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Isolation and Identification of Freshwater Microalgae Potentially as Antibacterial From Talago Biru, Koto Baru, West Sumatera

Zulkarnain Chaidir[1], Syafrizayanti[1], Prima F. Hillman[1] and Rahadian Zainul[2]

[1] *Andalas University*
[2] *Universitas Negeri Padang*

ABSTRACT

Microalgae have potential as antibacterial compounds, the content of microalgae which has the activity of amino acids, terpenoids, florotanins, steroids, phenolics, acrylic acids and halogenated ketones can be used as an antimicrobial. In this research, screening and isolation of microalgae which can be used as antibacterial has been done at Talago Biru, Koto Baru, West Sumatera and the content of secondary metabolites were identified. The isolation was done by using agar plate and serial dilution methods. Isolates were identified morphological and molecular, it is known as a type of Chlorella sp. Best harvest time was determined by Spectrophotometric method at 450 nm is obtained in day 20 on the stationary phase. Based on phytochemical test, this isolate positively contain phenolics, saponins, steroids and triterpenoids, then the antibacterial activity of microalgae extracted with methanol was tested. It is known that the isolates has potential as an antibacterial, were seen from the test results provided by the zone of inhibition against four isolates of bacteria test with extract concentrations of 10 mg / mL, 100 mg / mL and 500 mg / mL respectively E .Coli 8 mm, 10 mm and 12 mm, Salmonella bacteria typhii 10.5 mm, 9 mm, 9 mm, Staphylococcus aureus 9 mm, 10 mm and 10 mm as well as the bacteria Bacillus cereus 10 mm, 10.5 mm, and 11 mm with a comparator amoxycillin as a positive control and 0.1% DMSO as a negative control.

Keywords*:* Talago Biru, *Chlorella sp*., secondary metabolite, antibacterial

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INTRODUCTION

Microalgae have been used in the production of biomass, energy production, bioaccumulation of specific compounds as well as a variety of biotransformation processes. The products produced by microalgae is intracellular and extracellular, ranging from simple metabolites to antibiotic complex, toxin pigments as well as a number of other useful products [1].Has many biological active substance that has been extracted from microalgae such as antialga, antiviral activity, and antimicrobial activity such as antibacterial and antifungal.[2]

Antimicrobials are biologically active molecules which are increasingly being used for animal and human health to prevent or treat infections caused by microbes [3]. The content of microalgae which has the activity of amino acids, terpenoids, florotanin, steroids, phenolic, acrylic acid and halogenated ketones can be used as an antimicrobial and anticancer [4]. *Chlorella vulgaris* and *Chlamydomonas pyrenoidosa* has been shown to have antibacterial activity in vitro against gram positive and negative [5].

Other microalgae that has been investigated as antibacterial are*Pithophora oedogonium* extracted using ethanol can inhibit the growth of bacteria *Salmonella and Staphylococcus*sp [6].*Sargassum wightii, Chaetomorpha linum, Padina Gymnospora.* with methanol and acetone extracts can inhibit the growth of bacteria *P. aeruginosa,S. typhi*-B,*Erwinia amylovora, Proteus vulgaris, E. coli*and *S. aureus* [7].*Chlorella sp.*with ethanol extract can inhibit the growth of bacteria *Staphylococcus aureus, Bacillus cereus,Escherichia coli,Pseudomonas aeruginosa, Salmonella typhimurium*and *Yersinia enterocolitica* [8].In this research was conducted to obtain a superior kind of freshwater microalgae derived from Talago Biru, Koto Baru, West Sumatera and observe the antimicrobial capabilities possessed by microalgae which have been isolated.

