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Isolation and Identification of Microalgae from Harau Valley Payakumbuh, West Sumatra as One Agent Producing Compounds Antibacterial.

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ABSTRACT

Antibiotic resistance against pathogens is a major problem in disease management. One of the microorganisms capable of producing the active compounds as antibacterial is microalgae. This study was conducted to determine the antibacterial activity of secondary metabolites produced by microalgae isolated from Harau Valley, Payakumbuh, West Sumatera against *Escherichia coli*, *Salmonella typhi*, *Bacillus cereus* and *Staphylococcus aureus*. Screening and isolation of microalgae were done by diluting and the micropipette. Based on the results of molecular identification by using a primer ChloroF and ChloroR, it is known that the isolate of microalgae belongs to the genus *Chlorella sp*. A total of 1 g biomass of microalgae extracted by sonication using methanol to produce extracts of 0.3387 g. The Methanol extract of *Chlorella sp.* has antibacterial activity against Gram positive and Gram negative bacteria.

Keywords: Isolation, identification, extraction, antimicrobial

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INTRODUCTION

In this present, many diseases are caused by pathogen bacteria and fungi. One of the ways to inhibit or kill that pathogen bacteria or fungi is by using antibiotic. The available antibiotics have been in various of sources, however antibiotic resistance to bacteria and fungi, is one of the major problems in the handling of diseases are caused by bacterial and fungal pathogens. The declining in the efficiency of antibiotics against bacterial and fungal pathogens and increasing resistance to antibiotics require finding new antimicrobial compounds that can inhibit the growth of pathogenic microbes.

Microalgae is a natural source that is very attractive to produce new compounds, such as fatty acid, lipids, pigments, polyphenols, carbohydrates, and simple hydrocarbons, chlorellin, phenolic compounds, flavonoids, and tannins, as well as bioactive proteins. Moreover, microalgae can do biosynthesis, metabolic, accumulation, and secretion of various primary metabolites or secondary metabolites that are valuable in the pharmaceutical industry and microalgae can live in extreme conditions and therefore microalgae must adapt quickly and efficiently [1-4].

The aim of this research was to find a compound that can inhibit the growth of bacterial and fungal pathogens from microalgae which was isolated from Harau Valley, Payakumbuh, West Sumatera.

MATERIAL AND METHODS

Isolation of Microalgae

In this study, fresh water in Harau Valley was used as a sample. Harau Valley is located in Nagari Harau, Harau District, Lima Puluh Kota regency, West Sumatera Province (Figure 1). The sample was collected from three different pick-up points, namely in water was exposed to sunlight, the water was not exposed to sunlight, as well as at a depth of 1 meter from the surface of the water by using a plankton net. The sample was isolation followed Nutan et al method [5]. Furthermore, a single culture microalgae was molecularly identified.

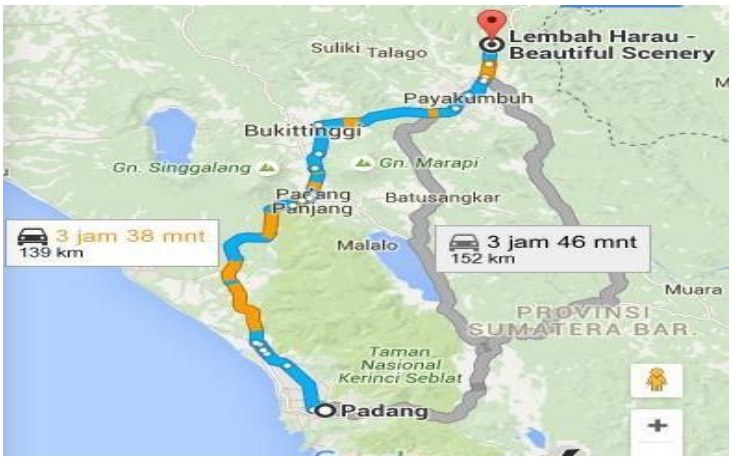

Figure 1. Sampling microalgae in Harau Valley, Payakumbuh using Google Map

Morphology Identification

A single microalgae culture was analyzed using optical microscopy and digital camera. The sample was observed at a magnification of 1000x. Taxonomic of microalgae species was identified by using *Algae Resource Database* [5].

