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Original Article

Effect of temperature and pH on primary metabolic and biomass productivity culture in *Euglena* sp.

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Abstract: *Euglena* is a microalga with the potential to be used as a renewable energy source. The biofuel-making potential is present in *Euglena* species biomass's proteins, lipids, and carbohydrates. Therefore, optimizing microalgal growth under various physiological conditions is crucial to obtaining more biomass. In this study, *Euglena* sp. was cultivated on medium Cramer-Myers (CM) and subjected to various temperatures and acidities. *Euglena* sp. cultures were optimized at different pH levels, including 2.5, 3.5, and 5.5, and at 29 and 32°C. Then, treatments were evaluated on the culture's pace of cell development, total biomass, and amount of carbohydrates, protein, and lipids. Based on the results, *Euglena* sp. at pH 5.5 and 29°C had the optimal growth rate, biomass, carbohydrate, protein, and fat content compared to the other treatments. In a pH 5.5 at 29°C, the average biomass was 0.382±0.173 g/L, and the resulting concentrations of protein, carbohydrates, and lipids of 0.288±0.12, 0.201±0.052, and 0.182±0.083 g/L, respectively.

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Introduction

Biofuel was a promising renewable fuel that could be processed into biodiesel, biogas, and bioethanol. Biofuel production was increasing, given the limited number of fossil fuels. Currently, biofuel energy sources have been used in various countries. The biofuel industry was spread across Europe, America, and Asia. Biofuels were produced from carbon sources and could also be produced from biomass. This biomass could come from living organisms such as plants, animals, or garden waste and harvest waste. Biomass could produce energy based on the carbon cycle. Therefore, biofuel from biomass was a promising alternative renewable energy source.

Microalgae are aquatic microorganisms that have a high potential to be used as an energy source. Microalgae have a lipid content of around 50-60%, protein of as much as 70%, and carbohydrate content that reaches 40% of the total microalgae biomass (Chisti, 2007). Microalgae with high lipid, protein, and carbohydrate content can be processed into renewable energy sources, reducing global warming. Therefore, the culture of microalgae is increasing. Microalgae are more environmentally friendly, the processing is cheap, and the productivity is high, but it only takes up a little space for microalgae cultivation (Becker, 1994).

Euglena sp. can be a biofuel or bioenergy source, including biodiesel and bioethanol (Gissibl et al., 2019). The high biomass in the *Euglena* culture is a source that can be processed into biofuels as renewable energy. Besides, the *Euglena* biomass contains carbohydrate, protein, and lipid components, which can be processed into profitable products. Researchers and companies have tried to find renewable energy sources using cheap but environmentally friendly and promising objects. It had begun to be cultivated and processed into various products because it had a high level of productivity (Harun et al., 2010). Therefore, *Euglena* was very

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suitable for development as a source of renewable bioenergy. However, the productivity of biomass and metabolite content needs to be increased. This increase can be accomplished by optimizing *Euglena* cultivation. As a result, if the cultivation process is successful, productivity will increase, as will selling value and potential utilization.

Several physiological conditions affect growth in *Euglena* culture, two of which are temperature and pH. Low temperatures inhibit the activity of photosynthesis-related enzymes (and other metabolic reactions). Moderately high temperatures may promote the rate of respiratory action, but extremely high temperatures will inhibit metabolic activity and respiration in microalgae (Breuer et al., 2013). Under low pH conditions, the increasing chemical gradient between the cytoplasm and the medium causes a more significant H^+ influx into the cells, necessitating active H^+ transport to maintain an internal pH suitable for normal metabolic processes (Visviki and Santikul, 2000).

The effects of physiological conditions on microalgae growth vary depending on the genus or strain. During a 10-day cultivation period, *Nannochloropsis* sp. MASCC 11 produced measurable biomass at pH levels of 3, 3.5, 4.0, 6.0, and 9.0 and temperatures of 20, 25, 30, 35, and 40ºC. The maximum biomass of Nanochloropsis (0.44 $g.L^{-1}$) was observed at pH 9.0, and the optimal temperature for proper growth was 35°C, with a biomass value of 0.63 $g.L^{-1}$ (Peng et al., 2020). At the same time, the highest final biomass concentration and initial biomass productivity of *Scenedesmus obliquus* were observed at pH 7, 27.5ºC, and temperatures of 20, 27.5, and 35ºC (9 combinations) (Breuer et al., 2013). In this research, an experiment was conducted with the culture of *Euglena* sp. under acidic conditions and at different temperatures. This research aims to determine which treatment can produce optimal *Euglena* sp. growth with high biomass and to assess the effect of the treatment on metabolite content such as carbohydrates, lipids, and protein contained in the culture of *Euglena* sp. isolated from Dieng Plateau, Central Java, Indonesia.

