Effect of Audible Sound Exposure on the Growth and Lipid Content of Chlorella DPK-01 Cultivated in Photobioreactor System using Different Growth Media

Rubiantin Mesha Nauli Tambunan
Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia Depok

Soekirno, Santoso
Department of Physics, Faculty of Mathematics and Natural Sciences, Universitas Indonesia Depok

N. Nasruddin
Department of Mechanical Engineering, Faculty of Engineering, Universitas Indonesia Depok

Nining Betawati Prihantini
Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia Depok

https://doi.org/10.5109/7172232

出版情報：Evergreen. 11 (1), pp.106-115, 2024-03. 九州大学グリーンテクノロジー研究教育センター
バージョン：

Effect of Audible Sound Exposure on the Growth and Lipid Content of *Chlorella* DPK-01 Cultivated in Photobioreactor System using Different Growth Media

Rubiantin Mesha Nauli Tambunan[1], Santoso Soekirno[2], N. Nasruddin[3] Nining Betawati Prihantini[1*]

[1]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Indonesia Depok, West Java, Indonesia

[2]Department of Physics, Faculty of Mathematics and Natural Sciences, Universitas Indonesia Depok, West Java, Indonesia

[3]Department of Mechanical Engineering, Faculty of Engineering, Universitas Indonesia Depok, West Java, Indonesia

*Author to whom correspondence should be addressed:
E-mail: nining@sci.ui.ac.id

(Received April 17, 2023: Revised December 12, 2023: Accepted February 19, 2024).

Abstract: Audible sound exposure affects the growth and lipid production of Indonesia's indigenous microalga *Chlorella* DPK-01 cultivated in Bold's Basal Media (BBM) and NPK Fertilizer Media. The NPK Fertilizer can also be considered a cost-efficient alternative medium to grow *Chlorella* DPK-01. Schierer-Ray Hare (SRH) test ($\alpha = 0.05$) shows that there is no significant difference between the average cell density of *Chlorella* DPK-01 grown in BBM (basal media) and NPK Media (alternative media). Hence, NPK media can be used as a cost-efficient alternative medium. However, based on eight days of observation, it is known that when cultivated in NPK Fertilizer Media with exposure to a 279.9 Hz sine soundwave, *Chlorella* DPK-01 has the highest cell density on the peak phase (2.060×10^6 cells/mL) and the highest growth rate (0.670 per day). However, it does not have the highest lipid percentage (23.4%). Meanwhile, even though the cell density on the peak phase (1.382×10^6 cells/mL) and growth rate value (0.419 per day) of microalga *Chlorella* DPK-01 exposed to 279.9 Hz square soundwave is not the highest, it has the highest lipid percentage (53.43%) among all other treatments. Different audible soundwave forms also affected the growth and lipid percentage of *Chlorella* DPK-01 cultivated in BBM, yet the results are not as varied as were cultivated NPK Fertilizer Media. Therefore, the exposure of audible soundwaves and the usage of NPK Fertilizer Media can be used as an improvement strategy for the photobioreactor system.

Keywords: audible sound, BBM, *Chlorella* DPK-01, growth rate, lipid percentage, NPK Media

1. Introduction and Background

Excessive use of fossil fuels in human activities increases the risk of ozone depletion and resource depletion on Earth. The concentration of carbon dioxide in the atmosphere has increased due to fossil fuel-based machinery gas emissions. This causes the depletion of the ozone layer and changes the composition of the atmosphere[1)]. These changes in the atmosphere might increase ultraviolet B (UV-B) radiation to the earth, resulting in ecosystem imbalance[2)]. In addition, fossil fuels as a resource are expected to run out in 2070 if alternative fuels cannot be produced[3)]. Therefore, alternative fuels from organic materials, or biofuels, are needed. The use of biofuels is expected to reduce the risk of ozone and resource depletion on Earth without hampering human activities in various sectors[4)].

Several studies have proven that prokaryotic and eukaryotic microalgae contain lipids that can be processed further as raw material for biofuels[4)]. Several potential prokaryotic microalgae to be processed as biofuels material are *Synechcococcus*[5)] and *Leptolyngbya*[6)]. Meanwhile, *Chlorella* and *Scenedesmus*[7)] are potential

eukaryotic microalgae genera to be processed as biofuels material. The advantages of biofuels made from microalgae biomass are their non-toxicity, bio-degradability, and no sulfur content. In addition, the residual materials after lipid extraction processes from microalgae biomass can be further processed into environmentally friendly fertilizers[8].

Chlorella is a cosmopolite eukaryotic microalgae genus that has the potential to be processed further as biofuel. Cosmopolite microalgae like *Chlorella* are known to be found easily, both in aquatic and terrestrial environment[9]. Reproduction of *Chlorella* happens asexually by forming autospores. Autospores are daughter cells that are identical to their parent cells[10]. Thus, *Chlorella* is considered a fast-grower eukaryotic microalga. *Chlorella* also has various fatty acid content with various carbon chains, i.e., C16:0, C16:1, C16:2, C17:0, C18;0, C18:1, and C18:2[11]. It is known that C16:0 (Palmitate Acid) and C18:0 (Oleic Acid)[4] from *Chlorella* biomass can be easily esterified, so that it can be processed further to be biofuel[12]. Therefore, this study uses Indonesia's indigenous *Chlorella* that was isolated from soil in Depok, West Java. The strain code of this Depok Isolated *Chlorella* is Depok number one or DPK-01. A previous study has shown that microalga *Chlorella* DPK-01 can produce lipids up to 31% of its biomass[13].

Cultivation of both prokaryotic and eukaryotic microalgae has to be done in the most efficient way, because huge amounts of microalgae biomasses are required to be produced in a short time to be processed further as biofuel[4]. Microalgae, including *Chlorella,* should be cultivated in a photobioreactor (PBR) system before their biomass can be processed further as biofuel material. Photobioreactor type to cultivate microalgae can be tube-shaped[14], rectangular[15], or another type as needed. The system of photobioreactor can be used to regulate several factors, such as light intensity, photoperiodicity, temperature, the acidity of growth media (pH)[16] and physical factors, such as aeration rate[17] and audible sound[18]. Regulating those factors will induce microalgae, including *Chlorella*, to produce a large amount of biomass with high lipid content in a short time[16].

