



The effect of lignosulfonate chemical stimulator to the metabolites of *Englena* sp.

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ABSTRACT

Englena sp. contains several metabolites that play a role in various sectors, likely biofuels, food, feed, and pharmaceuticals. Multiple treatments are required to increase the content of *Englena* sp. to support its use. In this study, a chemical stimulant in the form of lignosulfonate was added to *Englena* sp. in order to increase its natural potency as measured by lipid, carbohydrate, protein, paramylon content, and biomass productivity. Lignosulfonate is a macromolecular polyelectrolyte and anionic surfactant that is unique and multifunctional. This lignosulfonate can act as an emulsifier, dispersing agent, binder, and stabilizer of lipids in *Englena* sp. cells. In addition to the lignosulfonate chemical stimulator, it can affect the growth process of *Englena* sp. so that it can produce good-quality cells. The method used was a Completely Randomized Factorial Design (CRD) with three treatments and three replications. The results obtained indicated that the addition of lignosulfonate was significantly changed the cell and protein density tests. Therefore, the optimal concentration of lignosulfonate can be used as an agent to increase the growth rate and metabolites of *Englena* sp. significantly.

Introduction

Biomass or renewable resources can be used as biofuels for biodiesel and bioethanol, where the raw materials for these fuel products are available and renewable. The world has experienced a significant increase in biofuel production from 9.2 million tons in 2000 to 95.4 million in 2018 (Paminto *et al.*, 2022). Therefore, alternative energy diversification is needed to reduce oil use by implementing biofuels as an alternative source, especially in the transportation sector. Indonesia ranks third after the United States and Brazil in the use of biofuels in the world, with 98,000 barrels of oil equivalent per day in 2020. The use of biofuels has increased by 2.5% from 96,000 bopd in 2019. Indonesia's use of biofuels makes up 5.8% of the world's total use.

Meanwhile, the use of biofuels in the United States will reach 36 billion gallons by 2022 (Austin *et al.*, 2022). Of course, the use of biofuels can replace the need for oil from fossil energy. Biofuel is the result of processing organic matter into a renewable energy

source that can absorb carbon dioxide as a cause of the high greenhouse effect, as well as a potential alternative energy to be developed in Indonesia (Adhani *et al.*, 2020).

Based on their development, biofuels are divided into three types, including first, second, and third-generation biofuels. The first generation of biofuels is produced from food crops and feed for livestock, such as cassava, vegetables, sugar cane, soybeans, corn, and oil palm (Hussain *et al.*, 2021). However, the development of this first-generation biofuel causes problems because it is derived from food. In comparison, second-generation biofuels are produced from non-food plants containing lignocellulosic compounds, such as wood, and agricultural waste, such as biomass waste and wheat and corn stalks. In addition, second-generation biofuels are less effective because the manufacturing process is too complex (Aliyu *et al.*, 2021).

Furthermore, third-generation biofuels are microalgae-based. These microalgae are referred to as

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cosmopolitan plants because they can grow in fresh, waste, and marine waters (Pal et al., 2019). The use of first-generation biofuels has drawbacks, that their use is entirely overlapping with its use as food, especially since the current climate crisis has severely hampered the growth of food crops. Therefore, exploring the use of third-generation biofuels has better value. Furthermore, this third generation of biofuels is able to overcome problems with previous generations of biofuels by utilizing existing technology (Zakiyah et al., 2020; Alaswad et al., 2015).

Microalgae can be propagated easily and do not require large land areas compared to first and second-generation biofuel raw materials (Iqhrammullah et al., 2024). Some of the advantages of microalgae as a biofuel source agent are having a fast growth phase, being able to convert photons higher, being able to live in extreme environments, being non-toxic and highly biodegradable, not competing with food crops and agricultural land, being environmentally friendly, and accumulating oil. Also, high lipids in the cells (40-90% dry weight) can be used as fuel for making bio-jet and bio-avtur (Furuhashi et al., 2015). Microalgae play an important role in the production of biofuels because they contain lipids at a very high level so that they can be used for third-generation biofuels (Wood, 2021; Saad et al., 2021). Microalgae also have a higher absorption capacity for CO₂ than plants in general. So, the use of microalgae can contribute to SDGs-13 because it can reduce CO₂ emissions to combat climate change's effects (Harada et al., 2020).