Materials and Methods

Cultivation of *Euglena* sp.: *Euglena* sp. was cultured under different conditions. The treatment process of *Euglena* sp. cultivation was carried out using an Erlenmeyer flask of 250 ml. The medium used for the cultivation was the CM medium (Cramer and Myers, 1952). Culture conditions focused on environmental factors were pH and temperature. The pH factor was applied to the medium, which was conditioned at a different pH of 2.5, 3.5, and 5.5. Meanwhile, the temperature was set at 29 and 32°C; temperature adjustments were made using a heater placed in the water bath.

The growth rate of *Euglena* sp.: The cell growth rate can be determined by the cell density in each treatment. Optical Density (OD) was obtained by measuring absorbance using the spectrophotometric method at a wavelength of 680 nm (Suzuki, 2017). The 680 nm wavelength could determine the cell density from the chlorophyll content of the *Euglena* sp. cells in the sample (Harun et al., 2010). This OD measurement was done every day for 18 days of observation.

Measurement of biomass: The measurement of biomass was carried out using the gravimetric method. First, this method obtained cell biomass in a pure state after going through the separation process by centrifuge at 4000 rpm for 10 min (Olguín et al., 2013). Then, cell biomass was obtained from dry weight after drying at 30°C for 24 h. Dry weight was weighed using the analytical balance AL-204. Then, the result of dry weight was calculated using the formula of DW ($mg\ mL^{-1}$) = total weight- weight after drying/volume of samples.

Analysis of carbohydrate content: The analysis of carbohydrate content was carried out using the Phenol Sulfuric Acid method (Dubois et al., 1956). This method was done by adding 0.5 ml of 5% phenol and 1 mL concentrated sulfuric acid (H_2SO_4) into the supernatant of the centrifuged sample at 4000 rpm for 10 minutes at 4ºC. After that, absorbance readings were performed using a spectrometer with a wavelength of 490 nm. Then, a determination of total carbohydrate levels was done based on the standard

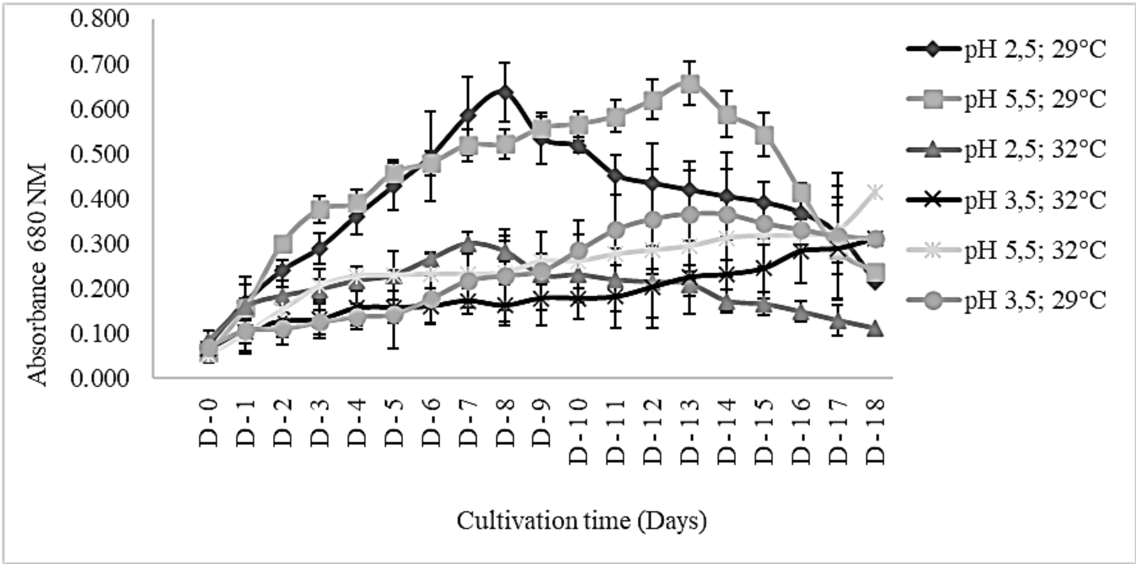

Figure 1. The growth rate of *Euglena* sp. in different treatments (Mujahidah, 2020).