Microalgae growth in the PBR system is affected by the chemical factor. One of the chemical factors is nutrient content in microalgal growth media[19]. Bold's Basal Media (BBM) is one of the most frequently used growth media for eukaryotic microalgae cultivation[20]. It consists of essential macronutrients and micronutrients needed by eukaryotic microalgae to grow optimally[21]. Nevertheless, using BBM in mass-scale production of microalgal biomass is considered not cost effective[22]. Therefore, several Indonesian researchers conducted several studies about the ability of microalgae to grow in alternative media. It is known that Indonesia's indigenous prokaryotic microalgae, such as *Synechococcus*[23], *Leptolyngbya*[24], *Stainieria*[25], and *Nostoc*[26], can grow in alternative media that was made from dissolved NPK fertilizer. However, studies on the growth of eukaryotic microalga *Chlorella* DPK-01 in NPK media have not been done.

Alongside chemical factors, microalgae growth in the PBR system is affected by physical factors, such as audible sound. It is known that differences in frequencies and intensities of audible sound might affect microalgal growth differently. The intensity of the audible sound wave is affected by its distance from the source of sound and its waveform[32]. Study of how the difference in distance from the source of sound affects the growth of microalga *Chlorella* DPK-01[27]. However, there is no study on how different sound waveforms affect the growth of eukaryotic microalga *Chlorella* DPK-01.

The interaction of physical and chemical factors is also known to affect the growth of microalgae[28]. However, the study to determine how the interaction of physical and chemical factors, such as audible sound and media composition in affecting the growth of *Chlorella* DPK-01 has not been done. Therefore, this study was done to determine the effect of audible sound exposure, in form of sine and square soundwaves (279.9 Hz), to the growth and lipid percentage of *Chlorella* DPK-01 in photobioreactor system, cultivated in different growth media, which are Bold's Basal Media (BBM) and NPK Fertilizer Media.

To our best knowledge, this is the first study of microalga *Chlorella* DPK-01 cultivation with exposure to audible sound in different growth media. This study was done to know the effect audible sound exposure on the growth and lipid content of *Chlorella* dpk-01 cultivated in photobioreactor system using different growth media, which are BBM and NPK Fertilizer media. The information from this study then can be used as a reference in selecting combinations of physical stimulants, in form of audible sound, and growth media to cultivate microalgae in a photobioreactor system.

2. Method and Experimental Setup

This study consists of several stages. Those steps include the preparation of the photobioreactor and incubation cabinet, preparation of microalgal growth media, preparation of *Chlorella* DPK-01 starter culture, photobioreactor system unit operation, data collection, and data analysis. Schematic workflow of this research can be seen in Fig.1.

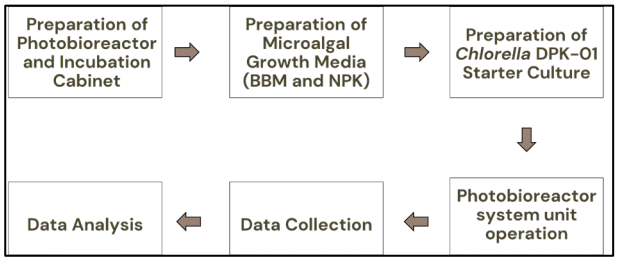

Fig.1: Schematic workflow

Several research equipment in this study are Microscope [Nikon SE], Autoclave [TOMY SX-500], Analytical Balance [Precisa XT-220A], Oven [Heraeus Instruments], Centrifuge [B-One], Sonicator [SAKURA], *Magnetic Stirrer* [Fisher Thermix], Cellphone Camera [Redmi Note 9 Pro], Laptop [Sony VAIO E Series], Infrared Thermometer [Krisbow], Soundproof Incubator, Music Player [RUIZU X-02], Speaker [NUBWO NSB16], Sound Level Meter [BENETECH GM1356], 18 W Tube Lamp [Phillips], Erlenmeyer Flask [Iwaki], Pasteur Pipette, Syringe, Photobioreactor Tubes, and Hand Tally Counter [Joyko]. All collected data then were analyzed by descriptive statistics method.

2.1. Photobioreactor and Incubation Cabinet

Photobioreactors (PBRs) and incubation cabinet in this study were designed and assembled identically with previous studies by Tambunan et al.[13] and Santoso et al.[29]. That system has been proven suitable for studies involving audible soundwaves exposure to microalgae, both prokaryotic[29] and eukaryotic[13]. The tube-shaped PBRs are made from 3 mm acrylic. Meanwhile, the soundproof incubation cabinet was made from iron, covered with 30 mm thick sound insulation foam and 3 mm thick glass wool carpet. The incubation cabinet was equipped with three Tube Lamps, each for every row; two music players, one for each row except in control row; two speakers, one for each row except in control row; three aerators, one for each row; and electric sockets to activate the whole system.

2.2. Preparation of Growth Media (BBM and NPK)

The growth media used to grow microalgae in this study were Bold's Basal Media (BBM) and NPK Fertilizer Media. The BBM was made by dissolving chemical substances in Table 1 into 1,000 mL of distilled water. Meanwhile, the NPK media was made by dissolving about 80 mg NPK (20:20:20) fertilizer into 1,000 mL or 1 L of distilled water, so the final concentration of NPK media would be 80 ppm.

Table 1. Composition of BBM (in 1 L Distilled Water)[30]

Chemical Substance	Amount (mg)
$NaNO_3$	250
KH_2PO_4	175
K_2HPO_4	100
$MgSO_4.7H_2O$	75
$CaCl_2.2H_2O$	25
NaCl	25
KOH	31
$FeSO_4.7H_2O$	4.98
H_3BO_3	11.42
$ZnSO_4.7H_2O$	8.82
$MnCl_2.7H_2O$	1.44
MoO_3	0.71
$CuSO_4.5H_2O$	1.57
$Co(NO_3)_2.6H_2O$	0.49
Na_2EDTA	50

Composition of NPK fertilizer can be seen in Table 2. The initial acidity value (pH) of NPK Media and the BBM in this study was adjusted to 7. After the chemical substances dissolved completely, both BBM and NPK media were sterilized using an autoclave in 121 ºC heat for 15 min.

