The enhancement of growth, biomass production and lipid productivity of microalgae *Choricystis* sp. LBB13-AL045 by the addition of hot water extract of its dried biomass

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Abstract. This study sought to evaluate the influence of hot water extract addition on cell growth, biomass production and lipid productivity during cultivation of microalgae *Choricystis* sp. LBB13-AL045 under different light intensity. The cellular growth of microalgae in terms of growth rate and produced biomass increased significantly with the addition of hot water extract into the microalgal culture under both low- and high-light intensity (3000 and 30000 lux, respectively), demonstrating that hot water extract of microalgal biomass positively interferes with the metabolism of microalgae and the production of biomass. Thus, microalgal lipid productivity was eventually increased almost three times when hot water extract (7.5 mg/mL) was employed in the microalgal cultivation with high light intensity. Hot water extract was prepared by the autoclaving varied concentration of dried microalgal biomass (2.5-7.5 mg/mL) in water. The cultivation of *Choricystis* sp. LBB13-AL045 showed the highest net increase of biomass production (975.05 mg) at hot water extract concentration of 7.5 mg/mL and light intensity of 30000 lux. The current investigation demonstrates that hot water extract of *Choricystis*’s dried biomass accelerate its growth and induce its lipid productivity for its application in biodiesel production.

Keywords: Hot water extract, growth, biomass production, lipid productivity, *Choricystis*

1. Introduction

Hot water extraction (HWE) is an extraction technique that uses liquid water as extractant (extraction solvent) at temperatures above the atmospheric boiling point of water (100°C, 0.1 MPa), but below the critical point of water (374°C, 22.1 MPa). Liquid water at elevated temperature is a solvent that enables faster mass transfer and improved wetting of the sample due to higher diffusivity and lower viscosity and surface tension [1]. At a certain temperature and applied pressure, the polarity of water would become similar to that of some common alcohols, such as methanol and ethanol [2]. In particular, the changes in temperature could affect the physicochemical properties of water and can also cause thermally labile compounds to decompose [3].

HWE is one of the most interesting techniques to isolates bioactives from plants, including microalgae, and other complex samples [1]. Hot water extract from microalgal biomass is rich in nutrients and contains amino acids, peptides, proteins, vitamins, polysaccharides, sugars and nucleic acids [4, 5]. Therefore, hot water extract from certain microalgae, such as *Chlorella pyrenoidosa*, was used as a supplement to support the healthy growth of living cells [6]. In the same manner, such
technique of adding microalgal hot water extract into the microalgal culture itself might be able to induce the microalgal’s cells growth and also its biomass production. Thus, the technique could be adapted to increase the lipid productivity value of biodiesel-producing microalgal strain, namely *Choricystis* sp. LBB13-AL045 [7], since such parameter was the determining factor for microalgal biomass utilization as biodiesel feedstock [8]. On the other hand, light intensity was known as a crucial factor for microalgal cultivation [9]. Therefore, its application in algal cultivation must have a synergistic effect with the other factor [10], including the hot water extract that was added into the microalgal culture, in inducing microalgal growth and biomass production.

This research was aiming at evaluating the influence of microalgal hot water extract addition on cell growth, biomass production, and lipid productivity during cultivation of microalgae *Choricystis* sp. LBB13-AL045 under different light intensity.

2. Methods

2.1. Microalgal cultivation

The culture of *Choricystis* sp. LBB13-AL045 was maintained and grown in the modified AF6 medium [7] containing (mM) 5.95 NaHCO$_3$; 1.65 NaNO$_3$; 0.47 (NH$_4$)$_2$SO$_4$; 0.12 MgSO$_4$·7H$_2$O; 0.074 KH$_2$PO$_4$; 0.029 K$_2$HPO$_4$; 0.008 C$_6$H$_5$FeO$_7$; and 0.008 C$_6$H$_8$O$_7$. Cells were grown in a 600-mL bottle containing 500 mL of the microalgae culture with aeration and under continuous illumination with low- and high-light intensity. In the low-light intensity (3000 lux) cultivation, a neon lamp was used to illuminate the microalgal culture. While in the high-light intensity (30000 lux) cultivation, white LED flood-light (50 Watt) was used as a light source.