linear regression equation of the standard glucose solution that had been made with concentrations of 25, 50, 75, 100, 125, 150, 175, 200, 225, and 250 g/L.

Analysis of protein content: The analysis of protein content in *Euglena* sp. was conducted using the Bradford method. This method was performed by adding an SDS solution to the supernatant of the separation process using a centrifuge. Then, incubation at 95°C was followed by incubation at 4°C each for 5 minutes. Incubation samples were taken and added Bradford's solution (Bradford, 1976). After that, absorbance measurement was performed using ELISA Reader Biotech with a wavelength of 595 nm. Protein content was calculated using standard linear curve regression equations from standard Bovine Serum Albumin (BSA) protein solutions with concentrations of 250, 500, 750, 1000, 1250, 1500, 1750, 2000, 2250, and 2250 ppm.

Analysis of lipid content: The analysis of the total lipid content of *Euglena* sp. was conducted using the Bligh and Dyer method. This method was an extraction method with the addition of chloroform and methanol with a ratio of 1:2, then chloroform and aquades with a ratio of 1:1 (Bligh and Dyer, 1959). Then, the solution was split using a centrifuge until there were three layers; the bottom layer was taken and incubated in the oven for 24 h at a temperature of 30ºC.

Statistical analysis: Statistical analysis was performed using SPSS ver.16 and the Two-way Analysis of Variance (ANOVA) test to determine the significance value between treatments at the 5% test level. Then, treatment means were separated using Tukey's test HSD.

Results

Growth rate *Euglena* sp.: The results showed that treatment with various pH mediums and ambient temperatures can affect cell growth in *Euglena*. The growth rate for the cell density in each treatment was measured using the spectrophotometric method. *Euglena* sp. had an optimal growth rate at pH 5.5 and temperature at 29°C (Fig. 1). At pH 2.5, *Euglena* sp. had a fast growth rate and began to experience death on the 9th day. In cultures with pH 3.5, *Euglena* sp. can grow with a reasonable growth rate but have a lower optical density value than pH 5.5. The *Euglena* sp. culture at 29°C had a more optimal growth than at 32°C. *Euglena* culture at a temperature of 32°C had slow growth and was still in the log phase on the last day of observation. This can affect *Euglena* sp. harvesting time. Harvesting can be done faster in the culture at 29°C because it can enter the stationary phase more quickly with a high cell density, while the culture at 32°C harvest time is slower.

Cell density of *Euglena* sp.: Based on cell density (Fig. 2), *Euglena* cultures at 29°C had a higher cell density than 32°C. At 32°C, cell growth lasts less

Table 1. Value of Biomass in *Euglena* sp.

Treatment	Mean of Biomass *Euglena* sp. (g/L) ± SD						
	D-0	**D-3**	**D-6**	**D-9**	**D-12**	**D-15**	**D-18**
pH 2.5; 29°C	0.106±0.113^{a}	0.246±0.011^{a}	0.319±0.019^{b}	0.487±0.030^{b}	0.414±0.046^{ab}	0.273±0.023^{ab}	0.133±0.041^{ab}
pH 3.5; 29°C	0.087±0.012^{a}	0.160±0.087^{a}	0.156±0.035^{a}	0.273±0.049^{a}	0.333±0.031^{a}	0.313±0.042^{bc}	0.220±0.053^{b}
pH 5.5; 29°C	0.990 ±0.020^{a}	0.270±0.010^{ab}	0.467±0.063^{c}	0.527±0.083^{b}	0.573±0.011^{b}	0.499±0.021^{c}	0.240±b0.040^{b}
pH 2.5; 32°C	0.113±0.121^{a}	0.180±0.020^{a}	0.386±0.030^{bc}	0.413±0.061^{ab}	0.263±0.095^{a}	0.153±0.023^{a}	0.053±0.012^{a}
pH 3.5; 32°C	0.113±0.012^{a}	0.186±0.046^{a}	0.353±0.041^{bc}	0.374±0.043^{ab}	0.323±0.027^{a}	0.399±0.052^{bc}	0.480±0.080^{c}
pH 5.5; 32°C	0.993±0.019^{a}	0.187±0.012^{a}	0.280±0.069^{ab}	0.327±0.064^{a}	0.320±0.030^{a}	0.360±0.120^{bc}	0.427±0.046^{c}