MATERIALS AND METHODS

Materials and Equipment

The materials were used in this study are water samples of microalgae from Talago Biru, Koto Baru, Gram-positive bacteria (*Staphylococcus aureus* and *Bacillus cereus*) and Gram-negative bacteria (*Escherichia coli* and *Salmonella typhii*), Medium BBM ($NaNO_3$(Merck) , $MgSO_4.7H_2O$ (Merck), NaCl(Merck), K_2HPO_4 (Merck), KH_2PO_4 (Merck), $CaCl_2.2H_2O$ (Merck), $ZnSO_4.7H_2O$ (Merck), $MnCl_2.4H_2O$ (Merck), MoO_3 (Merck), $CoSO_4$ $.5H_2O$ (Merck), $Co(NO_3)_2.6H_2O$ (Merck), Qiagen kits for the isolation of DNA, loading dye, DMSO, H_2SO_4,technical methanol,Dragendorff reagents, meyer reagents, chloroform, ethanol 70%, $FeCl_3$ 5%, magnesium powder, 37% HCl, 1 N NaOH, Mueller-Hinton agar, Nutrient Agar, discs, *amoxycillin.*

The tools were used are 30 micron filter plankton net, a light microscope (Olympus CX41), Incubators, aerator, autoclave, UV-Vis spectrophotometer (Genesys 20), PCR tools (Polymerase Chain Reaction), electrophoresis apparatus, centrifuges, low temperature refrigerator, micropipette, Eppendorf tubes, vortex.

Research procedure

Sampling Microalgae

Samples microalgae were collected from three withdrawal points with a depth of ± 1 meter from the surface of the water collected into onefrom Talago Biru, Koto Baru using a plankton net with 30 micron hole size.[9]

Isolation and Purification of Microalgae

Bold Basal Medium(BBM) was autoclaved at a pressure of 1.2 atm, temperature 121^{o}C. 10 mL water sample was inoculated into 200 ml of medium in a 500-ml bottle, and then incubated with aeration administration. Every two days, the samples that had been grown were checked to see the growth of microalgae using a light microscope, and the dilution series had been done to see the growth. [10]

Morphological Identification of Microalgae

Morphological identification of microalgae which has been isolated is done microscopically. Observations were carried out regularly under the microscope to make sure that had gotten a single cell.

Observation of Microalgae Growth
Isolated microalgae growth is determined by Optical Density (OD) and measured using a spectrophotometer at a wavelength of 450 nm. [10]

Molecular Identification
Cells were harvested from 10 mL of liquid culture and DNA was isolated using a Qiagen DNA isolation kit. DNA isolation results were amplificated using PCR amplificationwith 2 mL of genomic DNA, 0.2 mM deoxynucleotide trifospat, 1.25 units of Taq DNA Polymerase and use forward primerChloroF 5'-CCT TGG TGT ATC TTG TTG GTC-3 ' and reverse primer ChloroR 5'-GAA TCA ACC TGA CAA GGC AAC-3 '. For CHLORO amplification, program PCR consisted of 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 59°C for 1 minute, and 72°C for 1 minute, with the cycles of the 10-minute extra at 72°C.DNA amplification product was electrophoresed with 1% agarose gel with a voltage of 101 V using 1x TAE buffer was detected by staining using aloading dye. PCR products were purified with the additionGeneaid Gel / PCR DNA Fragments Extraction Kit [11] and sequencing was done in Macrogen, Korea.

Phytochemical analysis
Phytochemical analysis was conducted on the test alkaloids, triterpenoids and steroids, phenols, flavonoids and saponins. The analytical method used is based on Harborne (1987).

Biomass collection and Making Microalgae Extract
Liquid cultures of microalgae are dried on a predetermined harvesting, dry by the wind until all the water is gone. Dry biomass was added by ten parts technical methanol, then sonicated for 45 minutes. The filtrate obtained is filtered, the filtering is done several times until methanol is relatively colorless. Methanol was evaporated to obtain a dry extract of microalgae.