Microalgae are unicellular species that can live in colonies or solitary. Microalgae have a diameter between 2-2,000 µm and can live in both marine and fresh waters (Chaidir, 2016). Estimates of the diversity of microalgae in the world are in the range of millions of species with around 200,000-800,000 living species in nature, 35,000 species can be identified, and 15,000 species have known chemical components and biomass composition (Rahman et al., 2019). Microalgae are one of the natural resources that have high potential for solving world problems, namely being an alternative step in tackling the climate crisis. Microalgae are easy to cultivate, require nutrients and sufficient light to carry out photosynthesis, and can produce large amounts of various biomass products quickly (Ali et al., 2014). Several factors can increase the growth rate of biomass, namely light intensity, temperature, nutrients, carbon dioxide, oxygen, and pH. Microalgae have an efficiency level in fixing CO₂ of 10-50 times higher than that of other plants (Agustiyar, 2021). The carbon content in microalgae cells is 50%, of which 1.83 kg of CO₂ can be captured

to obtain 1 kg of microalgae biomass (Mendonca et al., 2021). Microalgae also have a high oil content, reaching 70-85% dry weight (Irhamni et al., 2014).

One of the microalgae species from the Euglenophyta phylum that has high potential is *Euglena* sp. *Euglena* sp. is a unicellular organism with no cell wall and a flagellum as a means of locomotion. *Euglena* sp. has a red eyespot to detect light and sunlight. Each type of microalgae has the ability to absorb carbon dioxide at different levels. Microalgae from the species *Euglena* sp. can absorb CO₂ better than other blue-green pigment microalgae. This is quite different from other microalgae, such as *Spirulina* sp. which have a lower ability and tolerance to absorb CO₂ (Abdurrachman et al., 2013). *Euglena* sp. includes autotrophic organisms, which mean that they can produce their own food photosynthetically by fixing CO₂ very well in large quantities to produce high biomass (Platt et al., 2003). Therefore, *Euglena* sp. It can live in both photoautotrophs and heterotrophs. It can be called a photoautotrophic organism because *Euglena* sp. can carry out photosynthesis during heterotrophs because they are able to eat organic substances available in their environment to be used as an energy source (Grimm et al., 2015). Based on its nature and way of life, *Euglena* sp. has high levels of biomass and nutrients because of its high productivity. The high natural content, such as lipids, proteins, and carbohydrates owned by microalgae, is also helpful as a food source. In addition, microalgae also play an essential role in improving food nutrition because it contains several sources of vitamins, including vitamins A, B, B1, B2, B6, B12, C, E, nicotinate, biotin, folic acid, and pantothenic acid (Harun, 2010).

The uniqueness of the metabolites produced by *Euglena* sp. from its biomass content, which is a source of food protein that reaches 70%, carbohydrates in the form of paramylon which account for 40% of the total biomass, and lipids in high amounts that reach 50-60% so that it has high prospects for large-scale commercialization in the cultivation process (Gissibl et al., 2019). It can also be integrated with waste recovery from the environment through phytoremediation (Mahapatra et al., 2013). The availability of *Euglena* sp. can be cultured to meet the needs of biofuel production and play a crucial role in the future (Dong et al., 2016). *Euglena* sp. is one of the potential microalgae in the lipid production process and can be used as raw material for biofuels. Lipid compounds produced by *Euglena* sp. include palmitic fat, linolenic acid, and linoleic acid (Asiandu et al., 2023). *Euglena* sp. can accumulate lipid content to a relatively high level. In addition,

Englena sp. can also produce high-value products that include 20 amino acids, long-chain polyunsaturated fatty acids, vitamins, minerals, and β -1,3-glucan, known as paramylon (Gissibl et al., 2019).

Englena sp. can accumulate large amounts of β -1,3-glucan in 20-75% of dry weight when cultured with adequate carbon and nutrient sources (Muchut et al., 2018). Paramylon is a linear β -glucan consisting of β -1,3 linkages and acts as an immunomodulator of β -glucan, it is lipophilic, and cannot be digested by humans due to a deficiency of β -1,3 glucanase in the human digestive system (Lam & Cheung, 2013). Therefore, β -1,3-glucan from *Englena* sp. can be an effective alternative to replace β -1,3/1,6-glucan derivative yeast in the pharmaceutical and nutraceutical fields (Barsanti & Gualtieri, 2019; Bhattad et al., 2021). Paramylon can be defined as a natural polysaccharide-derived compound that can be used as a food reserve for *Englena* sp. as well as an additive in food products and medicines. This paramylon compound is in great demand because it has several beneficial ingredients in the health sector. *Englena* sp. can produce paramylon content in very high amounts, reaching more than 60% of the total dry weight (Kim et al., 2019). The urgency of other uses owned by *Englena* sp. is that it is capable of producing carbohydrates and stored in the form of paramylon. This compound can potentially be used as a raw material for biofuels to produce energy (Asiandu et al., 2023).