2.2. Production of dried powder microalgal biomass

*Choricystis* sp. LBB13-AL045 was cultivated in 100 L aquarium with continuous bubbling aeration for 7 days at outdoor. After 7 days, the microalgae were harvested by using flocculation method with the addition of magnesium ions under alkaline condition [11]. The microalgal paste was then dried indirectly under the illumination of sunlight. After drying, microalgal biomass was then ground by using grinder until it turned into powder. This powder form of microalgal biomass was subsequently used as feedstock in the production of hot water extract.

2.3. Hot water extract preparation

The biomass of *Choricystis* sp. LBB13-AL045 in the dried powder form was used as the feedstock of hot water extract. Hot water extract was prepared by autoclaving (at 121°C for 15 minutes) dried microalgal biomass [12] soaked in the aqua distillate (20 ml) inside the glass bottle (100 mL). Hot water extract with varied concentration of dried microalgal biomass (2.5; 3.75; 5 and 7.5 mg/ml) was prepared. After cooling, the hot water extract of microalgal biomass was filtered through sterile filter paper and then was added to the microalgal culture for its cultivation.

2.4. Microalgal cultivation with hot water extract

20 mL of filtered hot water extract was added to the microalgal culture before cultivation. The microalgae culture for both conditions of light intensity (3000 and 30000 lux) was cultivated for 8 days. The light intensity was measured at the outer-surface of the bottle (in the middle of the culture’s height) by using a light meter. The laying distance of the microalgal culture from the light source corresponds to its value of light intensity used. Every light-intensity treatment of cultivation was conducted in duplicate.

2.5. Growth properties of microalgae

The growth of microalgae *Choricystis* sp. LBB13-AL045 was determined by using cell counting (haemocytometer) under light microscope. The specific growth rate and doubling time of *Choricystis*
sp. LBB13-AL045 in each treatment of different concentration of hot water extract was calculated based on the equation as follows:

\[ \mu = \ln \left( \frac{C_y}{C_x} \right) / (t_y - t_x) \] .......................... (1)

\[ t_d = \ln 2 / \mu \] .......................... (2)

\( C_x \): the cell amount at the start \((t_x)\) of the logarithmic growth phase

\( C_y \): the cell amount at the end \((t_y)\) of the logarithmic growth phase

Biomass productivity \((B_p)\) was determined as the dry biomass produced during the exponential growth phase and was stated in milligram per liter per day \((\text{mg/L/day})\). Lipid productivity \((L_p)\) was calculated according to the equation as recommended by Nascimento et al. [8]:

\[ L_p = B_p \times L_c \] .......................... (3)

\( L_p \): Lipid productivity \((\text{mg/L/day})\)

\( B_p \): Biomass productivity \((\text{mg/L/day})\)

\( L_c \): Lipid content \((\%\)

2.6. Microalgal lipid extraction

Microalgal lipid was extracted by adapting the modified method from Rykebosch et al. [13]. The extracted microalgal lipid was subsequently mentioned as lipid content and was reported as a percentage of the total biomass \((\% \text{ dry weight})\). Briefly, chloroform: methanol = 1:1 were used as solvent in the microalgal lipid extraction. 6 mL of solvent was added to 50 mg microalgal biomass, and the tube was vortex mixed for 30 s. 2 mL of solvent and water were then added and the tube was vortex mixed again and subsequently centrifuged at 2000 rpm for 10 min. The aqueous layer was removed and the solvent layer was transferred into the clear tube. The remaining-microalgal biomass was re-extracted with 4 mL solvent. The re-extraction of lipid was repeated until the remaining-microalgal biomass turned to be colorless. The solvent was removed by letting it evaporated in the open air, and the lipid content was determined gravimetrically. The extraction was performed in triplicate.

2.7. Statistical analysis

To determine the significant difference among groups \((p < 0.05)\), all average values of HWE addition-treatments were analyzed against the control \((\text{zero HWE addition})\) employing Analysis of Variance test \((\text{ANOVA})\) by using Microsoft Excel Software.

