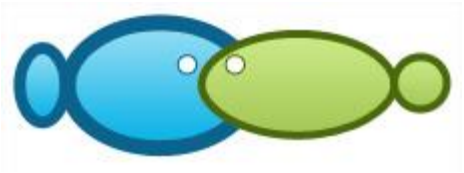

The effect of acidic pH on chlorophyll, carotenoids, and carotenoid derivatives of *Euglena* sp. as antioxidants

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Abstract. *Euglena* sp. is a microalgae species of the phylum Euglenozoa that lives in freshwater, in open, sunny areas. The potential of *Euglena* sp. as an innovative functional ingredient in the food and feed industry, as well as medicines and cosmetics, is closely related to the presence of pigments in *Euglena* sp. *Euglena* sp. contains chlorophyll pigments a and b, as well as carotenoids such as xanthophyll, astaxanthin, zeaxanthin, carotene, and beta-carotene. The pH is an essential factor in microalgal cultivation. The pH changes generally affect biomass, primary metabolites, and secondary metabolites in microalgae growth. In this study, *Euglena* sp. was cultivated on a Cramer-Myers medium and treated under different acidic conditions. This study aims to determine the effect of pH on the production of pigments in *Euglena* sp. Culture optimization of *Euglena* sp. was carried out on various acidic pH media at pH 2.5, 3.5, and 4.5. The production results of chlorophyll and total carotenoids, astaxanthin, and carotenoid derivatives of *Euglena* sp. were analyzed using One-Way ANOVA at a 95% confidence level, followed by Duncan's advanced test. Based on the analysis, the study results show that *Euglena* sp. at pH 4.5 has the most optimal production of chlorophyll and total carotenoids, astaxanthin, and carotenoid derivatives compared to the other pH treatments. In the pH treatment of 4.5, the average output of chlorophyll a was 12.898±0.357 μg mL^{-1}, and the productions of chlorophyll b, total carotenoids, and astaxanthin were 7.563±1.800 μg mL^{-1}, 3.244±0.235 μg mL^{-1}, and 1.187x10^{-3} ±0.078 g L^{-1}, respectively.
Key Words: acidity, Euglenozoa, peat swamp, pigments, xanthophyll.

Introduction. Microalgae are increasingly being used in a variety of applications, including food, biopharmaceutical industry materials, environmental improvement, and natural dyes. Natural pigments derived from microalgae are suitable for use as additives and cosmetics. Some microalgae can produce pigments other than green as a result of photosynthesis. Chlorophyll, phycobiliprotein, and carotenoids are examples of pigments commonly used in various industries (Nur 2014).

Euglena sp. is a freshwater microalgae of the phylum Euglenozoa that lives in open and sunny areas. The presence of pigments in *Euglena* sp. is closely related to its potential as an innovative functional ingredient in the food, feed, medicine, and cosmetics industries (Christaki et al 2015). Pigments are compounds found in microalgae that aid in light absorption and energy transfer during metabolic processes such as photosynthesis (Pagels et al 2020). *Euglena* sp. contains the pigments chlorophyll a and chlorophyll b, and carotenoids such as xanthophyll, astaxanthin, zeaxanthin, carotene, and beta-carotene (Azizullah et al 2014).

Chlorophyll is the primary green pigment, with a structure similar to hemoglobin (a pigment found in human blood), except that the central atom of Fe^{2+} in the blood is replaced with Mg^{2+} in chlorophyll. Chlorophyll is an unstable compound, being so sensitive to light that it is difficult to keep its molecules intact with a green color (Hutajulu et al 2008). Furthermore, chlorophyll is susceptible to heat, oxygen, and chemical degradation. The pH has an effect on chlorophyll degradation as well. Green plants, algae, and cyanobacteria all contain chlorophyll. Chlorophyll b is only found in green algae and plants (Merizawati 2008).

Carotenoids are a class of fat-soluble pigments found in a variety of plants, algae, microorganisms, and animals (Sachindra & Mahendrakar 2005). They are classified into two groups based on the chemical elements contained: carotenes, which have only the elements C (carbon) and H (hydrogen), and xanthophylls, which also contain the element O (oxygen). Carotenoids number more than 600 and can be categorized into 2 parts. The first part is xanthophylls (phylloxanthins), which have oxygen as their functional group, for example, xanthophylls (astaxanthin, lutein, and zeaxanthin). The second part is carotene which only has hydrocarbon bonds without functional groups, for example, β-carotene and lycopene (Saini & Keum 2018). Carotenoids play an important role as a precursor to vitamin A (Lila 2004). Vitamin A is extremely beneficial to the processes of vision, bone growth, reproduction, and immune defense. Carotenoid extracts have been applied in various food, pharmaceutical, and nutraceutical products.