Table 2. Composition of NPK Fertilizer Media[31]

Chemical Substance	Percentage (%)
Total Nitrogen	20
Ammoniacal Nitrogen	3,90
Nitrate Nitrogen	5,70
Urea Nitrogen	10,40
P_2O_5	20
K_2O	20
Ca	0,05
MgO	0,10
S (combined)	0,20
B_2O_3	0,02
CuO	0,05
Fe_2O_3	0,10
Mn (MnO)	0,05
Mo (MoO_3)	0,0005
Zn (ZnO)	0,05
Inert Ingredient	39

2.3. Preparation of *Chlorella* DPK-01 Culture

The starter culture was prepared by inoculating about 40 mL of *Chlorella* DPK-01 into 80 mL of BBM, which was available in a 200 mL Erlenmeyer flask. The culture was then incubated at 35 °C incubator for eight days. This process was repeated about ten times to obtain a sufficient amount of biomass ($2\pm0.05\times10^6$ cells/mL) to be inoculated into the photobioreactors. Photomicrograph of *Chlorella* DPK-01 starter can be seen in Fig. 2.

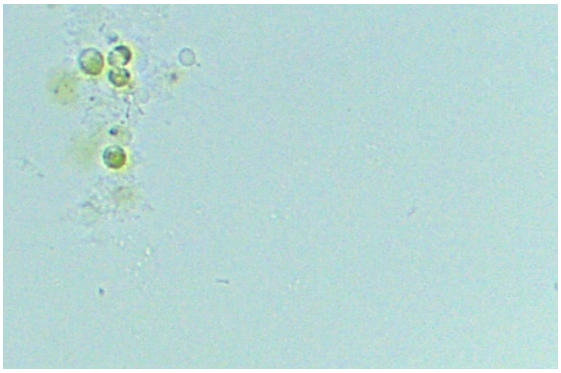

Fig.2: Photomicrograph of *Chlorella* DPK-01 (not to scale)

2.4. Inoculation and Operation of the System

About 400 mL of BBM was prepared into six PBRs; meanwhile, about 400 mL of NPK Media was also prepared into another six PBRs. There were twelve PBRs used in this study. The PBR groups division in the incubation cabinet and PBR labelling can be seen in Fig. 3. The numbers 1 and 2 on each PBR label indicate the unit number (PBR unit 1 or 2). A clearer explanation of this PBR labelling can be seen in Table 3.

As much as 80 mL of *Chlorella* DPK-01 culture was inoculated into each PBRs. Inoculation was done aseptically to prevent contamination. All PBRs then placed in an incubation cabinet. Aerators with an air speed of 1.5 liter per minute (LPM) were then connected to each PBR to mix nutrients in growth media and to provide Carbon Dioxide (CO_2) that *Chlorella* DPK-01 needed to undergo photosynthesis. The light intensity of LED lamps in the incubation cabinet was adjusted to 4,250±75 lux so that the temperature of the incubation cabinet could be set to 30±3 ºC

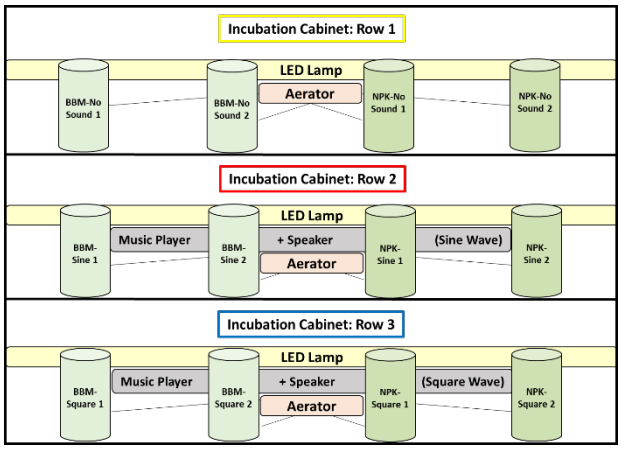

Fig.3: PBR labelling in incubation cabinet

Table 3. PBR labelling explanation

Label	Explanation
BBM-No Sound	Cultivated in BBM, Not exposed to any sound.
BBM-Sine	Cultivated in BBM, Exposed to 279.9 Hz Sine Wave
BBM-Square	Cultivated in BBM, Exposed to 279.9 Hz Square Wave
NPK-No Sound	Cultivated in NPK Media, Not exposed to any sound.
NPK-Sine	Cultivated in NPK Media, Exposed to 279.9 Hz Sine Wave
NPK-Square	Cultivated in NPK Media, Exposed to 279.9 Hz Square Wave

The audible sound in this study was 279.9 Hz sine wave and square wave. A frequency generator was used to create the sound, while the music player and speaker were used to adjust the sound level exposure. The music players' volume was 100%, and the speakers' volume was adjusted to level 5. The audible sound was played from 10 AM to 1 PM. Meanwhile, the control group was not exposed to any sound. This PBR system unit operation was done for eight days. Pictures of *Chlorella* DPK-01 culture in all PBR groups were taken every observation day. The sound level in every incubation cabinet row was measured by Sound Level Meter. The sound level (β) was then converted to sound intensity (I) by this following equation, where I_0 is the reference intensity which all types of sound in this world are being compared to[32].

$$\beta = (10) log \frac{I}{I_0} \quad (1)$$

2.5. Cell Density and Growth Rate Measurement

Chlorella DPK-01 cell counting was done every 24 h during the observation period. About 1 mL of culture was taken using a syringe, then shaken by hand for about 10 seconds. Cell counting was done using the direct count method with an Improved Neubauer counting chamber. All counted cells were then multiplied by 2,500 as a microalgal constant number[20].