Making suspension Bacteria
Test bacterias (*E. coli, Staphylococcus aureus, Salmonella typhii, Bacillus cereus*) were cultured on a nutrient agar medium for 24 hours at 37°C, then taken a loopful and suspended into 5 mL LB medium.[12]

Testing the antibacterial activity
SterilizedMueller-Hinton agar liquid put into petri dishes 20 mLper each and allowed to solidify at room temperature. Media drip with 200µL test bacterial suspension. Sterile paper disc with a diameter of 5 mm was dropped extract with a volume of 20 mL with a concentration of 10 mg / mL, 100 mg / mL and 500 mg / mL, and then placed on medium and incubated at room temperature for 24 hours. *Amoxycillin* 10 mg/mL was used as positive controland a negative control using DMSO 0.1%. Diameter of inhibition zone measured horizontally and vertically using a scale ruler. [12]

RESULTS AND DISCUSSION

Microalgae Morphology and Molecular Identification Results Isolation
Microalgae isolated from Talago Biru, Koto Baru, West Sumatera and obtained a single dominant isolates live in medium BBM. Isolates were identified morphological and molecular. Results of morphological identification of single isolates are then compared with the morphological data contained on the website Algabase.org data which gathering microalgae that have been studied worldwide in Figure 1.

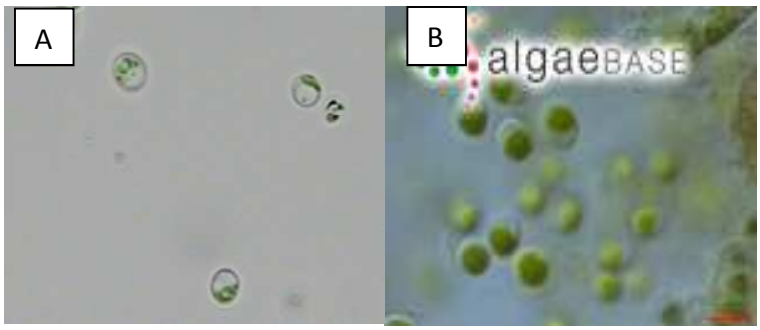

Figure 1. (A) Morphology of the isolated microalgae 1000x magnification, (B) Morphology Chlorella sp. of Algabase.org

Judging from their morphological similarity, it can be said that the superior microalgae have been successfully isolated is a type of microalgae *Chlorella sp. Chlorella sp.* is a kind of green microalgae, In Figure 1 (A) it can be seen that the color of isolates is green. The green color signifies *Chlorella sp.* have chlorophyll. *Chlorella sp.* have a size of 3-15 microns and round, a eukaryotic organism which means that already have a cell nucleus and also *Chlorella sp.* does not have flagell [13]. From the result of morphological test we can state that single isolates of microalgae which has been isolated is the type *Chlorella sp.*, The results of morphological identification is confirmed by the results of existing molecular identification. Where the results of the identification of molecular phylogenetic trees obtained stating closest kinship microalgae isolation results can be seen in Figure 2.

