Isolation, identification of fatty acids from *Spirulina platensis* as antibacterial

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**Abstract.** The main target of fatty acids is disrupting the electron transport chain and oxidative phosphorylation of bacterial cells. One of the fatty acid-producing microorganisms is *Spirulina platensis*. Therefore this study was aimed to determine the types of fatty acid compounds from *S. platensis*, which have the potential as antibacterial. Biomass is extracted by soxhlet using different polarity solvents (n-hexane, chloroform, ethyl acetate, and ethanol). An antibacterial activity using the paper disc diffusion method against *Staphylococcus aureus* and *Escherichia coli*. Identification of fatty acid compounds using Gas Chromatography-Spectrometry Mass (GC-MS). The antibacterial activity showed that chloroform extract showed the largest inhibition zone. Furthermore, chloroform extract was fractionated and simplifying fractions. The results of the identification of the best fraction showed that there were several types of fatty acids namely Hexadecanoic acid, methyl ester (10.99%), 1,2-Benzenedicarboxylic acid (0.74%), heptadecanoic acid, methyl ester (0.09%), 6.9.12-Oxiraneoctanoic acid (8.84%), 9,12-Octadecadienoic acid (0.52%), Octadecanoic acid, methyl ester (2.28%), Oxiraneoctanoic acid (8.86%), Eicosanoic acid, methyl ester (0.46%), Hexanedioic acid, diocyl ester (6.46%) and Docosanoic acid, methyl ester (0.26%). Based on this study, the fatty acids found in *S. platensis* have an ability to be naturally antibacterial.

**Keywords:** Fatty acids, antibacterial, *Spirulina platensis*

1. **Introduction**

In the past five decades, increased resistance of bacterial strains to drugs, including antibiotics, has been a significant factor in increased morbidity, mortality and health care costs for bacterial infections. It is urgent to find and develop new infection control strategies to address the alarming increase in arbitrary and abusive use of synthetic antibiotics.

*Spirulina platensis* is one of the microorganisms that have the potential to produce antibacterial compounds. Spirulina is included in blue-green algae and has now become a health food throughout the world. Spirulina is microscopic, multicellular, filamentous, alkalophilic, and photoautotrophic, belonging to the microalgae of the Cyanophyta class, known as a rich cell protein source. The protein content of *Spirulina* sp. varies between 50% -70% of its dry weight [1-3]. Besides, *Spirulina* sp. possesses high contents of vitamins, lipids, minerals, and fatty acids showing functional biological activities; hence, it may be promising drug treatment and prevention of many diseases [1, 4, 5].

The fatty acids from Spirulina are usually found to be bound to other compounds such as glycerol, sugar, or phosphate headgroup to form lipids. Fatty acids can be released from lipids, generally
through the action of enzymes, into free fatty acids, which have diverse and influential biological activities such as antibacterial, antifungal, antiprotozoal, antiviral, cytotoxic, etc [6]. The biological activity of fatty acid has a role in host defense against potential pathogenic or opportunistic microorganisms [7].

An essential aspect of this is the direct inhibition of growth or killing of bacteria. Now there is extensive literature on the antibacterial effects of various fatty acids from various biological sources, including algae, animals, and plants [7-9]. Indeed, fatty acids are often identified as an active ingredient in ethnic and herbal medicines [8]. The study of fatty acid isolation from *Spirulina platensis* was carried out using organic solvents, which differed in polarity. The purpose of this study was to determine the ability of organic solvents to isolate the body of fatty acids, which have the potential as antibacterial.

2. Materials and methods

2.1. Cultivation of microalgae *S. platensis*.

Microalgae *S. platensis* used in this study is a collection of microbes belonging to Bioenergy and Bioprocess Laboratory, Research Center for Biotechnology-LIPI. Microalgae were cultivated in a technical medium consisting (g/L) 0.2 MgSO4 g/L; 0.12 CaCl2; 0.64 EDTA; 0.31 Urea 0.3; 0.18 trisodium phosphate; 0.15 KOH; 2.5 K2SO4; 0.01 FeSO4; 16.8 NaHCO3; 21; micronutrient A5 1 mL/L under 3500 lux intensity and continuous aeration. The culture was evaluated based on its cell density using Spectrophotometer UV-VIS at 550 nm [10]. Microalgae were cultivated for 14 days and harvested a stationary phase by filtered with monil/nylon fabric. Biomass was oven-dried at 50°C.