Growth rate of *Chlorella* DPK-01 could then be counted based on the cell counting result. It was counted by dividing the natural logarithmic value (ln) of cell density on the peak phase (N_t) minus cell density on the first day of inoculation (N_0) to the interval (time) of the cultivation[20].

$$r = \frac{ln(N_t - N_0)}{\triangle t} \quad (2)$$

2.6. Lipid Extraction and Measurement

Lipid percentage of *Chlorella* DPK-01 biomass was measured on the last day of observation (t_8). The extraction was done using Bligh & Dyer (1959) method[33], which was modified to extract lipids from eukaryotic microalgae in this study. *Chlorella* DPK-01 culture from each photobioreactor was taken by syringe for this extraction. The biomass was then centrifuged at 6,000 rpm for 10 minutes. The result of this process are two phases: supernatant and pellets. The supernatant was separated from the pellets; then the pellets were then dried in an oven at 40 ºC for 2.5 h. The dry pellet obtained from each treatment was about was then transferred to a 100 mL Erlenmeyer flask, and 15 mL of chloroform and 30 mL of methanol were added gradually. The mixture was then sonicated for 15 minutes. Sonication was carried out so that the cell walls of *Chlorella* DPK-01 could be broken down. Distilled water and chloroform, 15 mL each, were added gradually to the centrifuged sample. The sample was then sonicated for another 10 minutes, and then transferred into several 15 mL centrifugation tubes. Centrifugation at 6,000 rpm for 10 minutes was done for the second time to obtain a mixture of lipids and chloroform phase. The mixture was then dried in an oven at 40 ºC for 2.5 h. The drying process was done until the chloroform evaporated, and lipid fraction was obtained. After dry lipid was obtained, lipid percentage in the dry biomass of *Chlorella* DPK-01 can be calculated using the following equation[34], DLP is dry lipid weight (in gram) and DCW is dry microalgal cell weight (in gram).

$$\%\ \boldsymbol{Total\ Lipid} = \frac{\boldsymbol{DLP\ (g)}}{\boldsymbol{DCW\ (g)}} \qquad (3)$$

3. Results and Discussion

Data obtained in this study are cell density (cells/mL), growth rate/***r*** (per day), sound intensity (W/m^2), culture color comparison, and lipid percentage (%). All collected data were explained with descriptive statistic method.

3.1. Growth Rate Analysis of *Chlorella* DPK-01

The average cell density of *Chlorella* DPK-01 in all PBR groups was measured every 24 h. The result can be visualized by a growth curve in Fig 4.

Based on Fig 4, *Chlorella* DPK-01 in all PBR groups undergo the logarithmic growth phase from the inoculation day (t_0) until the peak, which was happened on the fifth day after inoculation (t_5). However, each PBR group has a different trend of growth. *Chlorella* DPK-01 in PBR BBM-No Sound (light green line), BBM-Sine (red line), and BBM-Square (purple line) were undergoing cell density decreasement on the first day of inoculation (t_1), when the other groups were not. This might happen because *Chlorella* DPK-01 in those PBRs underwent an adaptation phase.

It is common for microalgae to underwent an adaptation when grown in photobioreactor system. Prokaryotic microalgae *Stanieria* HS-48 underwent an adaptation phase for about 2 days when transferred from Erlenmeyer flask filled with BBM into airlift photobioreactor filled NPK Fertilizer Media, with and without the addition of bean sprout extract[25]. *Chlorella* DPK-01 itself, as eukaryotic microalgae, also underwent an adaptation phase for about 2 days when exposed to different photoperiods[13].

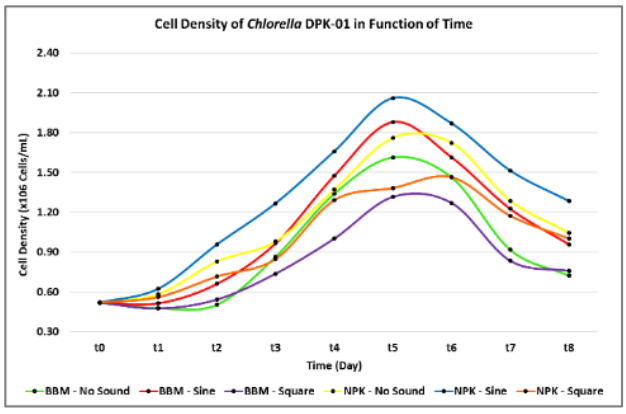

Fig.4: Graph of *Chlorella* DPK-01 Cell Density in Function of Time, based on 8 Days of Observation

Adaptation phase or lag phase takes place before the growth process takes place. It happens when microorganisms are inoculated into new environment to grow, such as new growth media[35], or new photoperiods. The adaptation phase is needed by microorganism cells to produce the growth constituents required by cells to carry out a series of growth processes[36].

The adaptation phase of *Chlorella* DPK-01 in PBR BBM-No Sound (light green line), BBM-Sine (red line), and BBM-Square (purple line) happened, because before being inoculated into these PBR groups, the starter culture of *Chlorella* DPK-01 was prepared in Erlenmeyer flask filled with NPK Fertilizer media. This is confirmed by looking at the graph of *Chlorella* DPK-01 in PBR NPK-No Sound (yellow line), NPK-Sine (blue line), and NPK-Square (orange line). *Chlorella* DPK-01 in these other three groups did not undergo the adaptation phase. On the first day after inoculation (t_1), the cell density value of *Chlorella* DPK-01 in these groups was inclining rapidly.

After t_5, it can be seen that *Chlorella* DPK-01 in all PBR groups undergo a decline in growth, until the end of the observation period (t_8). Cell densities declining of *Chlorella* DPK-01 in all PBR groups after their peak phase indicates that all the nutrients in PBR have been used optimally by the cells to grow. It could happen because the photobioreactor system is equipped with simple aeration. Aeration is known to act as an agitator, which maximizes contact between cells and nutrients in the medium, so that all the nutrients in PBR can be used optimally in a shorter time[17].

The growth rate of *Chlorella* DPK-01 in all PBR groups was measured to strengthen the cell density data. Measurement was done based on Equation 1. The result can be seen in Table 4.

Table 4. Growth Rate of *Chlorella* DPK-01 in each Photobioreactor Group

PBR	Growth Rate (per day)
BBM-No Sound	0.457
BBM-Sine	0.552
BBM-Square	0.165
NPK-No Sound	0.479
NPK-Sine	0.670
NPK-Square	0.419

Based on Table 4, the growth rate of *Chlorella* DPK-01 from the highest to the lowest are *Chlorella* DPK-01 in PBR NPK-Sine (0.670 per day), BBM-Sine (0.552 per day), NPK-No Sound (0.479 per day), BBM-No Sound (0.457 per day), NPK-Square (0.419 per day), and BBM-Square (0.165) respectively. *Chlorella* DPK-01 that have the highest growth rate among the same growth media is exposed to the sine wave. Meanwhile, among the same audible sound exposure, microalga *Chlorella* DPK-01 have the highest growth rate when cultivated in NPK media.