Astaxanthin is a carotenoid pigment that belongs to the terpenoid group (Butler et al 2018). This compound is helpful for photo-oxidation protection from UV rays, and it has some properties against inflammation, cancer, aging, and age-related diseases; it also enhances the immune response, liver function, heart function, eye, joint, and prostate health (Helly de Fretes et al 2012). This study aims to determine the production of various pigments in *Euglena* sp. under different pH values. Some pigments to be studied are chlorophyll a, chlorophyll b, total carotenoids, astaxanthin, and ten types of carotenoid derivative pigments.

Material and Method

Description of the study sites. This research was conducted at the Biotechnology and Falitma Laboratory, Universitas Gadjah Mada, Yogyakarta, Indonesia. 84 mL of *Euglena* sp. samples were collected from the peat swamp at the Dieng plateau, Central Java, and then cultured in Cramers Mayers (CM) medium for 18 days.

Cultivation of Euglena sp. Table 1 shows the medium used for the cultivation, namely Cramer-Myers (CM) medium (Cramer & Myers 1952). A 500 mL culture vial that included 300 mL of CM medium was added to the culture medium. The water medium used was distilled water. *Euglena* sp. was cultured at 28°C with an illuminance of 1000 lux. The pH in the medium was conditioned at different values: 2.5, 3.5, and 4.5 by using H_2SO_4 to change the pH to acidic, while KOH was used for alkaline. The pH was measured every morning by using a pH meter before conducting metabolite tests.

Table 1

Composition of Cramer-Myers (CM) medium (Cramer & Myers 1952)

Composition	*Concentration (mg L^{-1})*
$(NH_4)_2SO_4$	1000
KH_2PO_4	1000
$MgSO_4 \cdot 7H_2O$	200
$CaCl_2 \cdot 2H_2O$	20
$Fe_2(SO_4)_3 \cdot 7H_2O$	3.0
$MnCl_2 \cdot 4H_2O$	1.8
$CoSO_4 \cdot 7H_2O$	1.5
$ZnSO_4 \cdot 7H_2O$	0.4
$CuSO_4 \cdot 5H_2O$	0.02
$Na_2MoO_4 \cdot 2H_2O$	0.2
Vitamin B1	0.1
Vitamin B12	0.0005

Measurement of chlorophyll and total carotenoids. 2 mL of microalgae sample were inserted in a microtube. The sample was centrifuged at a speed of 3300 rpm at 70°C for 5 minutes. The sample supernatant was discarded and replaced with 2 mL of 100% methanol. Furthermore, the sample was incubated at 40°C overnight in the dark. The

next day the sample was centrifuged at 10000 rpm for 5 min and the supernatant was measured using spectrophotometry for pigment determination (Pruvost et al 2011). Absorbance was measured at wavelengths of 480, 652, and 665 nm:

Chl-a (μg mL^{-1}) = -8.0962 x A652 + 15.6169 x A665

Chl-b (μg mL^{-1}) = 27.4405 x A652 – 12.1688 x A665

Carotenoids (μg mL^{-1}) = 4 x A480

Measurement of astaxanthin. Astaxanthin calculation followed the method of Boussiba et al (1992) with a slight modification. 2 mL of the sample were centrifuged at a speed of 8000 rpm for 5 minutes at 22°C. 2 mL of 5% KOH in 30% methanol were added to the samples, without prior removal of pellets. The sample was vortexed for 3 minutes to break down the cells. The sample was placed into a water bath (70°C) for 10 minutes, to eliminate chlorophyll. Next, the sample was centrifuged at 10000 rpm for 5 minutes and pelletized combed. Furthermore, the remaining pellets were extracted through the addition of 500 μL of acetic acid and 1.5 mL of dimethyl sulfoxide (DMSO) solution. The sample was placed back in a water bath with a temperature of 70°C for 10 minutes, then in a centrifuge at 10000 rpm for 10 minutes, and left with supernatant. The extraction process was repeated until the debris cells were close to colorless. Next, the sample was transferred to a glass cuvette, placed in a spectrophotometer, and its absorption was calculated at a wavelength of 490 nm. Based on the absorbance from the calculation results of the spectrophotometer, astaxanthin concentrations were obtained using the following equation (Zheng et al 2017):

C (mg L^{-1}) = 4.5 x A490 x Va/Vb

Where: A490 nm - absorbance of the extract used; Va - volume extract; Vb - volume of culture sample.