2.2. Extraction of *S. platensis* biomass

Twenty g of dry biomass from *S. platensis* extracted in stages by soxhletation for 7 hours. The solvents used were petroleum ether, chloroform, ethyl acetate, and ethanol (2g: 15mL). The extract obtained is concentrated by vacuum rotary evaporator, and then evaporated to get a dry extract.

2.3. Fatty acid extraction

The extraction process based on the method developed by the National Bureau of Standards Indonesia/Indonesian National Standard [11]. Each 200 mg dry extract petroleum ether, chloroform, ethyl acetate, and ethanol added with 12 mL of 0.5 N NaOH shaken until homogeneous, then flowed by nitrogen gas. After that, it heated for 5 minutes at 100°C. After cooling, add 16 mL of BF3 (Boron trifluoride) in methanol (14% w/v) and re-flow with nitrogen gas, tightly closed and heated at 100°C for 5 minutes. After the extract has cooled to 30-40°C, add 8 mL of n-hexane and shake until it is homogeneous. Then, add a layer of n-hexane with Na2SO4 (sodium sulfate) anhydrous, and then extract flowed again with nitrogen gas. Crude fatty acids are ready to be used to test antibacterial activity.

2.4 Preparation of tested bacteria (*S. aureus* and *E. coli*)

*S. aureus* and *E. coli* used in this study is a collection of microbes belonging to Bioenergy and Bioprocess Laboratory, Research Center for Biotechnology-LIPI. *S. aureus* and *E. coli* were cultured at NA (Nutrien Agar) medium. The culture incubated at 37°C for 24 hours.

2.5 Antibacterial activity

An antibacterial activity using the paper disc diffusion method [12]. The regenerated *S. aureus* and *E. coli* were cultivated in a liquid medium consisting of 0.5% peptone, 0.3% yeast, and incubated at 37°C for 24 hours. Ten µL inoculum was added to the NA media and poured into a petri dish. After solidifying, place the Whatman paper on top, then drop 20 µL of each crude fatty acid extract from petroleum ether, chloroform, ethyl acetate, and ethanol. Positive control uses Chloramphenicol at a concentration of 20 ppm, while negative control uses petroleum ether, chloroform, ethyl acetate,
ethanol, and n-hexane. All Petri dishes were incubated in an incubator at 37°C for 24 hours. The inhibition zone that forms around the paper disk is measured using a caliper.

2.6. Fractionate the extract by column chromatograph
Fractionation is carried out on extracts that have the largest inhibitory zone, namely chloroform extract. Fractionation using column chromatography, silica gel as a stationary phase, while as a mobile phase using a mixture of 2 kinds of solvents namely n-hexane and chloroform in a ratio of 3: 7. After that, simplified fractions were analyzed with Thin Layer Chromatography (TLC) GF254. Each faction has the same profile points combined into one.

2.7. Identification of antibacterial compounds by Gas Chromatography-Mass Spectrometry (GC-MS)
The GC-MS analysis was carried out to identify the chemical components of the most active fraction of S. platensis. Specifications of GC-MS used is Agilent Technologies 5973 N, Type of column is Capillary Column DB 5 with length 60 mm 0.25 µm. Volume of injection: 2µL Inlet temperature: 290 C, Aux temperature: 290°C, Program temperature: 70°C (15 / minute) – 290°C (20 minutes), Mode of gas flow: constant. Helium was used as carrier gas with a flow rate of 1 mL/min. A chromatogram obtained, and the mass spectrum of the unknown component compared with the spectrum of the known components stored in the WILEY 10 library.

3. Results and discussion
3.1. The Growth of microalgae S. platensis
As shown in figure 1, S. platensis does not undergo an adaptation phase, because S. platensis stocks are inoculated into new media in a logarithmic phase, so that microalgae cells can directly grow and multiply in new media. Observation of cell growth patterns aims to determine the growth phase of microalgae so that it can be known when it is appropriate to harvest.

S. platensis experiencing an exponential phase from day 1 to 9, which is characterized by a significant increase in the growth curve. This exponential growth occurs as a result of the availability of nutrients and environmental conditions such as pH, temperature, light intensity, and aeration sufficient to produce the maximum population. After that, starting from 10 to 14 days, the cells are in the stationary phase condition, which is characterized by the absence of cell growth.