3. Results and discussion

3.1. Effect of hot water extract on microalgal growth

The growth curves of Choricystis sp. LBB13-AL045 in the modified-AF6 medium with different concentrations of hot water extract and light intensity is shown in figure 1 and 2. Microalgae cultures were grown for 8 days under the illumination of low- \((3000 \text{ lux})\) and high-light intensity \((30000 \text{ lux})\) with the addition of varied hot water extract concentration, ranging from 2.5 to 7.5 mg/ml. In low light intensity cultivation, the influence of hot water extract treatment, especially for the treatment of 3.75 and 7.5 mg/ml concentration, in increasing Choricystis’ growth start to be seen within initial 48 h. The difference in growth between the treatment and control were conspicuously seen start at the fourth day of cultivation and onwards, except for the treatment of 2.5 mg/ml \((\text{figure 1})\). The microalgal culture of 2.5 mg/ml treatment showed a similar pattern of growth with the control until the sixth day of cultivation \((\text{figure 1})\), but the microalgal growth of control treatment started to decrease compared to
the 2.5 mg/ml treatment in the next 48 h of cultivation. However, based on the growth curve (figure 1), the initial concentration of hot water extract that starts to give a significant influence on Choricystis’ growth in low-light intensity cultivation was 3.75 mg/ml.

The high-light intensity cultivation demonstrated different results regarding hot water extract concentration and its correlation to the given effect on Choricystis’ growth. Based on its growth curve (figure 2), the increase of Choricystis’ growth given by all treatments of hot water extract concentration could be distinctly seen compared to control. The best enhancement of Choricystis’ growth in high-light intensity cultivation were demonstrated by the treatment of 7.5 mg/ml concentration. Such treatment gave a positive impact on microalgal growth compared to control, namely shorter time to reach initial stationary growth phase and higher biomass production. Those results would eventually conduce to the enhancement of lipid productivity, which is an essential characteristic for choosing microalgal species as biodiesel feedstock [14]. Based on the growth curve in figure 1 and 2, it was known that optimum concentrations of hot water extract between low- (3.75 mg/ml) and high-light (7.5 mg/ml) intensity cultivation for enhancing Choricystis’ growth were different.

![Figure 1](image1.png)

**Figure 1.** Growth curve of Choricystis sp. LBB13-AL045 with the addition of varied concentration microalgal hot water extract illuminated at 3000 lux of light intensity.

![Figure 2](image2.png)

**Figure 2.** Growth curve of Choricystis sp. LBB13-AL045 with the addition of varied concentration microalgal hot water extract illuminated at 30000 lux of light intensity.
The measurement of growth kinetics, i.e., specific growth rate and doubling time, as seen in Table 1, demonstrated that the highest concentration of microalgal hot water extract (7.5 mg/ml) used in this study was the optimum concentration for stimulating the division of microalgal cells per units of time. This result still opens an opportunity to conduct an examination of hot water extract concentration higher than 7.5 mg/ml in order to search optimum concentration that could more stimulate the division of Choricystis' cells. In general, Choricystis' growth demonstrated better performance when they were cultivated under high-light over low-light intensity. This result was coherent with our prior study [7] that specifically studied the influence of light intensity on the growth of the same strain (Choricystis sp. LBB13-AL045). Another study used another microalgal genus (Scenedesmus and Nannochloropsis) also demonstrated the same results that high light intensity cultivation tends to conduce to better growth performance to microalgal culture [15, 16].

### Table 1. Specific growth rate and doubling time of Choricystis sp. LBB13-AL045 under the effect of varied hot water extract concentration and different light intensity.

| Hot water extract concentration (mg/mL) | Specific growth rate (day⁻¹) | Doubling time (day) |
|----------------------------------------|-------------------------------|---------------------|
|                                        | 3000 lux                      | 30000 lux           | 3000 lux          | 30000 lux          |
| Control                               | 0.075 ± 0.000                 | 0.484 ± 0.001       | 9.21 ± 0.03       | 1.43 ± 0.01        |
| 2.5                                    | 0.168 ± 0.004                 | 0.598 ± 0.013       | 4.13 ± 0.10       | 1.16 ± 0.03        |
| 3.75                                   | 0.287 ± 0.000                 | 0.748 ± 0.007       | 2.41 ± 0.00       | 0.93 ± 0.01        |
| 5                                      | 0.325 ± 0.005                 | 0.883 ± 0.003       | 2.13 ± 0.04       | 0.79 ± 0.00        |
| 7.5                                    | 0.406 ± 0.012                 | 1.035 ± 0.011       | 1.71 ± 0.05       | 0.67 ± 0.01        |