The result of Equation 1 shows that the sine soundwave of 279.9 Hz has 12,6 x 10^{-7} W/m^2 intensity. Energy from that kind of audible sound is considered favorable for the growth of *Chlorella* DPK-01. *Chlorella* DPK-01 was exposed to sine sound waves with a frequency of 279.9 Hz and 12.6 x 10^{-7} W/m^2 compared to those exposed to square

waves and not exposed to any sound. This can happen because sound wave carries energy in the form of vibrations. The right vibration intensity, which is not too low and not too high, is needed in cultivating microalgae, including *Chlorella*, so that cells can achieve maximum growth rate values[37].

In addition to the paragraphs above, growth media also affects the growth of *Chlorella* DPK-01. It might happen because NPK media main compositions are Nitrogen, Phosphorus, and Kalium. Nitrogen and Phosphorus are the two primary nutrients that promote microalgal growth[10], including *Chlorella* DPK-01. Therefore, *Chlorella* DPK-01 cultivated in NPK media have a slightly higher growth rate than what was cultivated in BBM.

However, Schierer-Ray Hare (SRH) test ($\alpha = 0.05$), which is the nonparametric equivalent test to Two Factor ANOVA with Replication shows that the difference in growth rate between *Chlorella* DPK-01 exposed to the same sound wave in different growth media is generally insignificant. Results can be seen in Table 5. The growth rate value of *Chlorella* DPK-01 in PBR NPK-Sine is 0.670 per day, while the growth rate in PBR- BBM-Sine is 0.552 per day. The growth rate of *Chlorella* DPK-01 in PBR NPK-No Sound is 0.479, while the growth rate in PBR BBM-No Sound is 0.457. The only significant difference is the growth rate of *Chlorella* DPK-01 in NPK-Square (0.419 per day) and BBM-Square (0.165 per day). It indicates that *Chlorella* DPK-01 is more influenced by the difference in audible sound than growth media in terms of growth. This phenomenon might happen because physical stimulant does not undergo as many as chemical substances in terms of diffusion. In addition, the growth-stimulating signals in microorganism cells are physical[38].

Table 5. ANOVA Table of *Chlorella* DPK-01 Cell Density Analysis

	SS	*df*	*MS*	*H*	*p-value*	*sig*
Rows	186.8	1		1.683	0.194	no
Columns	266.8	1		2.404	0.121	no
Inter	0.44	1		0.004	0.949	no
		3				
Within	3430	2				
		3	110.9			
Total	3884	5	714			

3.2. Comparison of *Chlorella* DPK-01 Culture Color

A comparison of *Chlorella* DPK-01 was done. It was done to *Chlorella* DPK-01 in all PBR groups on the day of inoculation (t_0), peak phase (t_4), and at the end of observation day (t_8). The standard color that was used in this comparison was Faber Castell standard. The comparison result can be seen in Fig.5.

Based on Fig.5, it is shown that *Chlorella* DPK-01 cultivated in BBM and NPK has the same culture color on t_0, t_4, and t_8. However, when exposed to different audible soundwaves, the difference in *Chlorella* DPK-01 culture color can be seen. *Chlorella* DPK-01 groups that were exposed to a sine wave and not exposed to any sound showed the change of culture color from light green to apple green on t_4. Meanwhile, the culture color of *Chlorella* DPK-01 groups exposed to square wave is still light green from t_0 to t_8.

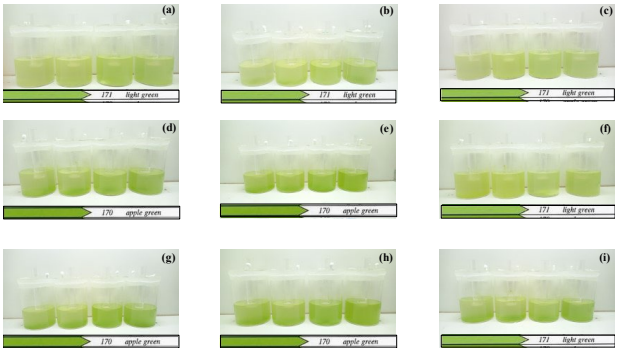

Fig.5: Culture of *Chlorella* DPK-01 (from left to right) in PBR BBM units 1 & 2 and PBR NPK units 1 &2 that were exposed to (a) no sound on t_0, (b) sine wave on t_0, (c) square wave on t_0, (d) no sound on t_4, (e) sine wave on t_4, (f) square wave on t_4, (g) no sound on t_8, (h) sine wave on t_8, and (i) square wave on t_8.

Alongside affecting the growth of *Chlorella* DPK-01, audible sound might also affect the chlorophyll-forming mechanism of *Chlorella* DPK-01, regardless of where it was cultivated. Even though measurement of chlorophyll levels was not carried out using the latest technology, comparing culture color to standard color is still the most simple and efficient way to estimate the chlorophyll level and cell density of microalgae culture. Since the control group (not exposed to any sound) and *Chlorella* DPK-01 in PBRs that were exposed to sine square showed the same trend, it can be surmised that exposure of square wave of 279.9 Hz with an intensity of 6.3×10^{-5} W/m^2 carried some energy that detained chlorophyll forming mechanism of *Chlorella* DPK-01. This phenomenon also might happen because the growth-stimulating signals in microorganism cells are physical[38]. The presence of physical signals, such as audible sound, that do not match the needs of cells to grow or undergo certain physiological processes, can inhibit these processes.

3.3. Analysis of *Chlorella* DPK-01 Lipid Percentage

Lipid percentage can be measured by dividing the Dry Lipid (DLP) by Dry Biomass Weight (DCW). All results are visualized by bar chart as seen in Fig. 6. Based on the graph, *Chlorella* DPK-01 cultured in the same growth media with different audible sound exposure has differences in lipid percentage. In addition to that,

Chlorella DPK-01 cultivated in different growth media with the same audible sound exposure also have differences in lipid percentage.