The stationary phase occurs when the microalgae growth rate is the same as the death rate. The balance of the total number of microalgae occurs due to a decrease in the degree of cell division. The decline in this cell division caused by the reduced level of nutrients and accumulation of toxic products that interfere with cell division [1, 10]. At the time of this phase, secondary metabolites such as fatty acids are formed. Secondary metabolites are formed in these extreme conditions and are necessary for self-defense to survive.

![Figure 1. The growth curve of microalgae S. platensis](image-url)
3.2. Harvesting Microalgae *S. platensis* biomass

The process of harvesting microalgae biomass is claimed to contribute 20-30% to the total cost of biomass production [13]. The harvesting technique of *S. platensis* biomass can be carried out by flotation with the addition of NaCl, which can maintain algal quality [14]. However, harvesting *S. platensis* was carried out by filtering using nylon cloth in this study (figure 2a). The advantage of this technique that it can maintain the quality of biomass (figure 2b), the resulting biomass is shiny greenish-blue. Besides, the supernatant separated from biomass can be reused as a culture medium, so that this technique can reduce the cost of production. Biomass obtained dried in an oven for 24-48 hours at a temperature of 40ºC. Biomass is dried at low temperatures to avoid damage to bioactive compounds in cells.

![Figure 2. The process of harvesting of *S. platensis* (a) and wet biomass of *S. platensis* (b)](image)

3.3. Extraction of *S. platensis* biomass

The ability of each solvent used is different; therefore, the extract obtained has a different yield value, namely 1.383 % of petroleum ether, 2.060 % of chloroform, 1.744% of ethyl acetate, and 12.859 % of ethanol (table 1). The highest extract yield was obtained using ethanol solvent, known that ethanol is a universal solvent, ethanol is a very polar molecule because its hydroxyl (OH) group, with high oxygen negativity allows hydrogen bonds to occur with other particles so that ethanol can dissolve both polar and non-polar.

| Weight of dry biomass (g) | Eluent                    | The weight of dry extract (mg) | Yield (%) |
|--------------------------|---------------------------|-------------------------------|-----------|
| 20.05                    | Petroleum ether           | 283.5                         | 1.383     |
|                          | Chloroform                | 422.5                         | 2.060     |
|                          | Ethyl acetate             | 357.7                         | 1.744     |
|                          | Ethanol                   | 2637.3                        | 12.859    |

The extraction method and solvent used can affect the quality and quantity of nutritional and bioactive compounds extracted from microalgae. In this study, the types of solution used for extraction...
have different levels of polarity. This is done to see the ability of each solvent to attract bioactive compounds and determine a suitable solvent in extracting bioactive compounds from *S. platensis*.

In this study, extraction was done by the soxhletation method. This method is used to isolate heat-resistant (thermostable) compounds such as fat compounds and fatty acids. Besides that, soxhletation considered a more efficient process because it increases surface contact between solvent and material, resulting in higher yields and more number of compounds extracted [15]. All stages of extractions, from the pre-extraction and extraction, are equally important in the study of medicinal plants. The sample preparation such as grinding and drying affected the efficiency and phytochemical constituents of the final extractions [16].

3.4. Antibacterial activity of crude extract fatty acids in petroleum ether, chloroform, ethyl ether, and ethanol

Antibacterial activity is carried out by the paper disc method. The principle of this method is to put disc paper containing extract on solid media that has been inoculated with the tested bacteria. The presence of antibacterial compounds characterized by the presence of clear zones that are not overgrown with bacteria. The advantages of this method are fast and easy, only requires simple tools, with small concentrations can provide results, and the interpretation of the results obtained is relatively accurate.

In table 2 the initial stage of the antibacterial activity test was carried out using extracts of petroleum ether, chloroform, ethyl acetate, and ethanol. The results showed that the antibacterial activity of all extracts provided a zone of inhibition against tested bacteria. But the exciting thing is the inhibition zone produced by chloroform extract. The chloroform extract showed similar inhibition zone against Gram-positive bacteria (*S. aureus*) is 18.33 mm and against Gram-negative bacteria (*E. coli*) is 18.81 mm. Therefore, to further research that fractionation and identification of compounds by GC-MS are focused on fatty acids contained in the extract of chloroform.