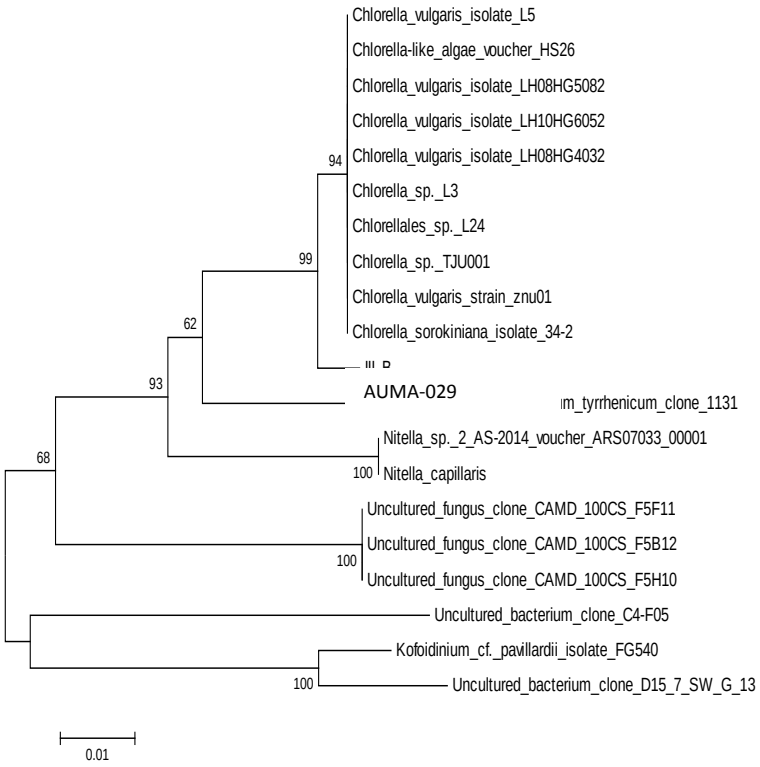

Figure 2. Results of analysis of phylogenetic trees with MEGA5.1

Phylogenetic trees obtained from it is seen that isolates AUMA-029 has the closest kinship with *Chlorella sp*. where the similaritypercentage that obtained from Figure 2 is equal to 99%. The percentage of similarity is so great that it can be said that isolates AUMA-029 which was isolated from Talago Biru, Koto Baru is a type of *Chlorella sp* ..

If the results of the identification of this molecular was compared with the result of morphological testit is proved that the isolates AUMA-029 is a group of *Chlorella sp* .. In the morphological testwas seen many similarities between isolates AUMA-029 with microalgae *Chlorella sp*.morphology which contained in the Algaebasedata.The more the similar characteristic, then the closer kinship.[15]

Microalgae Growth Curve

Growth is defined as an increase in cell mass and size accompanied by the synthesis of macromolecules that generate new structures[15]. Microalgae growth curve can be seen in Figure 3.

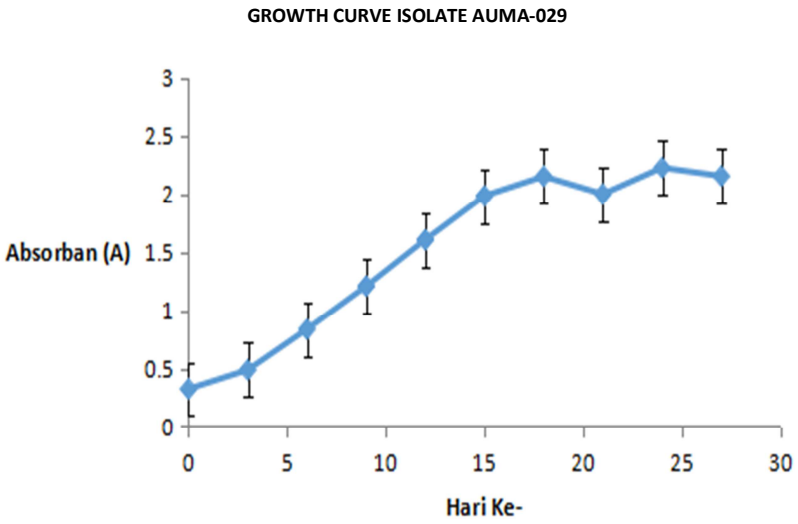

Figure 3. Isolate growth curve AUMA-029 (SD = 0.64573, n = 3)

During cultivation, the culture colour of isolated microalgae (AUMA-029) is green. The more dense the number of cell the more green that it looks, it means the microalgae have a lot of green pigment (chlorophyll) [16] in accordance with the results of morphological identification stating isolates AUMA-029 is a type of green alga *Chlorella sp.*

The isolates growth pattern possessed by AUMA-029 starting from the lag phase(adaptation) on days 0-3, followed by a log phase on days 4-15. Metabolism charactheristic during log phase is a high photosynthetic activity for protein formation and the composition of the plasma cells needed for growth. This condition is characterized byincreasing the culture green color than at the start of culture.[15]