* The average value followed by the same letter is not different significant, with a confidence level of α=5%.

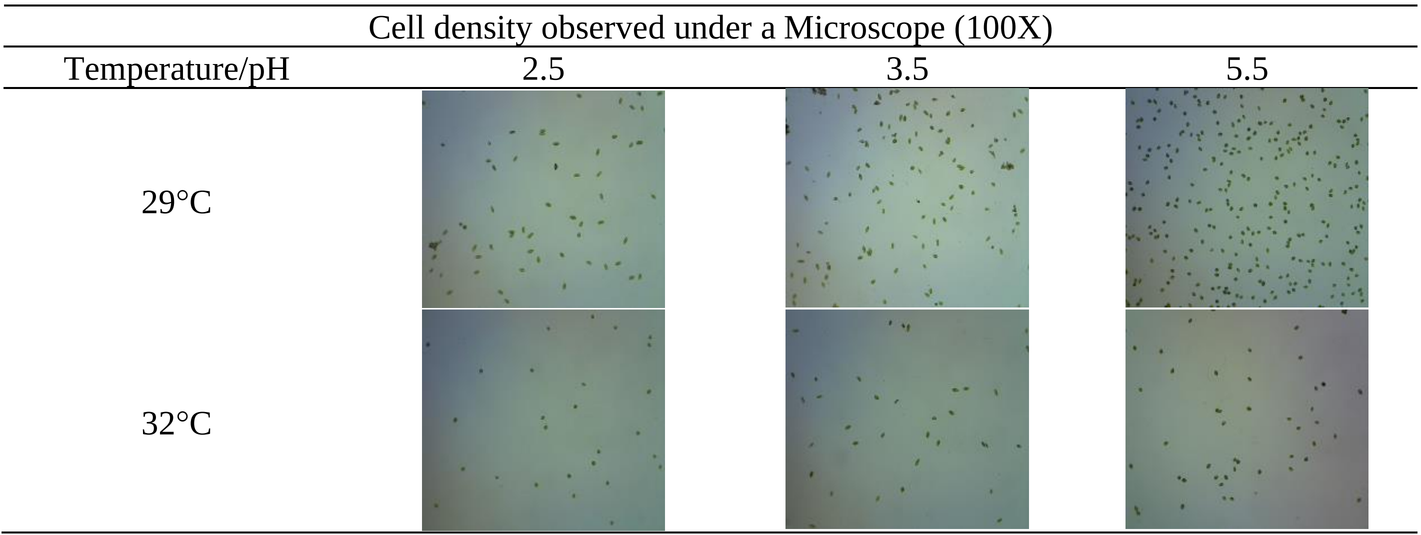

Figure 2. Cell Density of *Euglena* sp. in different treatments.

optimally, so the cell density was low. Cell density affects the amount of biomass and primary metabolites produced.

Biomass of *Euglena* sp.: Based on the results, the biomass at pH 5.5 with an environmental temperature of 29°C had an average of 0.382±0.173 g/L (Table 1). The produced biomass followed by pH 3.5 at 32°C, pH 5.5 at 32°C, pH 2.5 at 29°C, and pH 3.5 at 29°C with consecutive biomass of 0.318±0.130, 0.299±0.138, 0.283±0.133, and 0.229±0.106 g/L, respectively. The pH 2.5 treatment at a temperature of 32°C had a biomass content of 0.222±0.135 g/L, i.e., the least biomass.

Effect of pH and temperature on carbohydrate, protein, and lipid content of *Euglena* sp.: Based on the results, pH 5.5 and 29°C had the highest lipid and carbohydrate content (Fig. 3A). Protein content in *Euglena* (Fig. 3B) at treatment pH 5.5 and 29°C was the highest. Similarly, the lipid content was higher at 29°C than at 32°C (Fig. 3C). Metabolite content in *Euglena* sp. positively correlated with biomass, i.e., the metabolite content increased by increasing the biomass.