**Table 2.** Inhibition zone of crude fatty acids extract from *S. platensis* extracted using petroleum ether, chloroform, ethyl acetate, and ethanol

| Bacteria | Inhibitor zone (mm) |
|----------|---------------------|
|          | Petroleum ether | Chloroform | Ethyl acetate | Ethanol |
| *S. Aureus* | 1 | 21.90 | 19.00 | 25.30 | 23.00 |
| | 2 | 16.50 | 17.25 | 22.00 | 19.00 |
| | 3 | 16.90 | 18.75 | 21.50 | 21.00 |
| | Average | 18.43 | 18.33 | 22.90 | 21.00 |
| | Standard deviation | 3.01 | 0.95 | 2.00 | 2.00 |
| *E. coli* | 1 | 21.70 | 19.85 | 14.70 | 17.30 |
| | 2 | 23.65 | 18.34 | 15.25 | 17.45 |
| | 3 | 24.70 | 18.25 | 15.27 | 17.37 |
| | Average | 23.35 | 18.81 | 15.00 | 17.37 |
| | Standard deviation | 1.91 | 0.89 | 0.03 | 0.07 |

The diameter of the paper dish 6 mm
3.5. Fractionation of chloroform extract from S. platensis

The next step is fractionation of chloroform extract using a chromatography column. The stationary phase used was silica gel G60 by using an eluent mixture of two solvents are n-hexane: chloroform in the ratio 3: 7. The purpose of this fractionation is to obtain a more pure extract. After simplification of fractions, two fractions were produced namely fraction 1 and fraction 2. Based on qualitative analysis using TLC (Thin Layer Chromatography) on a fraction 1 obtained seven spots and fraction 2 obtained three spots (figure 3).

![Image](image_url)

Figure 3. The pattern of thin layer chromatogram from fraction 1 (F1) obtains 7 spots (1-7) and fraction 2 (F2) obtain 3 spots (a, b and c)

3.6. Antibacterial activity of fraction 1 and fraction 2

In table 3, the results showed that the inhibition zone in fraction 1 for both test bacteria (S. aureus and E. coli) was higher, compared to the inhibition zone in fraction 2. The average inhibition zone in fraction 1 against S. aureus was 20.0 mm, while that formed in E. coli was 18.8 mm.

| Samples  | Replicates | Zone of Inhibition in mm |
|----------|------------|--------------------------|
|          |            | S. aureus | E. coli |
| Fraction 1 (F1) | 1 | 22.00 | 19.00 |
|           | 2 | 20.00 | 19.00 |
|           | 3 | 21.00 | 18.45 |
| Average  |            | 21.00    | 18.8   |
| Standard deviation | | 0.57 | 0.45 |
| Fraction 2 (F2) | 1 | 7.50 | 0 |
|           | 2 | 6.35 | 0 |
|           | 3 | 6.35 | 0 |
| Average  |            | 6.61    | 0 |
| Standard deviation | | 0.66 | 0 |

Note: the diameter of the paper dish 6 mm
As shown in table 3, the inhibition zone fraction 2 against E. coli bacteria does not form an inhibition zone. Based on the results of the qualitative analysis using TLC, the spots produced by fraction 1 were more than those of fraction 2 (figure 3). These results indicate that the bioactive compounds such as fatty acids that are known to have antibacterial activity accumulate more in the fraction 1.

In table 3, the antibacterial activity in fraction 1 showed different results for the two tested bacteria. The inhibition zone produced by S. aureus was higher than the inhibition zone produced by E. coli. Different responses of the two types of bacteria to compounds in fraction 1 caused by differences in sensitivity to S. aureus and E. coli to the antibacterial compounds contained in fraction 1. S. aureus was more sensitive so that the test substance provided a more significant inhibition zone. S. aureus is possible because the S. aureus cell wall has a single layer (one layer) and low-fat content (1-4%) and has a peptidoglycan layer as a single layer of more than 50% dry weight so that the antibacterial compound is easily absorbed. Whereas the E. coli cell wall has three layers (multilayer) and high-fat content (11-22%) and has a peptidoglycan layer in a rigid sheet of about 10% dry weight so that antibacterial compounds are challenging to absorb [17]. Similar results were obtained from a previous study which showed that the dichloromethane extract of Lyngbya sp provide inhibition zone against Gram-positive bacteria (S. aureus) is higher than against Gram-negative bacteria (E. coli) [17]. Inhibitory zones produced by Gram-negative and positive bacteria due to differences in the sensitivity of fatty acids to the cell walls of Gram-positive and Gram-negative bacteria. Impermeability of the outer membrane of Gram-negative bacteria as an effective barrier against hydrophobic substances. Therefore, more Gram-negative bacteria are resistant to inactivation of long-chain fatty acids compared to Gram-positive bacteria [18].