Molecular Identification

Determination of microalgae species was done by molecular identification using 18s rDNA primer. DNA isolation used 10 mL of the liquid culture of microalgae and Qiagen DNA isolation kit [6]. PCR was performed using the pair of a primer of 18s rDNA of eukaryotic (ChloroF CCT TGG TGT ATC TTG TTG GTC and ChloroR GAA

TCA ACC TGA CAA GGC AAC) and for testing whether the DNA extract has been amplified, PCR product was electrophoresed by using Agarose gel 1 %. PCR amplification was performed using 2 µL of genomic DNA, 0.2 mM deoxynucleotide trifospat, 1.25 units DNA Taq Polymerase.The PCR reaction was consisted of 94°C for 3 minutes, followed by 35 cycles at 94°C for 1 min, 59°C for 1 minute, and 72°C for 1 minute, and a final extension at 72°C for 10 min [7]. The pure PCR product was shipped to Korea for sequencing. The results of sequencing were compared with GenBank data using the BLAST program. 18s rRNA gene was analyzed by using Bioedit and the phylogenetic tree was created by using MEGA5.1 software [8].

Cultivation of Microalgae

Cultivation of microalgae was carried out by using 153 mL of BBM medium that had been sterilized. Microalgae cultures were inoculated as much as 17 mL into the BBM medium and aerated at room temperature. The growth of microalgae was determined by measuring the optical density of the culture every day by using UV-Vis spectrophotometer at the wavelength of 450 nm. The experiment was conducted with three replications [9].The biomass of microalgae was harvested on day 24 by pipetting filtrate, then dried at room temperature [10].

Extraction of Secondary Metabolites

The dried microalgae biomass was sonicated and extracted by using a methanol in the ratio 1:10 (w/v). The mixture was extracted with ultrasonic waves at a frequency of 50 kHz for 40 minutes. The extract was poured into a petri dish. To obtain pure extracts, the solvent was removed by vaporizing at 60°C [11] The extract was stored in the refrigerator at a temperature of 4°C [12].

Phytochemicals Test

The phytochemical test was conducted on the polyphenol, flavonoid, terpenoid, steroid, and alkaloid followed Rajendran et al method, as well as the saponin, followed Geetha et al method [12-13].

Antibacterial Test

Antibacterial activity of methanol extract was performed by using disc diffusion method against *Escherichia coli, Salmonella typhi, Bacillus cereus* and *Staphylococcus aureus*. A total of 1 mg of extract was dissolved in 100 µL of 100% DMSO as a stock solution. To test antimicrobial solution was created in various concentrations. 20 µL of the extract solution was dropped onto sterile paper discs (diameter 6 mm) let until all the solution was absorbed and dried. A paper disc containing the extract was placed on the surface of the Mueller - Hinton medium that had been given strain of bacteria. Zones of inhibition were measured after incubation for 24 hours at 37°C [14].

RESULTS AND DISCUSSION

Morphology and Molecular Identification

Isolate microalgae were microscopically identified by using an optical microscope that can be seen in figure 2 (a). It can be seen that this is a unicellular microalgae, round, green colored. This result is supported by the image of the cell is accessed via Algae Resource Database (Figure 2 b). Based on the Algae Resource Database is known that isolate microalgae are *phylum Chlorophyta*, class *Trebouxiophyceae*, order *Chlorellales*, family *Chlorellaceae*, and *genus Chlorella*.

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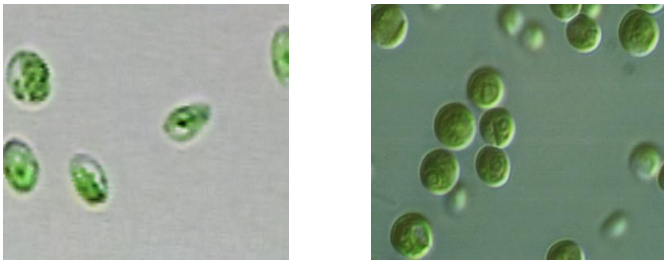
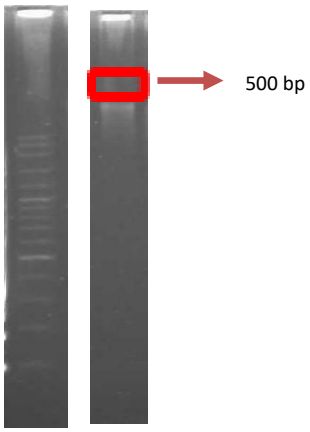


(a) (b)

Figure 2. (a) The result of the isolate microalgae microscopy (magnification 1000x), (b) was accessed via a cell image of *Algae Resource Database*

Molecular identification was conducted on DNA isolated from microalgae isolate. Isolation of DNA was done by using a Qiagen kit. The destruction of the cell wall was conducted by physical and chemical treatments. DNA purification was done by using buffer P3 which will lyse the cell. While ethanol is used to precipitate and purify DNA. The successful of isolation can be seen from bands that were resulting from the electrophoresis process (Figure 3).Based on the result of electrophoresis, the isolation of the DNA of isolate of microalgae has been successfully carried out.