Several treatments are needed to increase the content possessed by microalgae, especially in the species *Englena* sp. In this study, the addition of a chemical stimulator in the form of lignosulfonate was carried out to increase the natural potency of *Englena* sp. such as lipid, carbohydrate, protein, paramylon content, and biomass productivity. Moreover, this must be supported by several parameters that can increase cell density in *Englena* sp. Density is the number of cells from the growth of *Englena* sp. which is calculated every day to know and monitor the level of significance of the growth process of *Englena* sp. in the culture to be tested. In addition, the growth curve using optical density was also measured daily to determine the increase in *Englena* sp. cell growth. Lignosulfonate chemical stimulator in this study functions as a stimulant agent to increase cell metabolism and a growth regulator for *Englena* sp. (Zhu & Wakisaka, 2021). The lignosulfonate compound is in the form of brown powder obtained from making softwood sulfite pulp (Feizi et al., 2019). The availability of this compound is abundant in nature, the price is affordable, and it is environmentally friendly. Lignosulfonate is a

macromolecular polyelectrolyte and anionic surfactant which is unique and multifunctional. This lignosulfonate can act as an emulsifier, dispersing agent, binder, and stabilizer of lipids in *Englena* sp. cells. This can help improve the physical quality of pellets in *Englena* sp. cells. (Catargiu, 2015). In addition to the lignosulfonate chemical stimulator, it can affect the growth process of *Englena* sp. so that it can produce good-quality cells (Zhu & Wakisaka, 2021).

The research was conducted to test the effect of adding a lignosulfonate chemical stimulator to *Englena* sp. this has never been done before. This study uses microalgae candidates considered potentially suitable objects to be formulated using lignosulfonates, namely *Englena* sp. strain 33 from the Dieng Plateau Region, Central Java. The lignosulfonate addition formulation used in this study had a concentration of 2.5 g/L in the first treatment and 5 g/L in the second treatment. Meanwhile, a comparison based on research conducted by Zhu & Wakisaka, (2021) explained that in their research on *Englena gracilis*, they used a lignosulfonate addition formulation with concentrations of 10, 100, 500, 1000 and 5000 mg/L. At an optimal concentration of 5000 mg/L lignosulfonate, it can produce growth of *Englena gracilis* up to 1.95 times and reach the highest biomass.

Materials and Methods

Location and time of research

This research was carried out for three months, September-November 2022 in Biotechnology Laboratory and FALITMA (Fasilitas Penelitian Bersama), Faculty of Biology, Universitas Gadjah Mada, Yogyakarta.

Materials and tools of research

The research material used in this study was a sample of *Englena* sp. from Biotechnology Laboratory Faculty of Biology Universitas Gadjah Mada where sampled at Dieng (Central Java, Indonesia), Cramer-Myers (CM) Medium (Cramer & Myers, 1952), solution H_2SO_4 as a pH adjusting solution, 70% alcohol, distilled water, 10% SDS solution, chloroform, methanol, lignosulfonate, 5% phenol, Bradford reagent, Bovine Serum Albumine (BSA). The tools used in this research are TL lamp, magnetic stirrer, conical tube, microtube, ELISA reader, microplate 96-well plate, UV Vis spectrophotometer, tip sterile, centrifuge, beaker, hose, aerator, petri dish, Eppendorf micropipette, vortex, incubator, autoclave, pH meter, analytical balance, bunsen, dropper pipette, LAF (Laminar Air

Flow), inverted microscope, Optilab observer, Neubauer Improved, hemocytometer, glass cover, tissue, cuvette, marker, culture bottle, medium bottle, basket, glass object.

Design of research

This study used an experimental method to examine the effect of adding a lignosulfonate chemical stimulator to the parameters tested on *Euglena* sp. strains 33. The research design used is a Completely Randomized Design (CRD) Factorial, namely factors of lignosulfonate concentration and culture *Euglena* sp. with three repetitions (Table 1).

Table 1. In addition, treatment design lignosulfonate was given during the cultivation of *Euglena* sp.

lignosulfonate Concentration ($C_{11}H_{16}O_8S$) _n	Repetition		
	1	2	3
A1 (2,5 g/L)	A ₁ U ₁	A ₁ U ₂	A ₁ U ₃
A2 (5 g/L)	A ₂ U ₁	A ₂ U ₂	A ₂ U ₃
A3 (Control)	A ₃ U ₁	A ₃ U ₂	A ₃ U ₃

Procedure of research

1. Preparation and sterilization

All tools and materials are prepared. First, the tool is washed with water until clean. Then it is dry and wipe it using a tissue. Next, the perforated equipment is covered with aluminum foil. The materials were autoclaved at 121°C for 15 minutes. Furthermore, autoclave equipment is stored in a storage cupboard.