Measurement of carotenoid derivates. In addition to astaxanthin, the carotenoids tested in this study totaled 10 types (Table 2), namely pheophytin b, 9-cis-neoxanthin, violaxanthin, β,β-carotene, all-trans zeaxanthin, β-cryptoxanthin, peridinin, antheraxanthin, trans-diadinoxanthin, dinoxanthin. The method used to test the content of the ten types of carotenoids refers to the research of Thrane et al (2015). 5 mL from each sample were centrifuged at 3300 rpm for 10 minutes. Next, the supernatant was removed, and the pellets were soaked in 5 mL of 96% ethanol and then incubated for 24 hours in a refrigerator at 40°C. The absorbance of each sample was read using a spectrophotometer with the wavelengths of 437, 453, 448, 475, and 446 nm. The absorbance value was entered into the following Lambert-Beer law formula to determine the content of each type of carotenoid (Lichtenthaler & Buschmann 2001):

Cw = A/(αxd)

Where: Cw - weight concentration (g L^{-1}); A - absorbance; d - cuvette groove length (cm) usually 1 cm; α - specific absorbance coefficient (L g^{-1} cm^{-1}).

Data analysis. The study results were analyzed using One-Way ANOVA, followed by the Duncan Multiple Range Test (DMRT). The significance of the differences was determined at a confidence level of 95% ($p<0.05$). Statistical analyses were carried out using IBM SPSS Statistics 23.

Table 2

Absorbance peaks and absorbance coefficients of ten types of carotenoids

Types of carotenoids	*λ (nm)*	*a (L g^{-1} cm^{-1})*
Pheophytin b	437	141
9-cis-neoxanthin	437	233
Violaxanthin	437	254
β,β-carotene	453	262
All-trans zeaxanthin	453	245
β–cryptoxanthin	453	247
Peridinin	475	135
Antheraxanthin	446	235
Trans-diadinoxanthin	448	224
Dinoxanthin	442	210

Results

Chlorophyll and carotenoid content of Euglena sp. The pigment content of chlorophyll and carotenoids in *Euglena* sp. is presented in Figure 1. Based on the pigmentation analysis, chlorophyll-a has a higher content than chlorophyll-b and carotenoids. In this study, *Euglena* sp. cultivated at a pH of 4.5 had a higher content of chlorophyll-a, chlorophyll-b, and carotenoids than in other pH treatments. The highest chlorophyll-a content was observed in the treatment with the pH of 4.5, namely 12.898±0.357 μg mL^{-1}. At a pH of 3.5, the content was 11674±2.575 μg mL^{-1}. The lowest chlorophyll-a level was observed at the pH of 2.5, namely 6.525±1.950 μg mL^{-1}. *Euglena* sp. cultivated at a pH of 4.5 resulted in a higher chlorophyll-b content than in the other pH treatments, with 7.563±1.8 μg mL^{-1}. Like chlorophyll-a and chlorophyll-b, the carotenoid content is also higher at the pH of 4.5 (3.244±0.235 μg mL^{-1}). Based on One-Way ANOVA and Duncan's advanced tests, the pH change treatment had a significantly different effect on chlorophyll-a, chlorophyll-b, and carotenoid content of *Euglena* sp. ($p<0.05$). There are some cases where differences were significant (Figure 1).