(a) (b)

Figure 3. Results of electrophoresis of the isolated DNA, a. Ladder, b. AUMA 02

Microalgae DNA sequences are obtained after sequencing process by using a primer Chloro F and Chloro R generate forward sequence of 478 bp and reverse sequence of 467 bp. Both of sequences are combined with contig. Contig is done by using a Bioedit software.

Generated sequence from the contig analysis is identified and compared with the NCBI database. Comparative sequences are used for phylogenetic analysis. Alignment and made of a phylogenetic tree is performed by using MEGA5.1software [15]. From the result of sequence alignment, the phylogenetic tree is obtained as shown in Figure 4.

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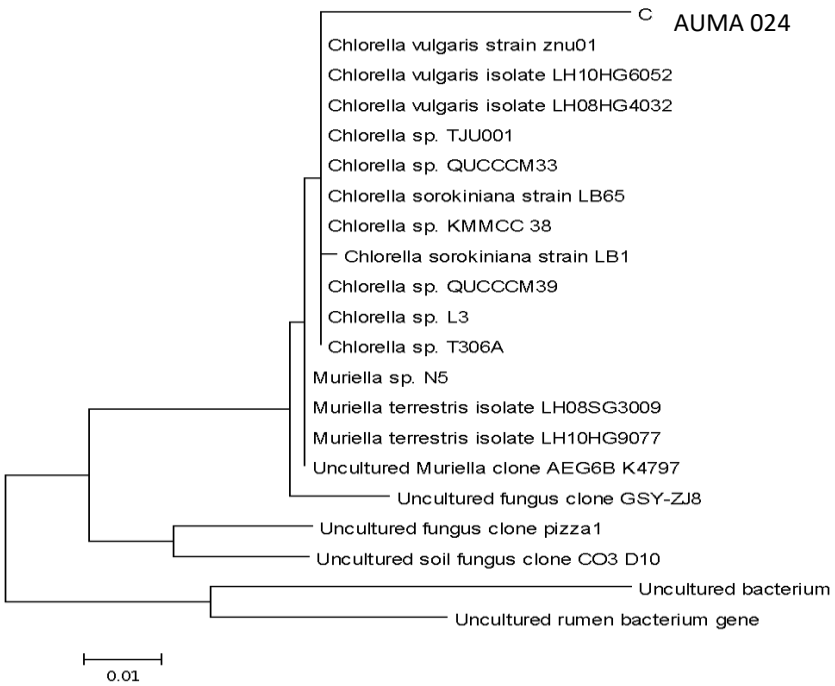

Figure 4. The result of phylogenetic analysis of microalgae isolate

The genetic distance of the isolate microalgae amounted to 0,040 with a level of similarity is 96%. Based on the degree of similarity is high shows that the high levels of trust of branch formed. Thus it can be estimated that isolate microalgae belongs to the genus *Chlorella.*

Microalgae Cultivation

The growth of microalgae *Chlorella sp* can be observed by measuring optical density using UV-Vis spectrophotometer at a wavelength of 450 nm every day until the culture reached the death phase. Based on the measurement, the growth curve is obtained which can be seen in Figure 5.

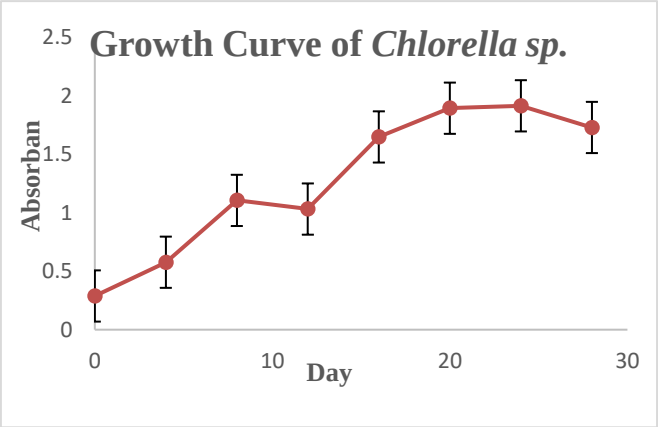

Figure 5. The growth curve of *Chlorella sp.* was measured every four days. Error bars represent the standard deviation n = 2

The results showed that the cultivation of *Chlorella sp.* increases from day 0 to day 8. Whereas after day 8 to day 12, a declined in the growth of *Chlorella sp.* is done. This shows that *Chlorella sp.* has the ability to perform cell division though the amount is not too fluctuating. At this stage, *Chlorella sp.* through a phase of

adaptation. Observations of the growth of *Chlorella sp.* cultivation increases until the 24th day. Subsequently, the growth was decreased at longer day. The declining in the growth of *Chlorella sp.* can be caused by the formation of autoinhibitory compounds which is a secondary metabolite in microalgae, which can occur because of changes in environmental conditions (culture) such as changes in pH, a decrease in light intensity and decrease the concentration of nutrients [2]. Similar results have been reported by Max et al [2] which reported the maximum growth of *Chlorella sp.* on the 24th day. Moreover, Zuliyana et al [16] and Ilavarasi et al [9] had obtained the maximum growth of *Chlorella sp*. on the 25th.