Discussions

Based on the results, temperatures of 29°C promote more optimal development in microalgae cultures, whereas 32°C is an extreme and relatively high temperature for *Euglena* sp. Extreme temperatures can inhibit the growth of *Euglena* cells. This result correlated with Buetow's (1962) findings that 25-28.5°C had a higher growth rate than 13-17°C and 30-32°C. *Euglena* can grow above 30°C but cause death and low growth rate. Temperature treatment can affect cell metabolic processes, and increasing the environment's temperature to some extent can increase cell activity. The increase in activity leads to a faster metabolic rate, and rapid metabolic activity

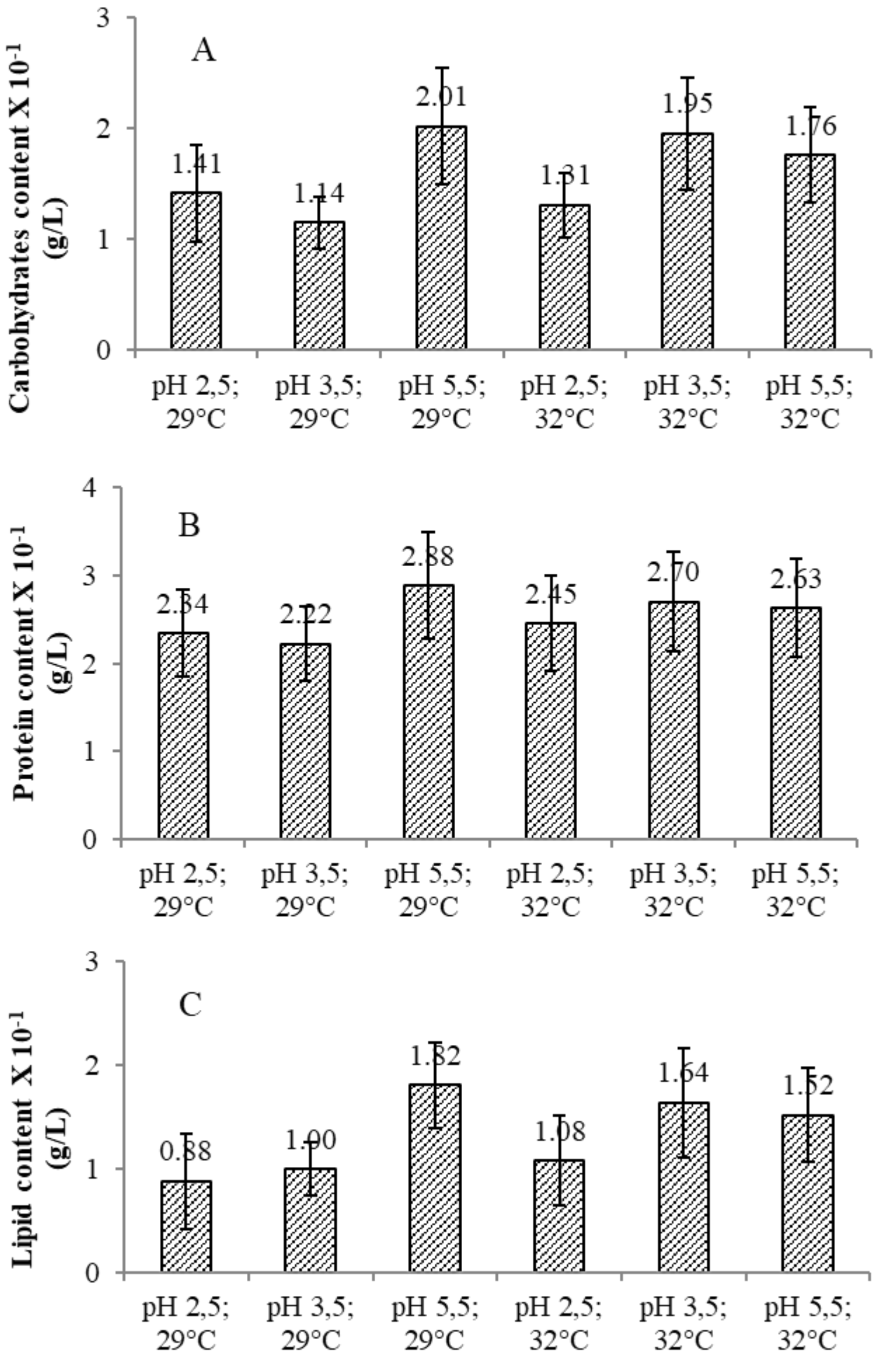

Figure 3. The result of the content of Euglena sp. A: carbohydrate content, B: protein, and C: lipids in different treatments.

causes the rate of cell diffusion. However, if the temperature is too high and exceeds the maximum temperature, it can cause the denaturation of proteins and enzymes. This causes the metabolism to stall, and microalgae cells will experience death. While at low temperatures, enzymes in cells cannot activate, so cell growth is inhibited.

Euglenoids have a high tolerance for acidic environments or low pH (Olaizola, 2003). Based on our results, *Euglena* sp. at pH 5.5 had the most significant growth, which agrees with previous studies that Euglena cell growth was high at pH 4-7 (Olaizola, 2003). At pH 2.5, *Euglena* sp. could not live for a long time and experienced faster death in the present work.

The degree of acidity or pH describes the presence of hydrogen ions in the culture medium. The pH factor can influence microalgae culture cells' metabolic processes and growth. Drastic pH changes can affect the activity of enzymes in cells. In addition, pH changes can inhibit photosynthesis and the growth of some microalgae. The low salinity of the cultural environment affects the pressure of osmosis and osmoregulation of cells. If the pressure of osmosis and osmoregulation of cells changes, it will affect metabolism, respiration, and microalgae cell density. In the present study, the treatment at 5.5 pH and 29°C had the maximum biomass concentration, and the increase in the number of cells is optimal in the long-lasting stationary phase. Biomass directly correlates with cell density and count (Masojidek, 2004).