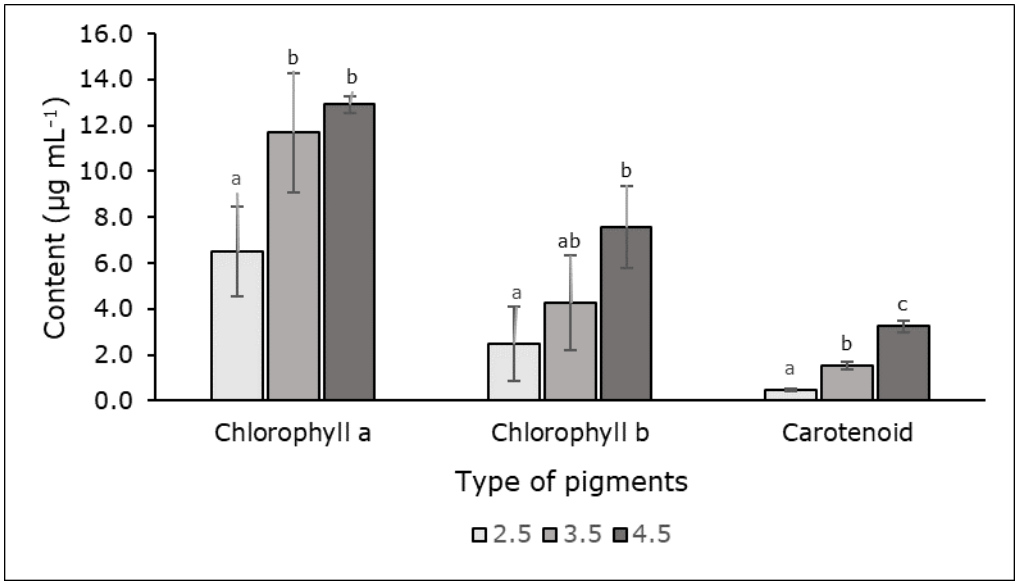

Figure 1. The chlorophyll and carotenoid content of *Euglena* sp. was cultivated for 18 days on CM media with pH treatments of 2.5, 3.5, and 4.5 pH; different letters above columns represent significance differences between treatments ($p<0.05$).

***Astaxanthin content of Euglena* sp**. Based on the results of astaxanthin calculations (Figure 2) at different pH treatments, the highest astaxanthin content was $1.187x10^{-3}$ ±0.078 g L^{-1}, in the 4.5 pH treatment, followed by $0.687x10^{-3}$±0.039 g L^{-1} in the 3.5 pH treatment, and $0.073x10^{-3}$±0.005 g L^{-1} in the 2.5 pH treatment. The 4.5 pH treatment had the highest astaxanthin content because the pH was at the threshold of astaxanthin stability (pH 4). Astaxanthin production is lowest at pH 2.5, this pH value likely causing astaxanthin to degrade (Azizah et al 2012). The astaxanthin production at different pH treatments is significantly different ($p<0.05$).

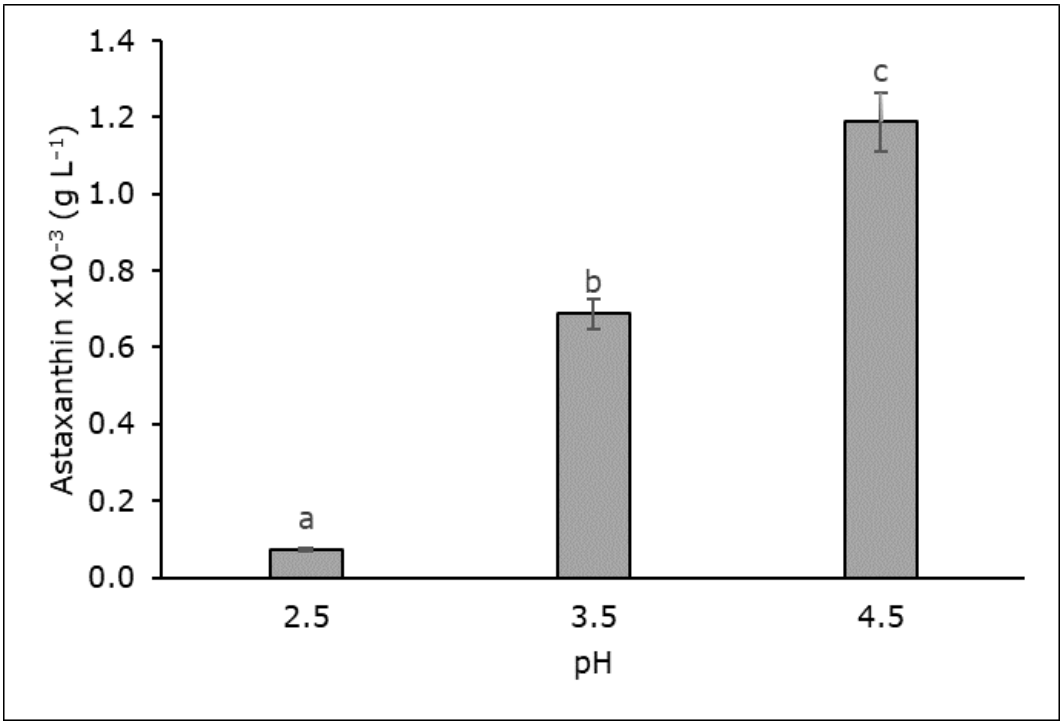

Figure 2. The astaxanthin content of *Euglena* sp. was cultivated on CM media with pH treatments of 2.5, 3.5, and 4.5 for 18 days; different letters above columns represent significance differences between treatments ($p<0.05$).