Stationary phase cultures experienced at the age of day 16was marked by the increase of cell relatively fixed. In this phase harvesting is done because in this phase secondary metabolites such as organic carbon most commonly produced. Stationary phase that occurs at day 16-24 phase interspersed with the growth rate decreased on day 21, the phase of growth rate decline usually occurs when nutrients, pH, and CO_2 contained in the medium began to reach its limits. [17]

Culture began to reach a phase towards death that is on day 25, seen in this phase the amount of precipitation and culture change color to yellow. The cell death caused by nutrient exhaustion and accumulation of specific metabolic or toxic materials.[15]

Phytochemicals profile

Phytochemical test against these secondary metabolites are performed for samples that have antimicrobial effects.The samples are containing hydrogen peroxide, terpenoids and bromo ether and according to previous studiesof *Enhalus acoroides*, the compounds were significantly identified as antimicrobial compounds are fatty acid, acrylic acid, halogen aliphatic , terpenes and phenols [18]. When compared with the test results of phytochemical that has been done against the microalgae *Chlorella sp.*whichextracted with methanolin Table 1.

Table 1. Phytochemicals Test Results Isolate AUMA-029 and *Chlorella vulgaris* (Adhoni et al, 2016)

No.	Secondary metabolites	isolates Microalgae AUMA-029	*chlorella vulgaris* (Adhoni et al, 2016)
1	flavonoids	-	+
2	phenolic	+	+
3	saponin	+	+
4	steroids	+	+
5	triterpenoids	+	+
6	alkaloids	-	-

There are differences in the results of flavonoids which in previous studies [19]was detected the presence of flavonoid compounds in microalgae *Chlorella sp.* while in this study flavonoid compound was expressed negative. This difference could have occurred because of secondary metabolites are not always shared by all organisms. Environmental conditions and the stage of development of an organism will induce the formation of secondary metabolites. Secondary metabolites are not directly involved in the growth and development of an organism or the reproductive system. Secondary metabolites appear and work in defense of life and the process of adaptation of an organism. [20]

Bacterial resistance mechanism by phenolic secondary metabolites can be toxic protoplasm, damage and penetrate the cell wall of bacterial cells and precipitate protein. Causing damage to the bacterial cell ends bacterial death. Triterpenoids as antibacterials can react with Porin (transmembrane protein) in the outer membrane of the cell walls of bacteria, forming a strong bond polymer resulting in damage Porin. Damaging Porin which is the doorway to the compound would reduce the permeability of cell walls of bacteria and bacteria will result in nutritional deficiencies, so that bacterial growth is inhibited and even death. Steroids inhibit the growth of bacteria by destroying the bacterial cell membrane [21]. While saponins inhibit bacterial growth by lowering the surface tension of the cell wall and when interacting with the bacterial wall, the wall will be broken. When disturbed surface tension, saponin will enter the cell easily and will disrupt bacterial metabolism and eventually death. [22]

Antibacterial activity

Antibacterial activity test performed against Gram-positive bacteria such as *Staphylococcus aureus* and *Bacillus cereus* as well as Gram-negative bacteria, namely *Escherichia coli* and *Salmonella typhii.* The results are presented in Table 2.Observations were made after incubation for 24 hours.

Table 2, The antibacterial activity of isolates AUMA-029 with methanol

Concentration of Extracts	Inhibition Zones			
	E.coli	*Salmonella typhi*	*Staphylococcus aureus*	*Bacillus cereus*
10µg / mL	8 mm	10.5 mm	9 mm	10 mm
100µg / mL	10 mm	9 mm	9 mm	10 mm
500µg / mL	12 mm	9 mm	10 mm	11 mm
control + (Amoxycillin 10µg / mL)	10 mm	9 mm	10 mm	10 mm
control -	7 mm	0 mm	7 mm	6 mm

