ulva lactuca* [2]. This seaweed is a healthy food source for humans because it contains high nutrients. Furthermore, dried *U. lactuca* contains 7-8% water, 2.85% protein, 2.24% fat, 56.4% carbohydrates, 7.54% crude fiber, 30.89% ash, and also contains polyunsaturated fatty acids (PUFA) omega-3 and omega-6 fatty acids that could prevent heart disease, inflammation, and nervous system disorders [3].

The fatty acids are insoluble in water (hydrophobic), therefore they require a carrier system when applied to a hydrophilic system, namely in the form of an oil in water (o/w) microemulsion. In this system, *U. lactuca* fatty acids oil phase will be dispersed as droplets surrounded by surfactants in the aqueous phase. The advantage of microemulsions compared to other emulsion systems is that they are more stable in storage because the interfacial tension in microemulsions is very low [4]. The important thing in the stability of the microemulsion during storage is that the temperature and length of storage time can affect the intensity of the bond between surfactants, oil, and water in the microemulsion. The high content of unsaturated fatty acids in microemulsion causes it to be susceptible to damage. PUFA is
very easily oxidized, mainly due to sensitizers, light, temperature, and oxygen. One way to prevent oil damage in the microemulsion system is by adding ascorbic acid as an antioxidant [5].

The formulation of *U. lactuca* indicated that the highest concentration of fatty acids that could be added stably to the microemulsion formula was 200 ppm with a surfactant percentage of 20% (Tween 20, Tween 80, and Span 80) [6]. The stability of omega-3 fatty acid nanoemulsions by storing samples at refrigeration (4±1°C), room (28±1°C), and oven temperatures (40±1°C) was for 16 days [7]. The omega-3 fatty acid nanoemulsion was damaged with peroxide values exceeding the standard on the 12th day of storage [8]. Ruandra (2015) investigated the addition of ascorbic acid antioxidants into fish oil microemulsions and showed that ascorbic acid of 200 ppm was effective in inhibiting deterioration during storage at room temperature. Microemulsions with the addition of ascorbic acid as an antioxidant are expected to have more stable results at various temperatures during storage. Therefore, this study aims to determine the effect of ascorbic acid as an antioxidant to prevent fatty acid in microemulsion systems under different temperature conditions [8].

2. Materials and Methods

2.1. Materials

The materials used were green algae *Ulva lactuca* obtained from Trenggole Beach, Gunungkidul, Yogyakarta in November 2019, methanol (Merck, Germany), internal standard nonadecanoic acid (C19:0) (Sigma-Aldrich), nitrogen gas (CV. Perkasa), aquades (CV. Progo), n-hexane (Merck, Germany), Na2SO4 (Merck, Germany), surfactant Tween 20 (Merck, Germany), Tween 80 (Merck, Germany), and Span 80 (Merck, Germany), NH4SCN or ammonium thiosianat (Merck, Germany), FeCl2 (Merck, Germany), Trichloroacetic acid (Merck, Germany), Thiobarbituric Acid (Merck, Germany), technical grade ethanol 96%, 1,1-diphenyl-2-picrylhydrazil (DPPH) (Merck, USA), and ascorbic acid (Merck, Germany).

2.2. Macroalgae Extraction

The extraction was carried out using the LRC (Lepage and Roy) method with modification [9]. The step used 25 grams of fresh algae extracted with 250 ml solution with a ratio of hexane and ethanol (1:19 v/v), then nonadecanoic acid was added as internal standard (100 µl) and vortexed for 1 minute. Subsequently, the samples were macerated in a water bath at 70°C for 3 hours. The sample was cooled, and then 50 ml of distilled water and 100 ml of hexane were put in. The sample was then homogenized for 1 minute and centrifuged at 3000 rpm for 7 minutes. The organic phase (fatty acid layer) was collected using a syringe, then Na2SO4 was added to separate the fatty acids and distilled water. The fatty acids were evaporated using nitrogen and 2,000 µL of hexane was added, then analyzed by GC-MS [10].

2.3. Microemulsion Preparation

Microemulsion preparation was carried out by mixing surfactant, water, and fatty acids volume of 10 ml [6]. The preparation of microemulsion surfactant (20 %) used hydrophilic (Tween 80 and Tween 20) and hydrophobic (Span 80) types with a ratio of 7.0:7.5:5.5 (% v/v), without oil as a carrier. The water was added drop by drop until 80% of total volume, and *U. lactuca* fatty acids 0, 100, and 200 ppm were added to the microemulsion system. Subsequently, the mixture was mixed with a magnetic stirrer at 70 ± 5°C. *U. lactuca* fatty acid microemulsion was then stored at 10°C, 30°C, and 50°C for 20 days, and observations were conducted on days 0, 4, 8, 12, 16, and 20.

