ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Screening, Identification and Fatty Acid Composition: Analysis of Mercury Resistance Microalgae from Freshwater Pond in Kuranji, Padang, West Sumatera, Indonesia

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ABSTRACT

The use of microalgae for the bioremoval of heavy metals has been observed for last decades. This encourages the investigation of biomolecular study of microalgae which absorb the heavy metals. This study concerns on the biomolecular effects of mercury treatment in microalgae: growth rate, fatty acids composition and identification of the microalgae species which is resistance of mercury treatment. This research aims to analyze the mercury effect comparison to the growth and fatty acids composition between the mercury and non mercury treated microalgae species. The microscopy identification showed that morphologically, both control and mercury treated microalgae species is *Chroococcus dispersus*, but 28S RNA sequencing data showed that the two species were tentatively related to *Poterioochromonas malhamensis*. The growth rate mercury treated species was lower than non mercury treated species. The fatty acid composition analysis by Gas Chromatography-Mass Spectrometry (GC-MS) showed that both control and mercury treated microalgae had the similar but different amount of fatty acids composition. Both control and mercury treated microalgae had C16:0 as the highest fatty acid content. Most of fatty acid contents in mercury treated microalgae were lower than control, except for C16:1, C16:3 and C18:0.
Keywords: Microalgae, mercury, fatty acids, *Chroococcus dispersus*

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ISSN: 0975-8585

INTRODUCTION

The rapid growth of industrial has led the increasing of the industrial waste. Many industry activities discharge waste with high content of heavy metal. Most of these waste containing heavy metals bring serious threat to the environment, especially the water system.[1] Even though, there are many metal ions which are essential for living organism but toxic in high concentration. High concentration heavy metal can't be degraded biologically or chemically. Mercury is one of the most heavy metal used in global industry. The use of mercury compounds for industrial has led to the accumulation of this metal in the environment. This accumulation causes the damage for the living organisms in the polluted environment. The damage can be neurological effect, reproductive effect or behavioral effect. [2]

There are number of studies to analyze the effect of mercury or any heavy metals on water organisms. The use of microorganisms and plants for detoxification of heavy metals were observed for last decades because living organism cause less environmental problems.[3] The heavy metal uptake ability by microalgae has been established from many algae species.[4] Microalgae possess molecular mechanisms that eliminate the heavy metals which are not essential for their growth.[5] These mechanisms can affect the biomolecular system of microalgae such as cell, fatty acids content and protein composition.

Microalgae as the lower class plant have been discussed as the alternative source of biofuel. Many researchers have observed the potential of microalage for biofuel production. Microalgae have an economic value because of high fatty acids content and short cultivation time.[6-8] Mostly in microalgae, the long carbon chain fatty acids carbon are produced such as linoleic and linolenic acid which are included in omega 3 which is also good for human body.[7] These fatty acids content can be influenced by the presence of heavy metal. For example, the use of cadmium and copper to marine algae cause to decrease the polyunsaturated fatty acids level.[9] Chromium has significant impact to the cell and fatty acids alteration of *Euglena gracilis.*[10] The study of microalgae fatty acids is essential for the production of sustainable energy.[11] Mercury effects in microalgae's fatty acids composition and growth analysis of microalgae. Microalgae species were screened and isolated to obtain the mercury resistance species.

METHODS

Sampling, screening and isolation of mercury resistance microalgae

Microalgae were collected from freshwater ponds in Kuranji district, Padang, Indonesia. The *Bolt Bassal Medium* (BBM) medium was used for growth medium. Initial isolate microalgae were grown in microplate for 5-7 days and kept in incubator at 25-30^{o} C with continuous light. Microalgae were isolated into new microplate by capillary pipetting technique. The isolated microalgae were divided into two conditions: control which was not treated by mercury and mercury treated microalgae. Mercury (25 mg/L) was added into the well including microalgae. The strong resistance species was isolated then into new BBM medium in order to grow only

one species. The isolated microalgae were grown in 48 well microplate for 1-2 weeks. The grown microalgae were cultured in petridish for 1-2 weeks. The microalgae were put into culture flask for mass culture. The grown microalgae were observed by Zeiss Axiovert Erc5s microscope and Zeiss Laser Scanning Microscope 710.

