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Growth and secondary metabolites content of chloroform extract of *Chlorella* sp. and *Chlorella sorokiniana* cultured on chicken *broiler* waste media

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Abstract. *Susanty D, Oksari AA. 2020. Growth and secondary metabolites content of chloroform extract of* Chlorella *sp. and* Chlorella sorokiniana *cultured on chicken broiler waste media. Nusantara Bioscience 12: 28-32.* Intensive chicken broiler farms create waste in the environment. Chicken Broiler Waste (CBW) was used as culture media for *Chlorella* sp. InaCC M39 and *Chlorella sorokiniana* InaCC M38 at various concentrations (2, 4, 6, 8, and 10%), and compared to AF6 media. The growth of *Chlorella* was observed every 48 hours for 10 days by counting the number of cells using a hemocytometer. The chloroform extract of *Chlorella* sp. and *C. sorokiniana* were analyzed for their phytochemical content to determine the presence of alkaloids, flavonoids, saponin, steroids, and tannin. The results showed that 2% of CBW media was the best medium for the growth of *Chlorella* with nitrogen (N), phosphorus (P), and potassium (K) content was 0.01% N; 0.01% P; 0.06% K respectively. The highest specific growth rate of *Chlorella* sp. on 2% CBW medium was on the 8th day of culture (0.8133) with cell density of 1.6 x 10^6 cells/mL, while the highest specific growth rate of *Chlorella sorokiniana* was on the 6th day (0.8907) with cell density of 2.99 x 10^7 cells/mL. The chloroform extract of the biomass of *Chlorella sorokiniana* contained steroid and saponin

Keywords: *Chlorella* sp, *Chlorella sorokiniana*, chicken broiler waste media, the phytochemical content

INTRODUCTION

Waste is an unutilized organic or inorganic material, causing serious problems in the environment if not handled properly. Waste can come from various sources of the production process. Waste treatment should be done properly so that it can be utilized and continuously and reduce environmental damage.

Some waste can be utilized as a microalgae culture medium in some previous studies such as liquid waste of palm oil (Mahdi et al. 2012), urban liquid waste (Djaghoubi et al. 2015), tofu liquid waste (Syaichurrozi and Jayanudin 2016; Simamora et al. 2017), and anaerobic processed swine waste (Cheunbarn and Peerapornpisal 2010). Liquid waste of palm oil was used as a culture medium for *Spirulina platensis*, *Nannochloropsis* sp, and *Chlorella* sp. (Mahdi et al. 2012). *Spirulina platensis* is one of the microalgae that has been cultured on several wastes, i.e, urban liquid waste (Djaghoubi et al. 2015), tofu liquid waste (Syaichurrozi and Jayanudin 2016; Simamora et al. 2017), and anaerobic processed swine waste (Cheunbarn and Peerapornpisal 2010).

Chlorella is a microalga that can grow on the waste medium. *Chlorella pyrenoidosa* grew on rubber waste (Zulfarina et al. 2013) and cow manure (Azhar et al. 2017). *Chlorella* sp. is able to grow in brewing waste (Farooq et al. 2013), fisheries waste (Lestari et al. 2014), industrial liquid dairy waste (Garno et al. 2014), and coal polluted environment as potential organisms for coal bioremediation (Selvika et al. 2016), fisheries waste (Lestari et al. 2014), and dairy liquid industrial waste (Garno et al. 2014). *Chlorella sorokinia*na can grow in liquid waste and exhaust gases (Lizzul et al. 2014).