2.4. Turbidity Test

The turbidity test was executed using a UV/Vis spectrophotometer at a wavelength of 502 nm and a cuvette with a width of 1 cm and observed for its appearance [11]. The formula used to calculate turbidity was: turbidity index x cuvette length = 2,303 x absorbance.

A stable turbidity index was indicated by a number less than 1% with a non-cloudy and transparent appearance.
2.5. **Moisture Content**
The water content test was carried out using the Moisture Content tool, namely MB 120 moisture analyzer OHAUS.

2.6. **Antioxidant Activity**
The antioxidant activity was performed out using the radical scavenging activity (RSA) DPPH (1,1-diphenyl-2-picrylhydrazyl) method [12]. The solution was made in a falcon tube wrapped in aluminum foil. 1.6 mL of ethanol solution was added with 0.4 mL of 0.76 nM DPPH into the cuvette (A), in another cuvette (B), 1.6 mL of the sample was added to 0.4 mL of DPPH solution. The solution was incubated for 30 minutes at room temperature and dark conditions. Furthermore, the absorbance was measured at a wavelength of 517 nm using a UV-Vis spectrophotometer. The antioxidant activity was calculated using the percentage of inhibition (percentage of inhibition) with the formula:

\[
\% \text{ Inhibition} = \frac{(A - B)}{(A)} \times 100
\]

2.7. **Peroxide Value**
The peroxide value was carried out by mixing 0.05 ml of sample, 10 ml of benzene: methanol (70:30), 0.05 ml of 0.038 M FeCl\(_2\), and 0.05 ml of 30% ammonium thiocyanate, that a red color was formed [13]. The mixture was vortexed for ± 5 seconds and incubated at 50°C for 2 minutes, and the mixture was read for absorbance using a spectrophotometer at a wavelength of 510 nm. The peroxide value was calculated as milliequivalents of peroxide per kg of fat with the following equation:

\[
\text{Peroxide Value} = \frac{(A \times B \times DF)}{(C \times 55.84)}
\]

Note: A = μg Fe per 10 ml; B = solution volume; C = sample weight; DF: dilution factor

The standard solution was prepared by dissolving 2.5 g of FeCl\(_3\) until the volume reached 25 ml, then the solution was diluted with water to a volume of 250 ml. 0.5ml of the mixture was taken, then diluted with benzene methanol solution to a volume of 100 ml, and used as a standard. The solution was pipetted as a series of dilutions of 0.5; 1.0; 2.0; 3.0; 4.0; and 5.0 ml and then placed into a test tube and diluted until the volume was 10 ml. Each tube was added with 50 μl of ammonium thiocyanate solution and 50 μl of FeCl\(_3\) solution, then homogenized by vortex for 5 seconds. Also, the solution was incubated in a shaker at 50°C for 2 minutes, and the absorbance value was measured at a wavelength of 510 nm. The relationship between the absorbance value and Fe (μg) concentration was expressed using regression equation.

2.8. **Thiobarbituric Acid (TBA) Value**
One-gram sample was weighed, then 5 ml of 10% TCA solution was added and shaken until homogeneous. The solution was filtered using a filter paper to obtain a clear filtrate, then 1 ml of clear filtrate was taken and put into a test tube. After that, 5 ml of 0.2 M TBA reagent was added and heated at 100°C for 45 minutes. The solution was then cooled and diluted using 10 ml of distilled water. Furthermore, the solution was vortexed until homogeneous, and then the absorbance was read using a spectrophotometer at a wavelength of 528 nm [14]. In addition, the TBA number was calculated in units of malonaldehyde per kg of fat with the following equation:

\[
\text{TBA number} = \frac{(\text{Abs} \times \text{dilution factor} \times 7.8)}{\text{Sample weight}}
\]