RNA extraction, PCR amplification and sequencing

RNA of microalgae was extracted using DNeasy mini plant KIT (QIAGEN). The extract of RNA was amplified with PCR kit (Takara, Japan) with these certain conditions: denaturation at 96^{o}C for 30 s, annealing at 57^{o}C for 30 s, extension at 72^{o}C for 30 s. All reactions were set for 35 cycles using universal eukaryotic fw1 (5'-AGCGGAGGAAAAGAAACTA-3') as forward primer and rev1 (5'-TACTAGAAGGTTCG-ATTAGTC-3') as reverse primer. Electrophoresis was run in agarose gel (1.5%). The bands of DNA were extracted and cloned using pGem-T Easy cloning KIT and purified by Wizard *Plus* Minipreps DNA purification system. The RNA sequencing was done in Division of Genomics Research, The Life Science Research Centre, Gifu University, Gifu, Japan. Ribosomal RNA gene sequences from the isolates were searched using NCBI GenBank using BLAST.

Microalgae growth analysis

The growth rate of control and the mercury treated microalgae were analyzed everyday by microplate reader by using Envision Reader UV-Vis Spectrophotometer (Perkin Elmer) at 570 nm.

Fatty acids composition analysis

The isolated microalgae (10 mL) was centrifuged at 3000 rpm for 10 min by TOMY LC-200 and separated from the supernatant. Pellet was dried by nitrogen gas and added by 0.5 mg/mL of C19:0 as internal standard (5 µL). Methyl esterification was performed by addition of 0.5% HCl-methanol (0.1 mL) and dehydrated methanol 0.4 mL. Fatty acids were extracted with n-hexane and separated by centrifugation. n-Hexane layer was dried with nitrogen gas. For GC-MC analysis, hexane (20 µL) was added and samples were injected to DB WAXTR column (30 m, ϕ = 0.250 µm, Helium mobile phase, 131^{o} C). The Gas Chromatography was done by Agilent 6890 for 20 minutes of retention time and the Mass Spectrum was measured by JEOL-GC mate II.

RESULTS AND DISCUSSION

Screening and Isolation

Fig. 1 shows the image of initial sample of microalgae. This initial sample contains many species of microalgae. The diversity of microalgae species was isolated by capillary pipetting technique. Fig. 2 shows the resistance species of microalgae which was treated by mercury. There were two dominant unidentified species which survived by mercury treatment: the round

ISSN: 0975-8585

shape and sharp shape, respectively. Since the round shape was more dominant than the sharp shape, it was selected to be analyzed. The microalgae were isolated to two type's microalgae: none treated microalgae (control) and mercury treated microalgae. These two microalgae isolates had same shape which were observed by microscope and can be seen in Fig. 3. The initial microscopy identification of the species was *Chroococcus dispersus.* This morphological identification is supported by the comparison with the cell image from algaeweb.net which can be seen in Fig.4. There is similarity with the cell structure, so the species which is observed in this study can be identified as *Chroococcus dispersus.*

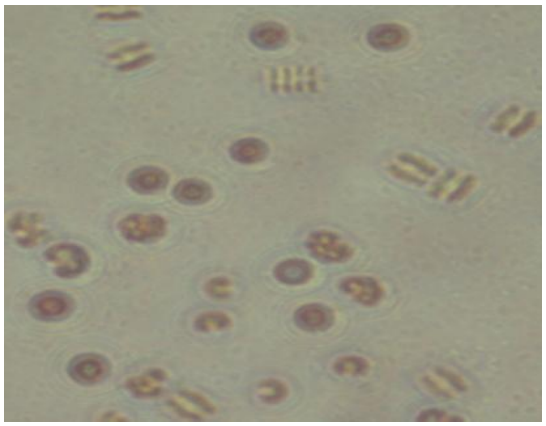

Fig 1: The image of initial sample by Zeiss microscope. The initial sample contains many species of microalgae.