Intensive broiler farmings create waste that greatly impacts the environment. West Java is the largest broiler chicken producing province in Indonesia, reaching 622,321 tons in 2017 (33.67% of contribution to national production) (BPS 2018). Broiler farming waste is chicken manure. "A chicken will produce about 33 grams of manure/tail per day" (Mulatsih, http://lppm.ipb.ac.id). In this research, chicken manure as a waste of broiler farming is utilized as the growth medium of *Chlorella* sp. and *Chlorella sorokiniana*. Febtisuharsi (2016) had compared the growth of *Chlorella* sp. on chicken manure media, cow manure media, and goat manure media. The result of that study was *Chlorella* sp. had highest cell and lipid production on chicken manure media. Broiler manure contains 2.2% Nitrogen, 1.41% Phosphor and 1.52% Kalium (Amanullah et al. 2010). The aims of the study were to determine the suitable waste concentration of chicken broiler for the growth of *Chlorella* sp. and *Chlorella sorokiniana* and to determine the secondary metabolites of the biomass of *Chlorella* sp. and *C. sorokiniana*.

Table 1. Secondary metabolites of *C. sorokiniana* (Sathasivam et al. 2017; Barkia et al. 2019)

Metabolites	Functions
Mycosporine-like amino acids (MAA)	UV-screening agent; sunscreen
α-carotene	Lower risk of premature death
β-carotene	Food colorant; antioxidant property; cancer-preventive properties; prevent night blindness; Prevent liver fibrosis; anti-allergic; pro- vitamin A
Lutein	Prevents cataract and age-related
Triglycerides and hydrocarbons	Biofuels
Vitamin E	Protect against toxic pollutants; premenstrual syndrome, protect against eye disorders; anti-Alzheimer's disease; antidiabetic property

MATERIALS AND METHODS

Materials

Microalgae used in this study were *Chlorella* sp. (InaCC M39), *C. sorokiniana* (InaCC M38), Chicken Broiler Waste (CBW) collected from poultry farms in Cijeruk, Bogor, AF6 media, chloroform, methanol, HCl, Mayer reagent, Wagner reagent, and Dragendorf reagent. Equipment used in this study were UV-Vis spectrophotometer (*Shimadzu*), Atomic Absorption Spectrophotometer (*PG990*), autoclave, hemocytometer, microscope, and other glassware.

Culture of *Chlorella* sp. and *C. sorokiniana*

Chlorella sp. and *C. sorokiniana* were cultured on AF6 and CBW media with various concentrations (2, 4, 6, 8, and 10%) with the initial cell density of 10^5 cells/mL. Cells were counted every 48 hours for 10 days. Microalgae-specific growth rate (k) was calculated by the formula:

$$K = 3.22 \frac{Log\left[\frac{n1}{n0}\right]}{T1-T0}$$

N1 is cell density at time T1
N0 is initial cell density (at time T0)

Nitrogen (N), phosphorus (P), and potassium (K) content of CBW media

The N, P and K content of CBW media were analyzed to determine proper CBW concentration for the culture medium of *Chlorella*. N content was determined using the Kjeldahl method, P content was determined using a UV-Vis spectrophotometer, and K content was determined using AAS based on SNI 7763:2018.

Extraction of secondary metabolites from microalgae

Biomass of *Chlorella* sp. and *C. sorokiniana* cultured on CBW media for 21 days were centrifuged at 1500 rpm for 10 minutes. The accumulated biomass was dissolved with chloroform at a 1:10 ratio (b/V) and shaken for 72 hours. The extract was filtered and evaporated to remove the solvent.

Qualitative phytochemical analysis of microalgae extracts

Qualitative phytochemical analysis was carried out to determine the presence of alkaloids, terpenoids, flavonoids, saponins, and tannins based on Harborne (1984) with modification.

Alkaloid

Chlorella sp. and *C. sorokiniana* extracts were taken as much as 1 mL, then added with 5 mL chloroform and 5 mL ammonia into the test tube. Three drops of H_2SO_4 were added subsequently into three divided solutions and kept for few minutes to separate. The upper part of each filtrate was taken and tested using Mayer, Wagner, and Dragendrof reagents. The sample was detected to contain alkaloid as producing a white color on the Mayer reagent, the red color on the Dragendrof reagent, and brown color on the Wagner reagent.