3.2. Effect of HWE on microalgal biomass production

Table 2 summarizes Choricystis' biomass production and biomass productivity after 8-day-cultivation under the influence of hot water extract addition in varied concentrations and two conditions of low- and high-light intensity. In term of biomass production, microalgal cultivation in high-light intensity condition could produce more microalgal biomass than in the low-intensity one. It was demonstrated, particularly in high-light intensity cultivation, that the higher the concentration of hot water extract the more microalgal biomass would be produced. While in low-light intensity cultivation, microalgal biomass would optimally be produced if the hot water extract concentration was 3.75 mg/ml. The same pattern of results was also applied for biomass productivity. The concentration of 3.75 mg/ml was an optimum concentration for the generation of microalgal biomass when it was cultivated under the illumination of low-light intensity, and in high-light intensity cultivation, the higher the concentration, the faster the microalgal biomass would be generated.

### Table 2. Biomass production and biomass productivity of Choricystis sp. LBB13-AL045 under the effect of varied hot water extract concentration and different light intensity

| Hot water extract concentration (mg/mL) | Biomass production (mg/L) | Biomass productivity (mg/L/day) |
|----------------------------------------|---------------------------|---------------------------------|
|                                        | 3000 lux                  | 30000 lux                       | 3000 lux          | 30000 lux          |
| Control                                | 448.38 ± 4.52             | 918.93 ± 3.66                   | 55.77 ± 0.28      | 115.09 ± 0.23      |
| 2.5                                    | 798.17 ± 5.70             | 971.18 ± 1.72                   | 100.13 ± 0.36     | 121.51 ± 0.11      |
| 3.75                                   | 1099.68 ± 3.55            | 1057.63 ± 3.01                  | 137.68 ± 0.22     | 132.02 ± 0.19      |
| 5                                      | 929.46 ± 4.95             | 1573.12 ± 4.95                  | 115.87 ± 0.31     | 196.33 ± 0.31      |
| 7.5                                    | 964.52 ± 1.51             | 2043.98 ± 4.19                  | 120.47 ± 0.09     | 255.24 ± 0.26      |
In both conditions of light intensity, all treatments of the addition of hot water extract were superior over the control conditions in terms of microalgal biomass production and biomass productivity. These results asserted that the addition of hot water extract into microalgal culture could significantly enhance biomass production and biomass productivity compared to control. This result of hot water extract treatment was similar with the treatment of CO$_2$ supplementation into the microalgal culture which also could enhance microalgal biomass productivity, as well as could realize the bioenergy production from microalgae to be feasible [17]. Thus, it could also be considered that microalgal hot water extract possesses high potency in realizing a techno-economically feasible conversion process of microalgal biomass into bioenergy, just like CO$_2$ supplementation. Such enhancements might be caused by the presence of phytohormone contained in the hot water extract as it had been proven by previous studies [18,19] that phytohormones, such as indole-3-acetic acid (IAA), could induce positive stimulatory effects on the growth, biomass production, as well as on lipid biosynthesis of microalgae *Scenedesmus quadricauda* and *Scenedesmus obliquus*. The presence of phytohormone (IAA or auxin) might alter microalgae membrane permeability since the hormone was exhibited to be capable of activating proton pump ATPase in the plasma membrane [20, 21] in vascular plants.

From table 3 which summarizes the net increase of microalgal biomass production for each treatment compared to the biomass produced by the control, it can be seen that the use of hot water extract with low concentration (2.5 to 3.75 mg/ml) would be more effective in increasing microalgal biomass production if the cultivations were carried out under the illumination of low-light instead of high-light intensity. However, if the microalgal cultivations were managed under the illumination of high light intensity, then the use of hot water extract with high concentration (5 to 7.5 mg/ml or possibly higher) would be more preferred in order to achieve the higher net increase of microalgal biomass production. These results demonstrated that the concentration of hot water extract would determine which type of light intensity should be used for microalgae cultivation to obtain optimum net-increase of produced biomass, such factors of microalgal growth would influence one to another.