From the measurement results shows that the clear zone on the test bacteria *E. coli*, *S. aureus* and *B.cereus*give the greatest inhibition zone is shown at a concentration of 500 ug / mL while on *S.typhii*the largest inhibition zone has been seen at a concentration of 10 ug / mL. Differences in increasing and decreasing zones of inhibition against the concentration of the extract is due to the component substances that contained in medicinal plants can be mutually weaken, strengthen, improve or change at all. Giving concentrations lower or higher than the concentration required to kill bacteria can also inhibit the antibacterial activity against the antibiotics given so that the bacteria will be resistence.This vulnerability is caused due to bacteria can produce enzymes that can perform antimicrobial inactivation and disruption of cell membrane permeability thus not achieving an effective antimicrobial concentrations in the cell as well as the modification of the molecules in the cell which is the target of antimicrobials. [23]

When compared with previous studies that have been conducted on microalgae *Chlorella vulgaris* were extracted by using methanol in Table 3 shows that the bacteria that form potential barriers are relatively low.

Table 3. Results of Antibacterial Activity of *Chlorella vulgaris* with Solvent Methanol[18,28]

No.	Bacteria Test	Inhibition Zones By *Chlorella vulgaris*
1	*E.coli (Syed, 2015)*	15 mm
2	*S.aureus (Salem, 2014)*	17 mm
3	*B.cereus (Salem, 2014)*	17.5 mm

This low potency can be caused by the extract which has been obtained is crude extracts and effectiveness are small. This smalleffectiveness presumably because least of the content from active compound has a similar structure to the antibiotics commonly used to inhibit the growth of the test bacteria used, the age of the bacteria and environmental conditions. [6]

Isolates AUMA-029which is a microalgae *Chlorella sp.* has potential as an antibacterial, because the content of metabolites contained in them either the content of primary and secondary metabolites. The most important antibacterial compounds owned by *Chlorella sp.* which is a mixture of fatty acids known as *chlorellin*, this compound is able to inhibit the growth of Gram positive and Gram negative. The methanol extract of *Chlorella sp*. can inhibit the growth of bacteria *Bacillus cereus* and *E. coli*[24]. The mechanism of inhibition of bacterial growth by a fatty acid that is by attacking and destroying the cell membrane of the bacteria causing the lysis of the bacterial cell, and also these fatty acids will reduce nutrient and inhibits cellular respiration. [25]

Besides containing *chlorellin* as an antibacterial agent, *Chlorella sp.*also containing secondary metabolites such as phenolic , terpenoids, steroids and saponins as an antibacterial agent. Chlorophyll that contained in *Chlorella sp*. can also act as an antibacterial compound. Chlorophyll does not have a bactericidal effect (kill microorganisms) but chlorophyll has the ability bacteriostatic (inhibits the growth of microorganisms), but that under the suitable environmental conditions chlorophyll can also have a bactericidal effect. [24]

In Table 3 shows that the negative control also provides an inhibitory effect on *E. coli*, *Staphylococcus aureus* and *Bacillus cereus.* This is because DMSO is toxic to bacteria.*Dimethyl sulfoxide* (DMSO) is an aprotic solvent that can dissolve both polar and non-polar compounds, therefore it is widely used as a solvent on antimicrobial testing [26]. DMSO 1%can kill 2-10% bacteria, therefore it is recommended to use DMSO in low concentrations at the time of dissolving the antibacterial test extract, to not give effect against bacteria inhibition test. Recommended concentration of DMSO is 0.01% - 0.5% . [27]

CONCLUSION

The research shown the dominant type of microalgae that live in medium BBMwhich was isolated from Talago Biru, Koto Baru, West Sumatera is a genus *Chlorella sp.* and the best harvesting time for this microalgae is on the day 20 in the stationary phase. Profile phytochemicals result shown the contained in this isolate are positive phenolic, saponin, steroids and triterpenoid, while alkaloids and flavonoids give negative results. The isolate have potential as a an antibacterial, seen from the test results provided by the zone of inhibition against four bacterial isolates tested, but the activity is low.

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