The lipid percentage of *Chlorella* DPK-01 cultivated in BBM ranges from 42.19% to 51.43%. These results are quite higher compared to previous research where *Chlorella* DPK-01 were cultivated in BBM with exposure to sine soundwave under different photoperiods. The lipid percentage of *Chlorella* DPK-01 in previous research ranges from 11% to 31%[13]. The bar chart shows that *Chlorella* DPK-01, which had the highest lipid content among the BBM group, was cultured in PBR BBM-Square (51.43%). Meanwhile, the lowest among the same growth media group were cultured in PBR BBM-Sine (42.19%).

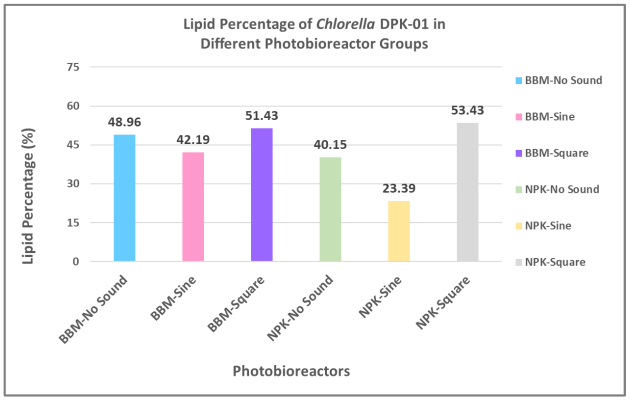

Fig.6: Graph of *Chlorella* DPK-01 Lipid Percentage in Function of Time, based on 8 Days of Observation

The range of lipid percentage of *Chlorella* DPK-01 cultivated in NPK is relatively wider. The bar chart in Fig.6 shows that the lipid percentage of microalga *Chlorella* DPK-01 cultivated in NPK ranges from 23.39% to 53.43%. *Chlorella* DPK-01, which had the highest lipid content among the NPK group, was cultured in PBR NPK-Square (53.43%). Meanwhile, *Chlorella* DPK-01 had the lowest lipid content among the same growth media group when cultivated in NPK-Sine (23.39%).

Compared to previous research, *Chlorella* DPK-01 which had the highest lipid percentage was exposed to audible sound in the light (31%). Meanwhile, the lowest was exposed to audible sound in the dark (11%)[13]. Results of the previous research and this current research are quite similar, in which the treatment that stimulate microalga *Chlorella* DPK-01 to produce the highest lipid was the same as the one that hinder the growth rate. However, the lipid percentage of *Chlorella* DPK-01 in this current research are higher. It indicates that audible sound can stimulate lipid production better than photoperiods regulation.

Both in BBM and NPK, *Chlorella* DPK-01 has the highest lipid content when exposed to square soundwave and the lowest lipid content when exposed to sine soundwave. It might happen because the maximum biomass growth of Chlorophyta cells, including *Chlorella*, occurs in optimum or favorable conditions for its growth. Meanwhile, lipid accumulation takes place in conditions that cause cell stress[39]. Based on the result of Equation 1, it is known that a square sound wave of 279.9 Hz with an intensity of 6.3 x 10^{-5} W/m^2 generates vibration that is too strong for the cell. The vibration was then responded to as a disturbance by the mechano-sensitive channel of the cell. The sound is thought to have too much energy for *Chlorella* DPK-01. Thus, *Chlorella* DPK-01 undergoes hydrodynamic stress and diverts potential energy that should be used for growth into lipid synthesis.

Different phenomena can be seen by comparing the lipid percentage of *Chlorella* DPK-01 cultivated in different growth media with the same audible sound exposure. Based on Fig.6, *Chlorella* DPK-01 exposed to a sine soundwave has a higher lipid percentage when cultivated in BBM (42.19%) than NPK (23.39%). *Chlorella* DPK-01, which was not exposed to any sound, also has higher lipid percentage when cultivated in BBM (48.96%) than NPK (40.15%). Meanwhile, when exposed to a square soundwave, *Chlorella* DPK-01 has a higher lipid percentage when cultivated in BBM (48.96%) than NPK (40.15%).

Based on the data above, it is known that the lipid percentage of *Chlorella* DPK-01 is also affected by its growth media. In this study, *Chlorella* DPK-01 cultivated in BBM generally has higher lipid content than cultivated in NPK. This might happen because BBM has relatively lower nitrogen (N) and phosphate (P) concentrations. This condition might increase the lipid production in *Chlorella*[40], including *Chlorella* DPK-01. Meanwhile, NPK Media's main compositions are Nitrogen and Phosphorus[31], which are favorable to the growth of photosynthetic microorganism[10], including microalga *Chlorella* DPK-01.

4. Conclusion

By cultivating microalgae in economic media such as NPK Fertilizer Media with a non-destructive physical stimulant such as an audible sound wave, humans are one step closer to achieve Sustainable Development Goal (SDGs) number seven, which is affordable and clean energy. Data obtained from this research can be used as initial information for bioprospecting activities of Indonesian indigenous microalgae, *Chlorella* DPK-01, as a biofuel raw material. However, improvement on further research is still needed.

It is suggested to do another study on the effect of exposure to sine and square sound waves with a frequency of 279.9 Hz to the growth of *Chlorella* DPK-01 in a photobioreactor system with a larger total capacity. In addition, it is also suggested to study the effect of exposure to sine and square sound waves on biomolecule synthesis of *Chlorella* DPK-01, such as proteins and carbohydrates. These things need to be done so that utilization of *Chlorella* DPK-01 for industrial scale can take place more optimally.

Acknowledgements

This study benefited from the support of Universitas Indonesia, by Hibah Publikasi Terindeks Internasional (PUTI) Pascasarjana (Batch 1), for the 2022—2023 Fiscal Year to Dr. Nining Betawati Prihantini, M.Sc., Grant no.NKB-285/UN2.RST/HKP.05.00/2022.