Steroids/terpenoids

Chlorella sp. and *C. sorokiniana* extracts were taken as much as 1 mL, then added with 3 mL ethanol, 5 mL anhydrous acetic acid, and 10 drops of concentrated sulfuric acid (Liebermann-Burchard). The color change from purple to blue or green indicated the presence of steroids, whereas the formation of brownish-red on the surface indicated the presence of terpenoids.

Flavonoids

Chlorella sp. and *C. sorokiniana* extracts were taken as much as 1 mL and added with 3 mL ethanol heated on the water bath, then 0.1 g Mg and two drops of concentrated HCl were added into the solution. The red color on the ethanol part indicated the presence of flavonoids.

Saponins

Chlorella sp. and *C. sorokiniana* extracts were taken as much as 1 mL and put into the test tube, then heated with 10 mL aqua dest on the water bath. Filtrates were shaken and kept for 15 minutes. Stabilized foam formed (sustained in a long period) indicated the presence of saponins.

Tannins

Chlorella sp. and *C. sorokiniana* extracts were taken as much as 1 mL and added with 10 mL aqua dest, then heated on the water bath and added with 2-3 drops of 1% $FeCl_3$. Green, dark blue, or dark green color formed indicated the presence of tannins.

RESULTS AND DISCUSSION

Based on the cell density at day 10th, it showed that 2% chicken broiler waste (CBW) was the most suitable culture medium for *Chlorella* sp. (Fig. 1) and *C. sorokiniana* (Fig. 2). Culture media of AF6 and CBW with concentrations >2% (4-10%) showed the growth *Chlorella* sp, however, it is not as good as the concentration of 2%. The highest specific growth rate of *Chlorella* sp. on 2% CBW medium was found on the 8th day (0.8133) with a cell density of 1.6 x 10^6 cells/mL, while the highest specific growth rate of *C. sorokiniana* was found on the 6th day (0.8907) with a cell density of 2.99 x 10^7 cells/mL.

The growth rate of *C. sorokiniana* was faster than *Chlorella* sp. in 2% CBW culture media. However, *C. sorokiniana* started the death phase earlier than *Chlorella* sp. because *C. sorokiniana* adapt more quickly to chicken broiler waste media than *Chlorella* sp. Research by Chen et al. (2017) showed that *C. sorokiniana* grew faster than *Chlorella* sp. on BG11 media. *C. sorokiniana* has the ability to grow fast on culture media with limited nutrients, therefore it can be utilized as a bioremediation agent (Edmundson et al. 2017; Lizzul et al. 2018).

The growth capabilities of two *Chlorella* species on broiler chicken waste media was associated with the nutrient content in the waste. Nitrogen (N), phosphorus (P), and potassium (K) were the main nutrients for microalgae growth. The 2% CBW media was the most suitable media for both *Chlorella* with 0.01% total N, 0.01% P (in P_2O_5), and 0.06% K (in K_2O) (Table 2).

Garno et al. (2014) showed that nitrogen-enriched milk industry waste equivalent to F/2 media (12.5 mg/L) was able to improve the growth of *Chlorella*. Triastuti et al. (2011) revealed that the addition of peanut root nodules fertilizer as a source of nitrogen and phosphorus source with a concentration of 4.5 ppm resulted in the highest population density of *Chlorella* sp. (1.4375x10^6 cells/ml) on the third day of culture. Xie (2018) reported that the suitable nitrogen source for *C. sorokiniana* should be 1 g/L glycine, 11.4 mg/L KH_2PO_4 at pH 9. In this study, *C.sorokiniana* has good growth in 0.01% nitrogen and phosphorus contained in Chicken Broiler Waste Media. It means that this species can grow in limited nitrogen.

Nitrogen is a macronutrient that plays a role in the formation of amino acids needed for protein biosynthesis and nucleic acids (Chen et al. 2017; Guerra-Renteria et al. 2019). The existence of adequate N in the media results in higher growth than media with limited N (Ikaran et al. 2015). *C. vulgaris* absorbs 7.19 mmol of NO_3 per gram biomass dry weight (Guerra-Renteria et al. 2019). The nitrogen source was in the form of NO_3 and NH_3 in the metabolism pathway. Nitrate is initially altered into ammonia for further microalgae metabolism, then ammonia is subsequently converted into glutamine and glutamate (Figure 3), followed by conversion into other amino acids for protein and nucleic acid biosynthesis (Chen et al. 2017; Guerra-Renteria et al. 2019).