Carotenoid derivatives content of Euglena sp. Figure 3 shows the content of the ten types of carotenoids determined for each sample. The highest content of all types of carotenoids was in the treatment with a 4.5 pH: pheophytin b ($5.571x10^{-3}$ g L^{-1}), 9-cis-neoxanthin ($3.371x10^{-3}$ g L^{-1}), violaxanthin ($3.092x10^{-3}$ g L^{-1}), β,β-carotene ($2.3x10^{-3}$ g L^{-1}), all-trans zeaxanthin ($2.459x10^{-3}$ g L^{-1}), β-cryptoxanthin ($2.39x10^{-3}$ g L^{-1}), peridinin ($2.608x10^{-3}$ g L^{-1}), antheraxanthin ($3.197x\ 10^{-3}$ g L^{-1}), trans-diadinoxanthin ($2.755x10^{-3}$ g L^{-1}), and dinoxanthin ($3.781x10^{-3}$ g L^{-1}). Based on statistical analysis tests using ANOVA, ten types of carotenoids at different pH treatments gave significantly different results at a confidence level of 95%. There are some cases where differences were significant (Figure 3).

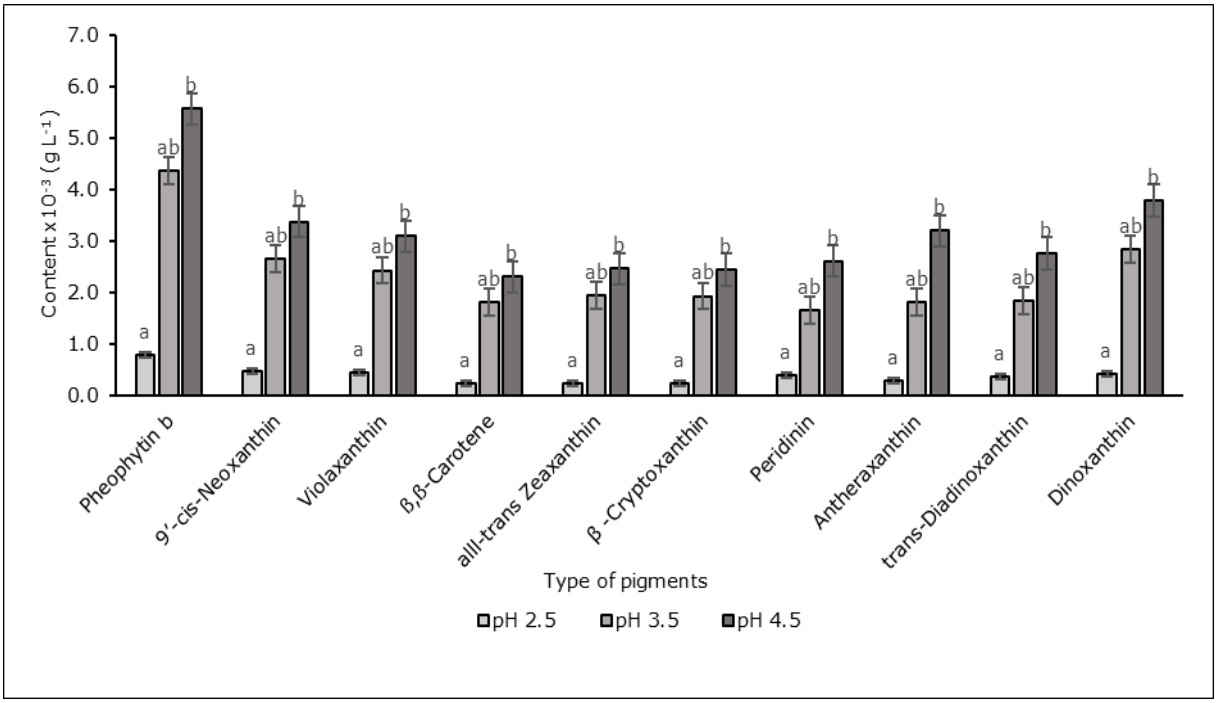

Figure 3. Carotenoid derivatives content of *Euglena* sp. cultivated on CM media with pH treatment of 2.5, 3.5, and 4.5 for 18 days; different letters above columns represent significance differences between treatments ($p<0.05$).