Nomenclature

DPK-01	Strain from Depok (DPK) number 1 (01)
PBR	Photobioreactor
No Sound	Not exposed to any sound
BBM	Bold's Basal Media
NPK	Nitrogen, Phosphorus, Kalium
β	Sound Level
I	Sound Intensity
I_0	Reference Intensity of Sound
W/m^2	Sound Intensity Unit
dB	Decibels (sound level unit)
d	Cell density (cells/mL)
r	Specific growth rate
ln	Natural logarithm
N_t	Cell density in a specific time
N_0	Cell density on the first day of inoculation
min	Minute
h	Hour
$\triangle t$	The interval of time (day)
t_n	Day of observation Period
pH	Power of Hydrogen
DLP	Dry Lipid Weight (g)
DCW	Dry Biomass or Cell Weight (g)

References

1) E. Kolbert, "The sixth extinction: An unnatural history," New York: Henry Holt and Company. (2022). https://books.google.co.id/books/about/The_Sixth_Extinction.html?id=Ra9RAQAAQBAJ&redir_esc=y
2) United States Environmental Protection Agency (US EPA). 2022. *Health and Environmental Effects of Ozone Layer Depletion.* https://www.epa.gov/ozone-layer-protection/health-and-environmental-effects-ozone-layer-depletion (accessed September 27, 2022)
3) E. Stephens, I. L. Ross, J. H. Mussgung, L. D. Wagner, M. A. Borowitzka, C. Posten. O. Kruse, and B. Hankamer. "Future prospects of microalgal biofuel production systems." *Trends in Plant Science* (15)10: 554—564. (2010). doi:10.1016/j.tplants.2010.06.003
4) R. B. Carpio, R. L. De Leon, & M. R. Martinez-Goss, "Growth, Lipid Content, & Lipid Profile of the Green Alga, *Chlorella vulgaris* Beij., Under Different Concentrations of Fe & CO_2, *Journal of Engineering Science & Technology,* Special Issue on SOMCHE 2014 & RSCE 2014 Conference, 19—30. (2015).
5) S. R., Ardiansyah, Nasruddin, W. Sjamsuridzal, W. Wardhana, and N. B. Prihantini, "Analysis of Carbon Dioxide Solubility Increasement Caused by Baffle Diameter Variation in Airlift Photobioreactor to Growth Rate of *Synechococcus* HS-9 Biomass," *Evergreen*, 8(4), 879–884. (2021). doi: 10.5109/4742136.
6) A. M. Orlando, Nasruddin, W. Sjamsuridzal, W. Wardhana, and N. B. Prihantini, "Biomass Production and Lipid Content of *Leptolyngbya* HS-16 cultivated in Bubble Column Photobioreactors (BCPBR) with Air Bubbler Pore Variation," *Evergreen*, (8)4, 885–889. (2021). doi: 10.5109/4742137.
7) T. Mathimani, M. Sekar, S. Shanmugam, J. S. M. Sabir, N. T. L. Chi, and A. Pugazhendhi, "Relative abundance of lipid types among *Chlorella* sp. and *Scenedesmus* sp. and ameliorating homogeneous acid catalytic conditions using central composite design (CCD) for maximizing fatty acid methyl ester yield," *Science of The Total Environment,* 771, 144700. (2021). doi: 10.1016/j.scitotenv.2020.144700.
8) A. Demirbas and M. Fatih Demirbas, "Importance of algae oil as a source of biodiesel," *Energy Conversion and Management*, 52(1), 163–170, (2011). doi: 10.1016/j.enconman.2010.06.055.
9) Heimann, K. & R. Huerlimann. "Microalgal Classification: Major Classes and Genera of Commercial Microalgal Species" in S.-W. Kim (Ed.) *Handbook of Marine Microalgae: Biotechnology Advances*. London: Elsevier Science & Technology. (2015).
10) Barsanti, L. & P. Gualtieri, "Algae: Anatomy, Biochemistry, and Biotechnology" 2nd Ed. Pisa, CRC Press. (2014).
11) F. Chu, J. Cheng, X. Zhang, Q. Ye, and J. Zhou, "Enhancing lipid production in microalgae *Chlorella* PY-ZU1 with phosphorus excess and nitrogen starvation under 15% CO2 in a continuous two-step cultivation process," *Chemical Engineering Journal*, 375, 121912. (2019). doi: 10.1016/j.cej.2019.121912.
12) A. Rahman, N. B. Prihantini, and Nasruddin, "Biomass Production and Synthesis of Biodiesel from Microalgae *Synechococcus* HS-9 (Cyanobacteria) Cultivated Using Bubble Column Photobioreactors," *Evergreen*, 7(4), 564–570. (2020). doi: 10.5109/4150507.
13) R. M. N. Tambunan, Y. A. Santoso, S. Soekirno, Nasruddin, and N. B. Prihantini, "Difference in Time of Audible Sound Exposure to *Chlorella* DPK-01 in Tubular Photobioreactors: A Strategy to Improve Photobioreactor System," *Journal of Advanced Research in Fluid Mechanics and Thermal Sciences*, 81(2), 82–88, (2021). doi: 10.37934/arfmts.81.2.8288.