3.7. Identification of antibacterial compounds at fraction 1 from S. platensis chloroform extract with gas chromatography - mass spectrometry

The identification of antibacterial compounds by Gas Chromatography-Mass Spectrometer was carried out on the most active fractions that could inhibit the growth of S. aureus and E. coli, namely fraction 1. Based on the WILEY 10 database system, the chromatogram in fraction 1 contained several dominant peaks.

In table 4, fatty acid compounds are identified in the form of fatty acid esters, because previously the extract was converted into esters by reacting BF₃ in methanol. Dominant peaks that have a similarity greater than or equal to above 90% there are 10 compounds namely Hexadecanoic acid, methyl ester, 1,2-Benzenedicarboxylic acid, Heptadecanoic acid, methyl ester (margaric acid), 6,9,12-Octadecatrienoic acid (γ-Linolenic acid-omega 3), 9, 12-Octadecadienoic acid (linoleic acid – Omega 6), Octadecanoic acid methyl ester, 7-Oxooctadecanoic acid methyl ester, Eicosanoic acid, methyl ester, Hexanedioic acid, dioctyl ester, Docosanoic acid, methyl ester (Behenic Acid).

Based on the double bonds, the types of fatty acids identified in chloroform extract from S. platensis are saturated fatty acids and unsaturated fatty acids. Fatty acids with more than 10 C atoms cause the protoplasm to become lysis by changing the permeability of the cytoplasmic membrane, creating an essential component to emerge from the bacterial cell. Damage to the layer results in the inhibition of bacterial growth or death in bacteria [19]. Long-chain fatty acids, such as linoleic acid, can inhibit the reductase bacteria carrying enoyl-acyl carrier protein reductase (FabI), an essential component of the synthesis of bacterial fatty acids, which has served as a promising target for antibacterial drugs. Other unsaturated fats such as palmitoleic acid, oleic acid, linolenic acid, and arachidonic acid also show inhibition of FabI. FabI is an enoyl-ACP reductase, which catalyzes the final and rate-limiting step of the chain elongation process of the type II FAS (Fatty Acid Synthesis) [20].

The GC-MS analysis of the volatile compounds of the extract of microalgae showed the presence of amide, long-chain hydrocarbons, aldehyde, alcohols, fatty acids esters, amines and sulfur-containing compounds [21].
### Table 4. The results of identification of fraction 1 of *S. platensis* using Gas Chromatography-Mass Spectrometry

| Retention time (minutes) | % Area | Similarity/Quality (%) | Name of Compound | Chemical formula |
|--------------------------|--------|------------------------|------------------|-----------------|
| 23.48                    | 10.99  | 99                     | Hexadecanoic acid, methyl ester | C_{16}H_{32}O_{2} |
| 24.14                    | 0.74   | 94                     | 1,2-Benzenedicarboxylic acid | C_{6}H_{12}O_{4} |
| 24.89                    | 0.09   | 96                     | Heptadecanoic acid, methyl ester | C_{17}H_{34}O_{2} |
| 25.36                    | 0.08   | 98                     | 6,9,12-Octadecatrienoic acid | C_{18}H_{30}O_{2} |
| 25.69                    | 0.52   | 99                     | 9,12-Octadecadienoic acid | C_{18}H_{32}O_{2} |
| 26.02                    | 2.28   | 99                     | Octadecanoic acid, methyl ester | C_{18}H_{36}O_{2} |
| 27.48                    | 8.84   | 99                     | 7-Octadecanoic acid methyl ester | C_{19}H_{38}O_{3} |
| 27.62                    | 0.46   | 99                     | Eicosanoic acid, methyl ester | C_{20}H_{42}O_{2} |
| 28.17                    | 6.46   | 97                     | Hexanedioic acid, dioctyl ester | C_{6}H_{10}O_{4} |
| 29.05                    | 0.26   | 95                     | Docosanoic acid, methyl ester | C_{22}H_{44}O_{2} |

### 4. Conclusion

Extraction of *S. platensis* using the Soxhletation method with chloroform solvent to produce compounds that have activity against test bacteria (*S. aureus* and *E. coli*). The identification of chemical compounds using GC-MS showed that chloroform extract from *S. platensis* contains fatty acid compounds that have antibacterial abilities, namely acidic Hexadecanoic, Oxiraneoctanoic acid, Hexanedioic acid, and Octadecanoic acid with a high percent area. Based on this, the chloroform extract from *S. platensis* can be an alternative to natural antimicrobial compounds.

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