Discussion. Chlorophyll is sensitive to heat, oxygen, and chemical degradation, in addition to pH. Chlorophyll degradation is also affected by pH. The pH of 2.5 had the smallest chlorophyll production because chlorophyll is unstable at a pH that is too acidic. The pH is one of the factors that cause the loss of chlorophyll; when it is too acidic, it has an impact on the loss of Mg, so there will be discoloration (Kimball 1992). Chlorophyll can degrade, beginning with the loss of Mg at the center of the chlorophyll molecule or loss of the tail part (phytol). According to Fennema (1996), acidic media conditions cause chlorophyll to be unstable. A decrease in pH water can occur when tissue heating results in the loss of chlorophyll pigment. In addition, pH also affects the color of the pigment. The chlorophyll color in treatments with pH 3.5 and 4.5 shows a clear greenish color. Meanwhile, at pH 2.5, it shows a brownish-clear color. The discoloration indicates that chlorophyll degrades into its derivatives (Dutton et al 1943; Rahayu & Limantara 2005; Nurcahyanti & Limantara 2007).

Carotenoids are organic pigments found in chloroplasts and chromoplasts of plants and other groups of organisms such as microalgae. They are formed from eight isoprene molecules, with 40 carbon atoms (Hirschberg et al 1997). Kiokias et al (2016) stated that carotenoids are secondary metabolites of fats that plants and microorganisms can produce. The content of carotenoids in microalgae reaches its peak when the cell growth reaches its maximum, in the stationary phase. Based on the results of the study, the highest carotenoid content was obtained in the treatment with a pH of 4.5. Ando & Tanaka (1996) stated that the pH of carotenoids is between acid and base (pH 2-8), pH stability affecting the color produced by carotenoids. Furthermore, according to Mortensen (2006), most carotenoids are stable under the effect of bases, but some carotenoids such as astaxanthin and fucoxanthin are sensitive. Carotenoids are unstable because the presence of oxygen and peroxide easily oxidizes them. In addition, they can undergo isomerization when exposed to heat, light, and acid, so the pH 2.5 treatment has the least carotenoid production. Astaxanthin is a 40-carbon tetraterpene consisting of linked isoprene units. The molecular structure of astaxanthin is composed of a linear polyene chain and two terminal β rings (Guerin et al 2003). The hydroxyl and ketone groups that astaxanthin have indicate that the compound is more polar compared to other types of carotenoids and has very high antioxidant activity.

In this study, extraction has been carried out by the maceration method using the organic solvent DMSO. DMSO is an aprotic polar solvent that can dissolve polar or nonpolar compounds. Extraction using DMSO solvent is also referred to as the "sucking process" extraction method because it can increase the permeability of thick microalgae cell walls and penetrate well without damaging their cell walls (Wang et al 2018). Before the extraction process, a saponification stage has been carried out by adding a 5% KOH solution (w/v) in 30% methanol (v/v). This saponification was carried out to eliminate chlorophyll, which was originally lipophilic. Adding alkalis such as KOH to chlorophyll will cause a change in chlorophyllin to chlorophyllin and phytol, whose properties tend to be more lipophobic (Ferruzzi & Blakeslee 2007). Based on this saponification reaction, chlorophyll will easily separate from other biochemical compounds of a lipophilic nature.

Carotenoids are pigments that are easily isomerized in acidic and alkaline conditions (Wahyuni & Widjanarko 2015). This isomerization can result in a change in the structure of carotenoids from trans to cis form. In addition, carotenoid pigments have a higher level of stability at neutral pH (Oktafia et al 2015). Xanthophylls are relatively hydrophobic molecules, so they are usually associated with membranes and/or are involved in non-covalent binding to certain proteins. They are typically localized in the thylakoid membrane, whereas secondary carotenoids are found in lipid vesicles, either in the stroma plastid or cytosol (Grossman et al 1995). All xanthophylls are synthesized by higher plants and can also be synthesized by green microalgae. Loraxanthin, astaxanthin, canthaxanthin, diatoxanthin, diadinoxanthin, and fucoxanthin can also be produced by brown algae or diatoms (Eonseon et al 2003). However, in most green microalgae, carotene and xanthophylls are synthesized in plastids and accumulate in them alone.