2. Medium making

The medium used for the initial culture of *Euglena* sp. is the CM medium (Cramer & Myers, 1952).

Table 2. CM medium composition in 1 L of distilled water

Materials	Concentration (mg/L)	Total (mg)
(NH ₄) ₂ SO ₄	1000	1000
KH ₂ PO ₄	1000	1000
MgSO ₄ · 7H ₂ O	200	200
CaCl ₂ ·2H ₂ O	20	20
	Volume (μL)	Volume (μL)
Trace metal MIX (10.000x)	100	100
Na ₂ MoO ₄ · 2H ₂ O solution (10.000x)	100	100

Vitamin B ₁ solution (50.000x)	20	20
Vitamin B ₁₂ solution (40.000x)	25	25

The steps taken were trace metal mix, Na₂MoO₄·2H₂O solution, Vitamin B1 solution, Vitamin B12 solution for CM medium, 100mL was made. Materials The chemical that has been prepared is weighed and put into culture bottles. Added distilled water and homogenized it with a magnetic stirrer. A stock solution of the media in the autoclave and saved in the refrigerator cooler.

3. Inoculation of *Euglena* sp.

Euglena sp. 100 mL was put into 300 mL CM medium in a labeled culture bottle. Culture transfer of *Euglena* sp. strain 33 was carried out using a 50 mL measuring cup in laminar airflow (LAF). First, *Euglena* sp. culture bottles were shaken to make them homogeneous. Then the culture of *Euglena* sp. was closed with cotton and a was prepared to be placed in the culture room.

4. Cultivation of *Euglena* sp.

Bottles measuring 500 mL were sterilized by autoclaving at 121°C, 1 atm for 15 minutes. Pre-cultivation was carried out three times before the primary cultivation was carried out. The temperature of the culture environment is set at 25°C. Aeration in the form of 95% O₂ and 5% CO₂ flowed into the culture with the help of a hose attached to a glass tube and given treatment light intensity of 3000 lux. The process of aeration and lighting is given for 24 hours.

5. Parameter test

Measurement the cell density of *Euglena* sp.

2 mL of sample was taken from the falcon. 0.1 alcohol 70% was added (if the density is calculated for one day after sampling). The sample was placed on a haemocytometer, and then cells were counted under a microscope. Measurement of cell density was carried out every day. The results of cell calculations are converted to the number of cells/mL using the following formula:

The number of cells (cells/mL) :

$$\frac{\text{The number of cells is counted} \times 10^4}{4}$$

In this cell density parameter, the doubling time calculation is also performed using the following formula:

Doubling time (G): $\ln 2 / \mu = 0.693 / \mu$
(Vonshak, 1997)

Description:

G = Doubling time
 μ = Specific growth rate

In addition, the cell density parameter is also calculated for the specific growth rate using the following formula:

Specific growth rate (μ):
$$\mu = (\ln N_t - \ln N_0) / t$$

(Vonshak, 1997)

Description:

μ : Specific growth rate (per day)
Nt : Density of *Euglena* sp. final (cells/mL)
N0 : Density of *Euglena* sp. initial (cells/mL)
T : Time interval from N0 to Nt (days)

Measurement of the growth curve of *Euglena* sp.

2 mL of sample was taken from the falcon and put it in the cuvette. 2 mL of distilled water was also placed into the cuvette as a blank solution. Absorbance was measured using a spectrophotometer with wavelengths of 680 nm and 860 nm. The results of the data obtained are tabulated in *Microsoft Excel*.

Testing the lipid content of *Euglena* sp.

Lipid testing in this study used the Bligh & Dryer (1959) method. The first step is to weigh the empty petri cup using an analytical balance. Next, 5 mL of the sample was centrifuged at 4000 rpm for 10 minutes at 4°C. The supernatant was discarded, and the pellet was added with 2 mL of methanol and 1 mL of chloroform, then homogenized using a vortex. Then, 1 mL of chloroform and 1 mL of distilled water were vortexed. Next, centrifuged at 4000 rpm for 10 minutes at 10°C. Centrifugation results formed three layers, and then the yellow layer (lipid) was taken using a dropper pipette. Next, it was placed in a dry petri dish that had previously been weighed, and then the petri dish was incubated at 40°C. Then the petri dish was weighed, and the lipid content was calculated by dividing the weight difference by the sample volume.

Testing the carbohydrate content of *Euglena* sp.