Note: Abs: absorbance; Dilution factor: 5; Sample weight: 1 g

3. **Result and Discussion**

3.1 **Ulva lactuca Fatty Acid Content**
The fatty acid content in this study was detected using the GC-MS (gas chromatography-mass spectrometry) test. Based on the results of the GC-MS test, U. lactuca was extracted by the LRC method using ethanol and hexane fatty acid concentration of 44.96%. The results showed there were saturated (SFA), unsaturated (UFA), monounsaturated (MUFA), and polyunsaturated fatty acids (PUFA). The fatty acids included palmitic acid, lignoceric acid, capric acid, palmitoleic acid, roughanatic acid, erucic acid, and...
acid, oleic acid, and linoleic acids. The *U. lactuca* fatty acid extraction had 29.55% SFA (palmitic acid 25.61% and lignoceric acid 3.94%) and 15.41% PUFA, which were 12.4% MUFA, namely palmitoleic acid (6.46%) and oleic acid (5.94%). Furthermore, PUFA had 3.01%, which were roughanic (0.67%) and linoleic acid (Omega-6) (2.34%), and the PUFA/SFA ratio was (0.1). This ratio was recommended because when the value was >1%, it resulted in increased total cholesterol and LDL (low-density lipoprotein) or increased risk of heart disease [15].

### 3.2. Turbidity

Turbidity is a change in color or cloudy appearance that occurs in liquids due to suspension sediments, the reaction between particles, or a decrease in quality [16]. Physical appearance showed that all samples were homogeneous and not gel. The microemulsion samples decreased in quality during 20 days of storage with different temperatures and ascorbic acid concentrations. This was evidenced by the high turbidity value (>1%) in the microemulsion sample without ascorbic acid antioxidant (0 ppm) at storage temperatures of 10 °C and 30 °C.

| Temperature (°C) | Ascorbic Acid (ppm) | Days 0 | 4 | 8 | 12 | 16 | 20 |
|-----------------|---------------------|-------|---|---|----|----|----|
| 10              | 0                   | 0.77 ± 0.11 | 0.83 ± 0.05 | 0.85±0.06 | 0.757±0.02 | 0.75±0.02 | 1.11±0.17 |
|                 | 100                 | 0.84 ± 0.00 | 0.80 ± 0.12 | 0.77±0.00 | 0.708±0.05 | 0.69±0.04 | 0.76±0.01 |
|                 | 200                 | 0.78 ± 0.08 | 0.75 ± 0.17 | 0.74±0.05 | 0.701±0.02 | 0.61±0.05 | 0.79±0.06 |
| 30              | 0                   | 0.91 ± 0.13 | 0.89 ± 0.02 | 0.91±0.24 | 0.879±0.13 | 0.88±0.11 | 1.06±0.16 |
|                 | 100                 | 0.86 ± 0.03 | 0.84 ± 0.03 | 0.71±0.02 | 0.664±0.02 | 0.64±0.02 | 0.78±0.04 |
|                 | 200                 | 0.71 ± 0.00 | 0.68 ± 0.02 | 0.62±0.02 | 0.588±0.06 | 0.47±0.10 | 0.62±0.00 |
| 50              | 0                   | 0.74 ± 0.07 | 0.70 ± 0.02 | 0.65±0.09 | 0.655±0.01 | 0.70±0.02 | 0.68±0.12 |
|                 | 100                 | 0.67 ± 0.00 | 0.67 ± 0.18 | 0.59±0.06 | 0.571±0.02 | 0.46±0.06 | 0.57±0.08 |
|                 | 200                 | 0.69 ± 0.17 | 0.59 ± 0.04 | 0.52±0.02 | 0.473±0.00 | 0.39±0.05 | 0.50±0.03 |

The increase in turbidity index was caused by the microemulsion instability induced by the deterioration of its components. Fatty acid deterioration, like oxidation of unsaturated fatty acids, would produce free radicals at the initiation stage. At the next stage, this compound would have propagation to form unstable hydroperoxide compounds during primary oxidation. These compounds then decomposed into more stable forms such as aldehydes, ketones, alcohols, esters, and acids, making the microemulsion cloudier [16]. The increase of turbidity index was also caused by the droplet size change on o/w microemulsion due to agglomeration or the incorporation of small colloids into larger ones. This condition was due to the decrease in droplet zeta potential, which was a parameter of electric charge between colloidal particles. The lower zeta potential allowed the droplets in the microemulsion to attract each other, and flocculation occurred [17].