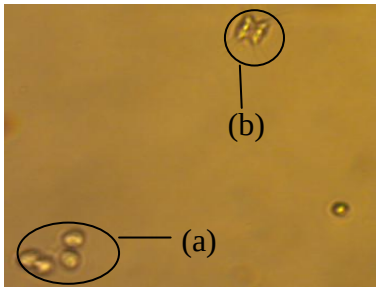

Fig 2: The initial samples treated by mercury (25 ppm) seen by Zeiss microscope. There are two dominant resistance species against mercury: (a) round shape and (b) sharp shape.

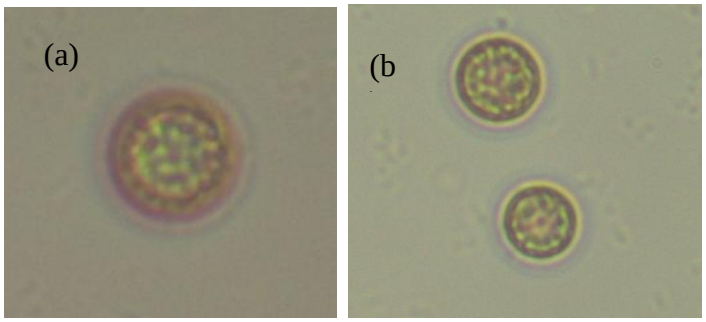

Fig 3: (a) The Microscope image of non mercury treatment microalgae (Control); (b) The microscope image of mercury treatment microalgae.

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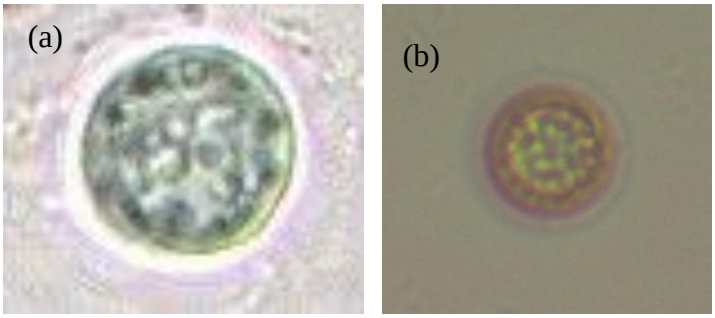

Fig 4: The comparison of cell image of *Chroococcus dispersus from* (a) algaeweb.net and (b) the control of this study.

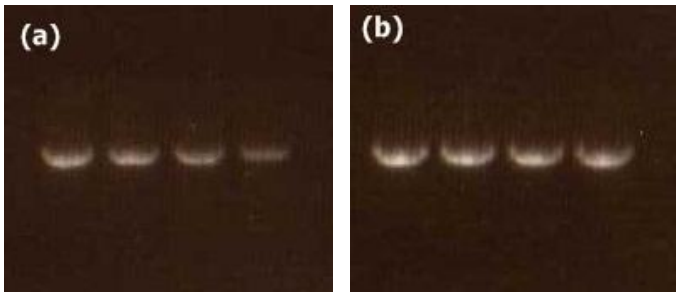

Fig 5: The Electrophoresis RNA bands: (a) Control; (b) Mercury Treated Microalgae.