Table 2. N, P, and K content of 2% CBW medium

Element	Content
Nitrogen	0.01 %
Phosphorus	0.01 %
Potassium	0.06 %

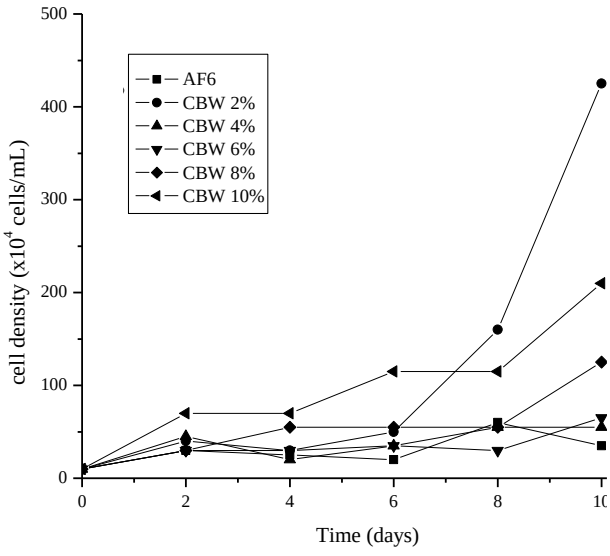

Figure 1. The cell density of *Chlorella* sp. cultured on various media

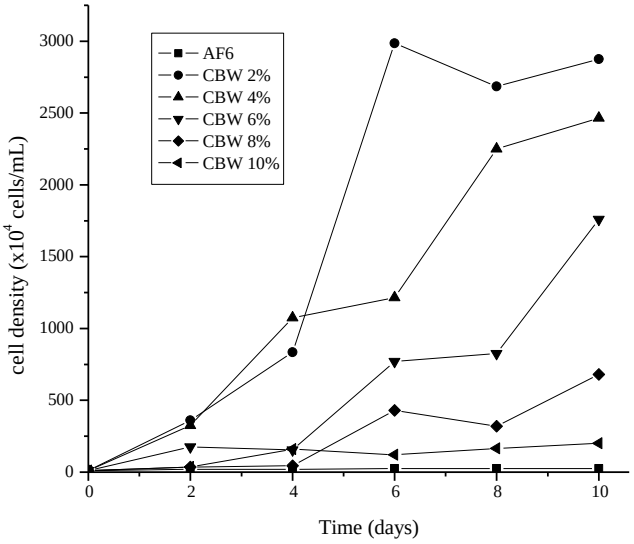

Figure 2. The cell density of *C. sorokiniana* cultured on various media

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Phosphorus plays a role in microalgae cell formation and energy transports (Manalu 2010). Phosphorus participates in the photosynthesis metabolism and biochemical reactions related to glycolysis, Krebs cycle, and oxidative phosphorylation (Guerra-Renteria et al. 2019). Growth and biomass of *C. vulgaris* decreased in proportion to the decrease in the amount of nitrogen and phosphorus in the media (Kalla and Khan 2016). Algal growth is influenced by the transport of soluble inorganic phosphorus, which passes through the cell membrane (Singh et al. 2018). Cell division is disrupted when algae suffer from the condition of P deficiency (Brembu et al. 2017).

Microalgae are microorganisms that are capable of producing metabolites compounds with biological activities that are beneficial for humans and animals (Patras et al. 2018). Diverse groups of carotenoids, fatty acids, amino acids, antioxidant compounds and other secondary metabolites in microalgae enhance the nutritional value of humans and animal food (Sathasivam et al. 2017; De Morais et al. 2015).