Extraction of Metabolites Compound

Microalgae are harvested at stationary phase because microalgae produce bioactive compounds. Harvesting is done on the 24th day, according to the growth curve obtained. A total of 1 g of biomass *Chlorella sp.* is extracted with 10 mL of methanol by using a sonicator and allowed to stand overnight to produce the crude extract of 0.3387 g. Sonication aims to increase the likelihood of collisions between the particles so the components that have been out can be bound and soluble in the solvent, and enhance the binding of a component with the solvent used. While maceration is done to increase the likelihood of reaction between the desired compounds with solvents [17].

The resulting extract is colored dark green and dense. Solvents used will destroy the cell membrane and dissolves the pigments are contained in the materials to produce these colors. Methanol extract which dark green suspected to be caused by chlorophyll extracted. The extract produced is a crude extract for the extraction solvent will generally produce mixtures of compounds contained in the sample [17].

Based on the test results of phytochemical are known that the methanol extract of *Chlorella sp.* is contained phenolic compounds, saponins, and steroids (Table 1). This result is slightly different from the result obtained by Shabudeen et al [18]. The content of secondary metabolic differences in the same genus can be caused by differences in the nutrients of the medium used, the environment, and the reduction in growth rate [19].

Table 1. Result of Phytochemicals Test of Methanol Extract from *Chlorella sp.*

Results	Compounds
Negative	Flavonoids
Positive	Phenolic
Positive	Saponins
Positive	Steroid
Negative	Triterpenoid
Negative	alkaloids

Table 2. Inhibition Zone Produced by Antimicrobial Compounds Isolated

Control (-)	Control (+)	Concentration			Bacteria
		0.5 mg / mL	0.1 mg / mL	0:01 mg / mL	
6.5 mm	9.5 mm	9 mm	9.5 mm	7.5 mm	*E. coli*
8 mm	9 mm	10.5 mm	9.5 mm	10.5 mm	*S. typhi*
7 mm	8 mm	9.5 mm	8 mm	8 mm	*S. aureus*
7.5 mm	9 mm	10 mm	10.5 mm	8 mm	*Cereus*

Based on table 2 the methanol extract *of Chlorella sp.* provides antimicrobial activity against all bacteria tested. This is due to their secondary metabolites (phenolic, saponins and steroids) are generated by the

methanol extract *of Chlorella sp.* Phenolic compounds have hydroxyl groups that act as toxic to microorganisms. Attachment side and the number of hydroxyl groups on the phenolic compounds associated with toxicity to microorganisms. The more hydroxyl group, toxicity will increase. In contrary, the stronger oxidized phenol will be inhibiting stronger. The mechanism reaction that responsible for the toxicity of the phenolic compounds in microbes by sulfhydryl groups reaction or interaction nonspecific protein [20].

The presence of saponins in the methanol extract of *Chlorella sp*. is responsible for the permeability cell walls as saponins have properties such as detergent. It is because saponins have carbohydrate molecules are soluble in water and sapogenin fat-soluble [21]. Thus, saponins can reduce the surface tension and increase the permeability membrane resulting in leakage of the cell [22]. Steroids reported having antibacterial activity due to steroid binds specifically with lipid membranes, causing leakage from the liposomes [23].

Based on table 2, it is known that the methanol extract of *Chlorella sp.* showed very low activity in inhibiting the growth of bacteria. Average clear zone produced against Gram-negative bacteria ranges from 7.5 mm to 10.5 mm, while the average clear zone produced in Gram-positive bacteria ranged from 8 to 10.5 mm. Responses were slightly different from the two groups of bacteria is caused by the difference in the sensitivity of Gram-negative and Gram-positive bacteria to antimicrobial compounds. This is caused by differences in the structure of the cell wall of the bacteria [24].

Low activity of clear zone measurement, it is caused by extract is not pure enough and still in the form of crude extract. In addition, the small effectiveness is due to least contain active compounds that have the same structure as the antibiotics commonly used to bacteria test, the susceptibility test bacteria, the age of the bacteria and environmental conditions [25].

CONCLUSION

Microalgae are isolated from waters of Harau Valley, Payakumbuh is *Chlorella sp.* and methanol extract of *Chlorella sp.* has antibacterial activity against Gram positive and Gram negative bacteria.

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