GGACCGCCATGGCGGCCGCGGGAATTCGATTAGCGGAGGAAAAGAAACTAACTAGGATTCCCTCAGTAACGG
CGTGTGAA
GCGGGAAGAGCTCAGACTGAAAACCTTCGGGGATGTAGTCTGGAGATGGGATATCGAATCTCAGGAAGCCGA
TAAAGTGG
TCTGGAACGACACGCCGTGGAGGGTGACAGCCCCGTTCGTATCGGTGGAAAGAGAAAGTAGATACCTATCAA
CGAGTCGA
GTTGTTTGGGATTGCAGCTCAAAGCGGGTGGTAAATTCCATCTAAAGCTAAATATGGATAGGAGACCGATAGC
AAACAAG
TACCGTGAGGGAAAGATGAAAAGCACTTTGAAAAGAGAGTGAAATAGTACCTGAAATTGCTGAAAAGGAACC
GCTGGGAA
GCAGTGTGCGGGCCGTAGAGTCTCTCTACGGCATGCAGGTGAGGCTTGCTTCGATGGTGAGGGAAAAGGCGT
GCTTGCAC
TGTCCTACCAATCCTCGGAGTGACTCTCACGAAATGCTTCTCATCAACCCGTCTTGAAACACGGACCAAGGAGT
CTAACA
TGTGTGCAAGTGTTCGGGAGTCAAGTCCCAAGCGCGAAATTAACGTAAAGGTGGGTTTCGGCCCGCTGAGGT
AGGAAGCT
TCGGCTGCACTATCGACCGACCATGATCCTTCGAGTGAAAGGTTTGAGTGTGAGCATACATGTTGGGACCCGA
AAGATGG
TGAACTATGCTTGAGTAGGACGAAGCCAGGGGAAACTCTGGTGGAGGTCCGTAGCGATTCTAACGTGCAAAT
TGATCGTC
GAACTTGAGTATAGGGGCGAAAGACTAATCGACCTTCTAGTAATCACTAGTGAATTCGCGGGCCGGCTTGCAG
GTCGACC

ISSN: 0975-8585

Fig 6: RNA amplified sequence data of microalgae control.

GCGCCCGCCATGGCGGCCGCGGGAATTCGATTAGCGGAGGAAAAGAAACTAACTAGGATTCCCTCAGT
AACGGCGAGTGA
AGCGGGAAGAGCTCAGACTGAAAACCTTCGGGGATGTAGTCTGGAGATGGGATATCGAATCTCAGGAA
GCCGATAAAGTG
GTCTGGAACGACACGCCGTGGAGGGTGACAGCCCCGTTCGTATCGGTGGAAAGAGAAAGTAGATACCT
ATCAACGAGTCG
AGTTGTTTGGGATTGCAGCTCAAAGCGGGTGGTAAATTCCATCTAAAGCTAAATATGGATAGGAGACC
GATAGCAAACAA
GTACCGTGAGGGAAAGATGAAAAGCACTTTGAAAAGAGAGCGAAATAGTACCTGAAATTGCTGAAAAG
GAACCGCTGGGA
AGCAGTGTGCGGGCCGTAGAGTCTCTCTACGGCATGCAGGTGAGGCTTGCTTCGATGGTGAGGGAAAA
GGCGTGCTTGCA
CTGTCCTACCAATCCTCGGAGTGACTCTCACGAAATGCTTCTCATCAACCCGTCTTGAAACACGGACC
AAGGAGTCTAAC
ATGTGTGCAAGTGTTCGGGAGTCAAGTCCCAAGCGCGAAATTAACGTAAAGGTGGGTTTCGGCCCGCT
GAGGTAGGAAGC
TTCGGCTGCACTATCGACCGACCATGATCCTTCGAGTGAAAGGTTTGAGTGTGAGCATACATGTTGGG
ACCCGAAAGATG
GTGAACTATGCTTGAGTAGGACGAAGCCAGGGGAAACTCTGGTGGAGGTCCGTAGCGATTCTAACGTG
CAAATTGATCGT
CGAACTTGAGTATAGGGGCGAAAGACTAATCGAACCTTCTAGTAAATCACTAGTGAATTCGCGGCCGC
CTGCAGGT

Fig 7: RNA amplified sequence data of mercury treated microalgae.

PCR Amplification and Sequence nalysis

The RNA electrophoresis for control (Fig. 5a) and mercury treated microalgae (Fig. 5b) resulted one band. The RNA amplified sequencing result for both control and mercury treated microalgae can be seen in Fig. 6 and 7. This biomolecular identification was supposed to prove the morphological identification by microscope. The nucleotide BLAST analysis from NCBI showed that both amplified sequences are tentatively *Poterioochromonas malhamensis* with Error value (E) is equal to 0 and the similarity approaching 99 %. This result is different than the morphological identification by microscope. This different may be caused by the lack of database information of 28s RNA sequencing of *Chroococcus dispersus* in NCBI so the sequencing identification is not matched with the microscope identification.