14) S. R. Ardiansyah, A. M. Orlando, A. Rahman, N. B. Prihantini, and Nasruddin, "Tubular Photobioreactor: A Preliminary Experiment Using *Synechococcus* sp. (Cyanobacteria) Cultivated in NPK Media for Biomass Production as Biofuel Feedstock," *Evergreen*, 6(2), 157–161. (2019). doi: 10.5109/2321011.
15) H. I. Muhammad, A. Rahman, Nining Betawati Prihantini, Deendarlianto, and Nasruddin, "The Application of Poly-dispersed Flow on Rectangular Airlift Photobioreactor Mixing Performance," *Evergreen*, 7(4), 571–579. (2020). doi: 10.5109/4150508.
16) C. U. Aguoru & P. O. Obike, "Content &Composition of Lipid Produced by *Chlorella vulgaris* for Biodiesel Production," *Advances in Life Science & Technology*, 36, 97–100. (2013).
17) ..Ardiansyah, S. R., A. M. Orlando., A. Rahman., N. B. Prihantini & Nasruddin, "Effect of Aeration in Simple Photobioreactor System for Biomass Production of *Synechococcus* (Cyanobacteria) HS-7 and HS-9 as Biofuel Feedstock," *E3S Web of Conferences* 67, 02006, (2018). doi: 10.1051/e3sconf/20186702006.
18) M. Christwardana and H. Hadiyanto, "The Effects of Audible Sound for Enhancing the Growth Rate of Microalgae *Haematococcus pluvialis* in Vegetative Stage," *HAYATI Journal of Biosciences*, 24(3), 149–155. (2017). doi: 10.1016/j.hjb.2017.08.009.
19) N. W. K. E. Yanti, A. T. Rochmanto, R. Damacena, S. Handayani, Nasruddin, and N. B. Prihantini, "Utilization of local fertilizer and spring water combination for growth medium of *Stanieria* HS-48 (cyanobacteria) as a biofuel feedstock," International Conference on Trends in Material Science And inventive Materials. (2020). doi: 10.1063/5.0013767.
20) R. A. Andersen. Algal Culturing Techniques. London: Elsevier Science. (2005). https://www.google.co.id/books/edition/Algal_Culturing_Techniques/9NADUHyFZaEC?hl=id&gbpv=0
21) S.-Y. Hsia and S.-K. Yang. "Enhancing Algal Growth by Stimulation with LED Lighting and Ultrasound" in *Journal of Nanomaterials* 2015:531325, 1–11. (2015).
22) S. Slocombe and J. Benemann, Eds., "Microalgal Production for Biomass and High-Value Products," (2016). doi: 10.1201/b19464.
23) J. D. Putra, A. Rahman, N. B. Prihantini, Deendarlianto, and Nasruddin, "Bubble Coalescence on Photobioreactor Bubble Columns by Using Horizontal Baffle for Microalgae," *Evergreen*, 8(4). 861–865. (2021). doi: 10.5109/4742133.
24) N. B. Prihantini, N. Rakhmayanti, S. Handayani, W. Sjamsuridzal, W. Wardhana, and Nasruddin, "Biomass Production of Indonesian Indigenous Leptolyngbya Strain on NPK Fertilizer Medium and its Potential as a Source of Biofuel," *Evergreen*, 7(4), 593–601. (2020). doi: 10.5109/4150512.
25) N. W. K. E.Yanti, A. T. Rochmanto, S. Handayani, Nasruddin, and N. B. Prihantini, "Optimization of NPK Medium with the Addition of Mung Bean Sprout Extract for Biomass and Lipid Production of *Stanieria* HS-48 as a Potential Biofuel," *Journal of Advanced Research in Fluid Mechanics and Thermal Sciences*, 82(1), 77–84, (2021). doi: 10.37934/arfmts.82.1.7784.
26) R. Damacena, A. T. Rochmanto, N. W. K. E. Yanti, S. Handayani, Nasruddin, and N. B. Prihantini, "Biomass production of indigenous strain *Nostoc* HS-20 in local NPK fertilizer solved in spring water of Lab. Alam FMIPA UI as alternative media for biofuel feedstock," International Conference on Trends in Material Science and Inventive Materials, (2020). doi: 10.1063/5.0013768.
27) R. M. N. Tambunan, Y. A. Santoso, S. Soekirno, Nasruddin, and N. B. Prihantini, "Cultivation of *Chlorella* DPK-01 using audible sound (music) as a potential strategy for improving photobioreactor system." *International Conference on Trends in Material Science and Inventive Materials*. (2020). doi:10.1063/5.0013759
28) H. Li, J. Wang, E. Zhang, Y. Shao, L. Yang, B. Yang, Y. Tan, and T. Gao, "Cumulative Effects of Physical, Chemical, and Biological Measures on Algae Growth Inhibition," *Water*, 14(6), 877. (2022). doi: 10.3390/w14060877.
29) Y. A. Santoso, R. M. N. Tambunan, S. Soekirno, Nasruddin, and N. B. Prihantini. "Sound Wave Exposure as a Strategy for Improving the Tubular Photobioreactor for Cultivating *Synechococcus* HS-9 as Biofuel Feedstock under Different Photoperiods." *International Journal of Technology*, (11)7, 1406. (2020).
30) National Institute for Environmental Penelitianes (=NIES). *Media list: Microbial Culture Collection at the National Institute for Environmental Penelitianes (NIES Collection).* (2001). http://mcc.nies.go.jp/medium/ja/ bg11.pdf (accessed November 28, 2022).
31) Grow More. *Water Soluble Fertilizer.* (2013). http://www.growmore.com/products/type/water-soluble-fertilizer.html (accessed November 28, 2022).
32) J. Walker et al. *Fundamental of Physics 10th ed.* (John Wiley & Sons, Inc., New York, 2014). (2016).
33) E.G. Bligh & W.J. Dyer, "A rapid method of total lipid extraction and purification" in *Canadian Journal of Biochemistry and Physiology*. 37, 911–917. (1959).
34) Y. Li, M. Horsman, B. Wang, & N. Wu, "Effect of nitrogen sources on cell growth and lipid accumulation of green algae *Neochloris oleoabundanss*," *Applied Microbiol Biotechnology*. 81, 629–636. (2008).
35) M. Madigan, J. Martino, K. Bender, D. Buckley, D. Stahl. *Brock Biology of Microorganisms*. 16th ed. New York: Pearson Education. (2020).

36) G. J. Tortora, B. R. Funke, & C. L. Case, *Microbiology: An Introduction* (13th ed.). San Fransisco: Pearson Benjamin Cummings. (2019).
37) D. A. Isiya & M. Sani, "Effects of Different Stirring Rates on the *Chlorella vulgaris* Growth for Wastewater Treatment Systems," *International Journal of Engineering Applied Sciences and Technology,* 4(11), 454–460. (2015).
38) G. Reguera, "When microbial conversations get physical," *Trends in Microbiology*, 19(3), 105–113. (2011). doi: 10.1016/j.tim.2010.12.007.
39) O. Savchenko, J. Xing, X. Yang, Q. Gu, M. Shaheen, M. Huang, X. Yu, R. Burrell, P. Patra, and J. Chen, "Algal Cell Response to Pulsed Waved Stimulation and Its Application to Increase Algal Lipid Production," *Scientific Reports*, 7(1), (2017). doi: 10.1038/srep42003.
40) B. S. Adam, B. M. Abubakar, L. Garba, and I. Hassan, "Effect of Growth Media and pH on Microalgal Biomass of *Chlorella vulgaris* for Biodiesel Production," *Bioremediation Science and Technology Research*, 10(1), 22–25, (2022). Doi: 10.54987/bstr.v10i1.684.