Our results were also supported by other study [22] who stated that microalgae growth is determined by the combined effects of many factors, including light intensity.

**Table 3.** The net increase of microalgal biomass production for each treatment of hot water extract addition and light intensity compared to the control’s biomass production.

| Hot water extract concentration (mg/mL) | 3000 lux | 30000 lux |
|----------------------------------------|----------|-----------|
| 2.5                                    | 299.79 ± 1.18 | 2.25 ± 1.94 |
| 3.75                                   | 576.30 ± 0.97 | 63.70 ± 0.65 |
| 5                                      | 381.08 ± 0.43 | 554.19 ± 1.29 |
| 7.5                                    | 366.14 ± 3.01 | 975.05 ± 0.53 |

**3.3. Effect of HWE on microalgal lipid productivity**

As shown in table 4, the lipid contents of HWE addition-treatment were slightly different compared to control under both light intensity conditions. However, statistical analysis between treatment groups by using ANOVA showed that the lipid contents were significantly different. The highest enhancement of lipid accumulation has occurred when HWE-concentration of 7.5 mg/mL was added into the microalgal culture, and when high light intensity was applied in the experiment. The light intensity had been proven as a critical factor in affecting lipid accumulation in *Choricystis* sp LBB13-AL045 [7], and also in many species of microalgae [23-25]. On the other hand, lipid productivities of HWE-additions treatment were quite superior over the control. In HWE concentration of 7.5 mg/mL, under both light intensity exposure, the microalgal lipid productivity was increased by almost threefold when comparing to the control condition. According previous study [8], lipid productivity is calculated by multiplying biomass productivity by a percentage of lipid content per dry-weight basis, or, on the other words, lipid productivity is determined by two factors, which are yield of biomass
production and also lipid content percentage. Thus, the actual role of microalgal biomass HWE towards microalgal culture of Choricystis sp. LBB13-AL045 was increasing the growth of microalgal cells so that its biomass would significantly be produced and its lipid productivity would eventually be increased. Lipid productivity of all HWE addition-treatments was higher than that of the control, and, generally, the higher the HWE-concentration, the higher the enhancement of lipid productivity would be.

Table 4. Lipid content and lipid productivity of Choricystis sp. LBB13-AL045 under the effect of varied hot water extract concentration and different light intensity.

| Hot water extract concentration (mg/mL) | Lipid content (%) | Lipid productivity (mg/L/day) |
|----------------------------------------|-------------------|-------------------------------|
|                                        | 3000 lux  | 30000 lux | 3000 lux | 30000 lux |
| Control                                | 22.06 ± 0.61 | 22.74 ± 0.77 | 12.30 ± 0.40 | 26.17 ± 0.94 |
| 2.5                                    | 28.13 ± 0.80 | 29.49 ± 0.08 | 28.16 ± 0.90 | 35.83 ± 0.12 |
| 3.75                                   | 22.11 ± 0.03 | 27.32 ± 0.11 | 30.44 ± 0.01 | 36.07 ± 0.20 |
| 5                                      | 25.91 ± 0.15 | 21.50 ± 0.27 | 30.02 ± 0.25 | 42.21 ± 0.60 |
| 7.5                                    | 27.37 ± 0.36 | 30.16 ± 0.20 | 32.97 ± 0.40 | 76.98 ± 0.59 |

4. Conclusion
We have applied HWE of Choricystis sp. LBB13-AL045 biomass into its culture to enhance the growth, biomass production, and lipid productivity under the influence of low- and high-light intensity. All treatments of HWE addition in microalgal cultivation had positive stimulatory effects on Choricystis sp. LBB13-AL045 cell growth, biomass production, and lipid productivity. To the extent to our knowledge, this is the first report on the use of HWE of (dried) microalgal biomass in the microalgal culture itself to enhance its growth, biomass production, as well as its lipid productivity. Our research recommends that a Hot Water Extract from an algal species could be used in its cultivation to enhance the growth and lipid productivity so that the process of biodiesel production could be more techno-economically feasible.

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