Growth Analysis

The growth rate of microalgae was increased since first day until 8 days. On the other hand, the growth rate of mercury treated microalgae increased in 2 days and start to decrease in 5 days. This stage probably was the stationary phase of mercury treated microalgae. This result showed that the growth of microalgae control was more stable than mercury treated microalgae. The growth of mercury treated microalgae may be inhibited because of the cell inhibition by mercury in the cells surface. Most mercury metals are found in vacuola or

cytoplasm. Mercury metal is forming organic metallic compounds in the cell surface. Probably, mercury adsorption in the cell causes the disturbance of microalgae cell metabolism and influence the growth.[12] Moreover this research used 25 ppm of mercury whereas 0.02-0.2 mg/L mercury concentration could inhibit the growth rate of microalgae. [13]

Fatty Acids Composition

The fatty acid analysis of both control and mercury treated microalgae showed that there were 10 major peaks which indicate 10 fatty acid contents. They were C16:0, C16:1, C16:2, C16:3, C16:4, C18:1, C18:2, C18:3 and C18:4. Most of these fatty acids were polyunsaturated fatty acids. Both control and mercury treated microalage had same but different amount of fatty acid contents. The comparison of each fatty acids amount can be seen in **Table 1.**

Table 1: Fatty acids concentration of control and mercury treated microalgae

Fatty acids	Concentration (nmol/µL)	
	Control	Mercury treated microalgae
C16:0	1.298	1.146
C16:1	0.609	0.834
C16:2	0.655	0.382
C16:3	0.402	0.573
C16:4	1.114	0.864
C18:0	0.138	0.261
C18:1	0.988	0.774
C18:2	1.080	0.784
C18:3	1.286	1.126
C18:4	0.540	0.322

The highest amount fatty acid in both control and mercury treated microalgae was C16:0. Most of fatty acids in control were higher than fatty acids in mercury treated microalgae. The use of heavy metal influences the production of microalgae lipid. Heavy metal such as mercury can influence the metabolism system in microalgae cells. The previous data reported the decrease of chlorophy level in concentrated heavy metal. This can affect the lipid production of organism, including microalgae. In this study, There are some higher fatty acids in mercury treated microalgae than control: C16:1, C16:3, and C18:0. The previous study reported that the some fatty acids production in microalgae treated by metal are high due to the formation of metal complexes in microalgae cell which can produce lipid peroxidative and change the fatty acid content in microalgae.[14] This hypothesis may explain the C16:1, C16:3 and C18:0 production in this study. The changes in fatty acid content caused by heavy metal treatment can also be used as an indicator of microalgae's defense mechanism to reduce the cellular damage by heavy metals.

ISSN: 0975-8585

CONCLUSION

There are two dominant species of mercury resistance microalgae in freshwater sample from freshwater pond in Kuranji, Padang, West Sumatera. The round shape species is the more dominant one and selected to be observed. This species was identified as *Chroococcus dispersus* by microscope identification but had different result with biomolecular identification by gene sequencing method. The biomolecular identification resulted the *Poterioochromonas malhamensis*. This difference probably caused by the lack information in NCBI database of 28s RNA *Chroococcus dispersus* sequence data. The microalage isolates were isolated by using capillary pipetting technique. The growth rate of control was more stable than mercury treated microalgae because mercury treatment caused the inhibition of microalgae growth rate. The GC MS analysis identified 10 major fatty acids in both control and mercury treated microalgae, which were C16:0, C16:1, C16:2, C16:3, C16:4, C18:1, C18:2, C18:3 and C18:4. Most of fatty acid content in control was higher than mercury treated microalgae except for C16:1, C16:3 and C18:0 due to the changes of fatty acids contents caused by mercury treatment.

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