The effect of treatments on the quality of the tested samples using the statistical regression method is shown in Table 2. The results of the turbidity regression equation during storage showed that the turbidity value decreased with increasing temperature and ascorbic acid concentration. Therefore, the higher the temperature used, the lower the turbidity value. This condition support Table 3 that *U. lactuca* changed color from green to yellow when stored at high temperatures.
**Table 2. Multiple linear regression analysis of turbidity.**

| Day | Sig Value | R square | Regression Equation       |
|-----|-----------|----------|---------------------------|
| 0   | 0.116     | 0.149    | Y = 0.885 - 0.02 T + 0.000 AA |
| 4   | 0.010     | 0.460    | Y = 0.924 - 0.004 T - 0.001 AA |
| 8   | 0.000     | 0.594    | Y = 0.944 - 0.005 T - 0.001 AA |
| 12  | 0.000     | 0.682    | Y = 0.924 - 0.004 T - 0.001 AA |
| 16  | 0.000     | 0.775    | Y = 0.889 - 0.004 T - 0.001 AA |
| 20  | 0.005     | 0.503    | Y = 1.090 - 0.008 T - 0.001 AA |

Note: T = temperature and AA = ascorbic acid

The turbidity acceptance limit was less than 1% [11]. Up to 20 days of storage, microemulsions without the addition of ascorbic acid had turbidity more than 1% with a cloudy yellow color. Meanwhile, microemulsions with ascorbic acid antioxidants could inhibit the increase in turbidity (turbidity values <1%) with a clear yellow color [16]. This was due to the mechanism of antioxidants that could scavenge metal ions that trigger free radicals, hence the microemulsion is more stable with low hydroperoxide formation and kept the solution clear [18]. A previous study declared that ascorbic acid in VCO microemulsions could act as SOQ (single oxygen quencher), ESQ (sensitizer quencher), and hydrogen ion donor, which could reduce the oxidation of fatty acids [5].

The appearance of fatty acid microemulsions before and after storage for 20 days was presented in Table 3. The observations of the physical appearance showed that all samples had a homogeneous appearance and did not form a gel. In general, the 200 ppm microemulsion with the addition of ascorbic acid changed color during storage but still had a translucent and homogeneous appearance. At higher temperatures, the color tends to fade to brown.

**Table 3. The appearance of *U. lactuca* fatty acid microemulsion after 0 and 20 days of storage.**

| Storage Duration | Storage Temperature (°C) |
|------------------|--------------------------|
|                  | 10       | 30       | 50       |

Day 0

Day 20

(a) (b) (c)  (a) (b) (c)  (a) (b) (c)

Note: Fatty acid microemulsion with ascorbic acid (a) 0 ppm; (b) 100 ppm; (c) 200 ppm

Note: T: temperature
3.3. Antioxidant Activity

Antioxidant activity can be defined as an inhibition of nutrient oxidation, especially lipids by restraining oxidative chain reactions. Figure 1 shows *U. lactuca* fatty acid microemulsion decreased in quality, which was indicated by the decreasing antioxidant activity during 20 days of storage. However, the greater the concentration of antioxidants added, the more the activity to allow a more stable microemulsion during storage. The microemulsions with the addition of ascorbic acid tend to be more stable than those without ascorbic acid because antioxidants could inhibit lipid oxidation.

*Ulva lactuca* fatty acid microemulsion without the addition of ascorbic acid (0 ppm) had high antioxidant activity (65-70%). This was because the extract contained 2,6-Di-tert-butyl-4-methylphenol (C15H24O) 0.3% based test. These compounds have phenolic groups and can be synthesized endogenously by some phytoplankton under oxidative stress conditions [19]. Tamat et al. (2007) revealed that *U. lactuca* contained phenolic compounds, flavonoids, and carotene compounds that had antioxidant activity. The phenolic content in the fatty acid extract of *U. lactuca* was also caused by the polar extraction solvent [20]. The results of this study are supported by previous research which showed that *U. lactuca* extracted using 96% ethanol solvent contained 4.59% of phenolic compounds and 0.59% of flavonoids [21]. In addition, the content of unsaturated fatty acids in the extract could also act as an antioxidant. Unsaturated fatty acids tend to be reactive as antioxidants because they have double bonds and donate 1 H atom to prevent free radicals [22].

Although the samples already have antioxidant activity, the addition of ascorbic acid made the activity higher, hence it could effectively inhibit *U. lactuca* fatty acids deterioration. Oxidation reactions caused microemulsion damage could be prevented by the addition of antioxidants [23]. This could inhibit the formation of free radicals at the initiation stage and inhibit the continuation of the autoxidation reaction at the propagation stage. This is because antioxidants have low activation energy to release one hydrogen atom to lipid radicals to prevent further oxidation steps.