Carbohydrate content is high when the biomass is high. The temperature can influence the hydrolysis process, accelerating the breakdown of complex sugars into simple sugars (Wang et al., 2011). Temperature does not have a significant effect on protein production. Protein production can be different at different temperatures (Wang et al., 2018). In the current work, the temperature showed a slight insignificant difference. According to Hayashi et al. (1994), pH 3-6 did not have a different effect on the protein content of *Euglena gracilis*. However, Wang et al. (2018) and Hayashi et al. (1994) pointed out that the pH can affect the protein content in *Euglena* when it is in the stationary phase. In the stationary phase, protein synthesis occurs optimally. In the stationary phase, cells no longer focus on cell division but on the formation of metabolites. Therefore, pH 5.5 and 29°C in our work had the highest protein content because the stationary phase lasts long, forming more protein.

The lipid content showed higher lipid content at 29°C than at 32°C. This is supported by previous work on *E. gracilis* culture at 25°C, which had more total lipids than at 30°C (Wang et al., 2011). When the temperature increases, the lipid or fatty acid ratio decreases. *Euglena*, which is at a lower temperature, produces more unsaturated total lipids and polar lipids. On the other hand, high temperatures cause an increase in the total fat content, especially esters (Kawabata and Kaneyana, 1989).

Light intensity influences the process of carbohydrate formation in *Euglena*. Light is closely related to chlorophyll in cells, where chlorophyll captures light for photosynthesis. In photosynthesis, carbohydrates will be obtained as food reserves for *Euglena* (Ferreira et al., 2019). According to Irhamni et al. (2014), the content and composition of lipids and fatty acids are also influenced by light intensity. Light can increase the formation of polyunsaturated fatty acids (PUFA) in the C-16 and C-18 chains and glycerolipids, sphingolipids, and phosphoglycerides in *Euglena* sp. In addition, when the light intensity is optimal, the photosynthetic process in *Euglena* will increase. When photosynthesis occurs, if the carbohydrate content is in excess, it will be stored as lipids (Irhamni et al., 2014).

Conclusion

The results show that the pH treatment 5.5; 29°C has the highest cell growth rate. Additionally, pH treatment 5.5; 29°C contains more carbohydrates, lipids, and proteins than other treatments.

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