According to Ibañez and Cifuentes (2013) *Chlorella* spp. contains several bioactive compounds such as carotenoids which have antioxidant activity, fatty acids can be used to reduce the risk of liver disease, sulfated polysaccharides as antitumor, antivirals, sterols to reduce LDL cholesterol and total cholesterol (Ibañez and Cifuentes 2013). The results of the phytochemical analysis of chloroform extract of *Chlorella* sp. and *C. sorokiniana* were presented in Table 3. The chloroform extract of *C. sorokiniana* contained steroids and saponins (Table 3). Steroid glycosides, known as saponins, are widely used in cosmetics, pharmaceuticals, food, and agriculture. Saponins generally possess antifungal and antibacterial activities (Feroz 2018).

Table 3. Phytochemical content of chloroform extract of *Chlorella* sp. and *C. Sorokiniana*

Test	Results	
	***Chlorella* sp.**	***Chlorella sorokiniana***
Alkaloid	-	-
Flavonoids	-	-
Steroids	-	+
Saponins	-	+
Tannins	-	-

Description: (+):present, (-) : absence

Chlorella sorokiniana contains phenolic compounds that have antioxidant activity (Azaman et al. 2017). Chloroform extract of *C. vulgaris* contains saponins (Adhoni et al. 2016; Syed et al. 2015), and have antibacterial activity against *Staphylococcus aureus*, *Corynebacterium*, *Bacillus subtilis*, *Streptococcus*, *Salmonella paratyphi B*, *Klebsiella pneumoniae*, *Aerobacter aerogenes*, *Candida albicans*, and *Aspergillus niger* but it showed no growth inhibition against *Escherichia coli* (Adhoni et al. 2016). The GC-MS analysis of chloroform extract of *C. sorokiniana* showed the presence of lauric acid, cyclotetradecane, butyl rubber, myristic acid, stearic acid, oleic acid, valeric acid, ethyl palmitate, lignoceryl alcohol, and perfluoroacetic acid (Adhoni et al. 2016). The chloroform extract of *C. vulgaris* cultured on bold basal medium and sewage medium contain alkaloids and flavonoids, showing antibacterial activity against some bacterial isolates, such as *S. aureus* and *S. typhi* (Dineshkumar et al. 2017).

In conclusion, the 2% CBW media contains 0.01% nitrogen, 0.01% phosphorus, and 0.06% potassium was the best media for culturing *Chlorella* sp. and *C. sorokiniana*. The chloroform extract of *C. sorokiniana* contains steroids and saponins.

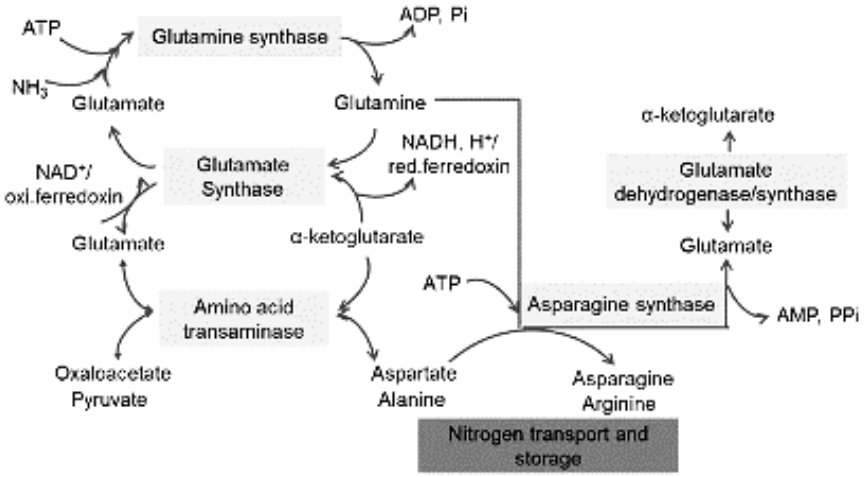

Figure 3. Glutamate-glutamine system on transaminase pathway in *Chlorella* sp. (Source: Chen et al. 2017)

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