**Figure 1.** The radical scavenging activity of *U. lactuca* fatty acid microemulsion with the addition of ascorbic acid 0, 100, and 200 ppm during storage for 20 days.

Note: T: temperature
3.4. Peroxide Value

The peroxide value is a parameter indicating the concentration of hydroperoxides formed due to the initiation and propagation steps of the oil/fat oxidation reaction. These peroxides and hydroperoxides were unstable and easily decomposed into secondary oxidation products that affected rancid odor and taste [24]. The addition of ascorbic acid into the microemulsion system could inhibit the deterioration of *U. lactuca* fatty acid microemulsion during 20-day storage at three different temperatures. Based on the results shown in Figure 2, it was known that the peroxide value tended to increase during 20 days of storage. This occurred because of the primary oxidation process of fatty acids into hydroperoxides.

The addition of antioxidants also made the increase in peroxide value slower because antioxidant compounds donated hydrogen atoms or electrons to free radicals (R*, R**), turning them into a more stable form, namely RH. Meanwhile, the antioxidant radical derivative (A*) was in a more stable state than the original radical (R*). Without additional antioxidant compounds, the reaction will continue until the termination stage to allow free radicals to react with each other to form complex compounds. The addition of antioxidants could inhibit oxidation at the initiation and propagation stages because they quickly gave hydrogen atoms to lipid radicals or converted them to more stable forms. Antioxidant radical derivatives have a more stable state than lipid [21]. The peroxide value measures the levels of peroxides and hydroperoxides formed in the early stages of the fat oxidation reaction [25]. A high peroxide value indicated that the fat or oil have been oxidized. However, a low peroxide value did not mean that the fatty acid or oil has not been damaged. The low peroxide value caused by the rate of new formation was smaller than the rate of its degradation into other compounds. This is because peroxide levels rapidly degrade and react with other substances.

3.5. Thiobarbituric Acid BA Value

Based on the results of testing the TBA value (Figure 3), it was known that the microemulsion sample with the addition of ascorbic acid antioxidant had a more stable value than those without ascorbic acid (0 ppm). During storage, the TBA value without the addition of ascorbic acid had a high increase on the
8th day and decreased on the 12th day of storage. Meanwhile, the TBA value with the addition of 100 ppm and 200 ppm ascorbic acid was relatively stable until the 20th day. This indicated the formation of secondary oxidation products. The TBA value, which fluctuated and tended to be higher than the peroxide value, was caused by the unstable primary oxidation product that degraded into secondary. The oxidation reaction begins with labile peroxides and hydroperoxides, which could be oxidized to short-chain organic components such as aldehydes, ketones, and other components [5]. The factors that affected the rate of fatty acid oxidation were the amount and type of oxygen, the chemical structure of lipids, the presence of antioxidant and pro-oxidant compounds, storage temperature, and the nature of packaging materials [26]. The reactions that occurred during the degradation of fatty acids are based on the decomposition of fatty acids [27]. The more double bonds of fatty acids, the faster the rate of oxidation would also increase. The results of the peroxide value and TBA number during 20 days of storage were still within the limits for food products, namely 5 mEq/kg for peroxide (based on IFOS) and 7-8 mg MDA/kg for TBA number [28]. The addition of ascorbic acid antioxidant to the fatty acid microemulsion of *U. lactuca* inhibits the formation of malonaldehyde compounds due to its antioxidant properties as free radical scavengers. The measurement of malonaldehyde compounds in a system could be an antioxidant activity standard. Furthermore, the compounds could inhibit the formation of malonaldehydes. Ascorbic acid in microemulsions also acted as a singlet oxygen quencher that inhibits the formation of peroxides. In addition, the mechanism of ascorbic acid-binding to singlet oxygen takes place physically by transferring energy from singlet oxygen and converting it into a triplet [5].

4. Conclusion

The fatty acid microemulsion sample of *U. lactuca* with the addition of 200 ppm ascorbic acid gave more effective results in inhibiting the deterioration rate of *U. lactuca* fatty acid microemulsion. Furthermore, temperature and storage time gave significant differences in turbidity, peroxide value, and TBA value. The higher the concentration of ascorbic acid added, the more effective the results in inhibiting the rate of deterioration.

Conflict of Interest

The authors declared no conflict of interest

Acknowledgments

The authors are grateful to Universitas Gadjah Mada for the Final Project Recognition Program. Letter of assignment number 2488/UN1.P.III/DIT-LIT/PT/2020. May 12th, 2020. This paper consists of data from the first and second author's undergraduate thesis.

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