Phaeophytin is a pigment derivative of chlorophyll formed by the degradation of chlorophyll. Phaeophytin is an important component of the photosynthesis reaction center and is involved in electron transfer, where light energy is converted into chemical energy (Dimara et al 2012). In this study, the pH treatments had a significant influence on phaeophytin. At a pH of 2.5, a fairly high amount of pheophytin was found, as the result of unstable chlorophyll degradation at acidic pH. According to Dimara et al (2012), chlorophyll extract can undergo degradation, which begins with the loss of Mg at the center of the chlorophyll molecule or loss of the tail part (phytol). The chlorophyll that loses its magnesium element is called phaeophytin.

β,β-carotene is an unsaturated hydrocarbon, contains no oxygen, and is usually orange in color (Hathcock 2004). β.β-carotene is very sensitive to oxidation reactions when exposed to air, light, metal, peroxide, and heat (Bauernfeind 1981). **T**he pH stability will affect the colors produced by carotenoids (Ando & Tanaka 1996). β,β-carotene can be converted into vitamin A in the body. The β ring of β–carotene in the body will be converted into vitamin A by the enzyme 15.15'dioxygenase into 2 retinal molecules, then the retinal molecule will be reduced to retinol (Lindqvist & Anderson 2002). In this study, the β.β-carotene content found was high. Limantara et al (2006) noted that β-carotene and lutein are compounds that are not as common as other carotenoids and are more resistant to acidic conditions.

Neoxanthin occurs mainly in two geometric isomers: all-trans-neoxanthin (also referred to as trans-neoxanthin), in which all double bonds have trans geometry, and 9’-cis-neoxanthin, in which the double bond at the 9’ positions has cis geometry, while all the others have trans (Märki-Fischer & Eugster 1990). The two isoforms can be distinguished with chromatographical separation due to different absorption spectra: in ethanol, trans-neoxanthin presents significant absorption peaks at 418, 442, and 471 nm (Baumeler & Eugster 1992), while 9’-cis-neoxanthin at 413, 437, and 466 nm (Baumeler et al 1994). Neoxanthin is particularly labile to acids due to its epoxide nature: a bland acid treatment (e.g., 0.002% ethereal or chloroformic hydrogen chloride) leads to the production of 5,8-furanoid oxide (called neochrome) as the main reaction product (Cholnoky et al 1969). The same reaction (reorganization of the 5,6-epoxide group of neoxanthin to the 5,8-furanoxide group of neochrome) occurs via thermal degradation. Neoxanthin was reported to have higher thermal stability than other 5,6-epoxy xanthophylls (violaxanthin and antheraxanthin) (Aparicio-Ruiz & Gandul-Rojas 2012).

The diversity of carotenoids in chloroplasts is generally low, with cyclical xanthophyll components (i.e. violaxanthin, antheraxanthin, and zeaxanthin), β,β-carotene, lutein, neoxanthin (Britton 1995; Britton et al 2004). The two-step xanthophyll cycle includes de-epoxidation of the pigment violaxanthin to zeaxanthin via antheraxanthin. The one-step xanthophyll cycle is called the diadinoxanthin cycle, in which the epoxide diadinoxanthin is converted into diatoxanthin (Stransky & Hager 1970). Violaxanthin de-epoxidase (VDE), located in the thylakoid lumen, is active at low pH and requires ascorbate as a co-substrate (Hager 1969). The study found that each pigment produced had the highest concentration at the pH 4.5 treatment. Grouneva et al (2006) stated that the concentration of carotenoid pigments (xanthophyll and carotene) is highly dependent on the process or cycle of formation of these pigments assisted by enzymes/catalysts. In contrast, the enzyme/catalyst can only work at a specific pH of 5-5.5, so that the concentration of the pigments will be abundant/high.

Conclusions. Cultured *Euglena* sp. in a medium with a pH of 4.5 shows higher total values of chlorophyll-a, chlorophyll-b, carotenoids, and astaxanthin than in other pH treatments. Ten types of carotenoids were detected in the study. The types of carotenoids are pheophytin b, violaxanthin, antheraxanthin, β,β-carotene, 9-cis-neoxanthin, β-cryptoxanthin, peridinin, all-trans zeaxanthin, trans-diadinoxanthin, and dinoxanthin.

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Conflict of Interest. The authors declare that there is no conflict of interest.

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