5 mL of the sample was put in a conical tube and centrifuged at 4000 rpm for 10 minutes. The supernatant was discarded and the pellet was added

with 5% phenol 500 μ L and a solution of H₂SO₄ by 1 mL. Samples were incubated at room temperature for 30 minutes. 2 mL of sample was put into a glass cuvette. The absorbance was measured with a UV Vis spectrophotometer with a wavelength of 490 nm. The blank solution is 0.5 mL of distilled water plus 500 μ L of 5% phenol and 1 mL of H₂SO₄. Calculations are made using a standard curve using the following formula,

A standard curve (X): $\ln (Y/0.58) / 0.0003$

Description:

Y : Optical density of the sample

Testing the protein content of *Euglena* sp.

Protein testing in this study used the Bradford (1976) method. The initial step was to weigh 2 mL of the sample and place it in a microtube. Then it was centrifuged for 10 minutes at 3000 rpm, and the supernatant was discarded. Next, the pellet was added with 1 mL of 10 % SDS solution. The sample was heated at 95°C for 5 minutes and then incubated at 4°C for 5 minutes. Samples were taken of 8 μ L and put into the microplate. Next, the sample was added to Bradford's solution of 200 μ L. Protein measurements were carried out using an ELISA reader with a wavelength of 595 nm.

Testing the paramylon content of *Euglena* sp.

Sample (5 ml) was put in the conical tube. Then d is centrifuged for 10 minutes at a speed of 4.000 rpm, and the supernatant is discarded to obtain pellets. The pellet was dissolved using a solution of 1% (w/v) SDS and 5% (w/v) Na₂ EDTA. Then the pellets were incubated at 37 °C in a water bath for 30 minutes. The treatment in the SDS-Na₂ EDTA solution was repeated without incubation. Then paramylon was washed using distilled water two times. Paramylon pellets were dissolved in 2 mL NaOH. Measurement of paramylon concentrations was carried out using the method of phenol-sulfuric acid. In a test tube, the extracted solution is mixed with 5% phenol and H₂ SO₄. The test tube was left in an upright position for 10 minutes. The solution was homogenized for 30 seconds before incubating for another 20 minutes at room temperature. After that, analysis was carried out using a spectrophotometer with a wavelength of 490 nm. A standard curve was prepared using the previous method by replacing the sample with pure glucose. Calculation using a standard curve can be done using the following formula:

A standard curve (X):

$\ln (Y/0.056) / 0.0003$

Description:

Y : Optical density of the sample

In addition, paramylon also calculates its productivity level using the following formula,

Productivity ($\frac{\text{mg}}{\text{L}}/\text{day}$):

$$\frac{\Delta x}{t}$$

Description:

Δx :Final metabolite level - initial metabolite level
 t :Total days

Testing the productivity content of *Euglena* sp. biomass

Microtubes were weighed, and the weight was recorded. 2 mL of sample was taken and put into a microtube and centrifuged at 3300 rpm for 5 minutes. The supernatant was discarded from microtubes. The microtubes were dried using an incubator at 30°C until their shape was constant. Biomass is weighed with an analytical balance. The result is reduced by the weight of the petri dish that was weighed at the beginning. The results are recorded as *Euglena* sp. biomass. Biomass productivity can be calculated using the following formula from Schagerl et al. (2022) with modifications.

Biomass productivity:

$$\frac{\text{Total weight of microtube- Early weight of microtube}}{\text{Sample volume}}$$

Data analysis

Data analysis in this study used Microsoft Office Excel 2013 to process calculation data in lipids, carbohydrates, proteins, paramylons, biomass productivity, cell density, and optical density. Furthermore, the ANOVA method used the SPSS v.25 (Statistical Program for Social Science) application. ANOVA is used to determine the significance of the treatment at the level of confidence $\alpha = 5\%$ ($p = 0.05$). If the results are significant, they will be followed by Tukey's HSD and DMRT tests which help know there are significant differences between lignosulfonate concentration. Finally, the parameters that have been tested are tabulated with the results, and the specific growth rate, doubling time, and productivity are calculated. Graphical data were obtained for the cell density parameter, results for specific growth rate and doubling time, and the average cell density from day 0 to day 12 was calculated using SPSS. On the optical density parameter, data is obtained in the form of a curve and the average optical density with a wavelength of 680 nm from day 0 to day 12 which is calculated using SPSS. Data on the parameters of

lipids, carbohydrates, proteins, paramylon, and biomass productivity were obtained in the form of productivity results and maximum value results on days 0, 4, 8, and 12 using SPSS.

Results

Growth curve of *Euglena* sp.

The results of the growth curve can be seen in Figure 1.

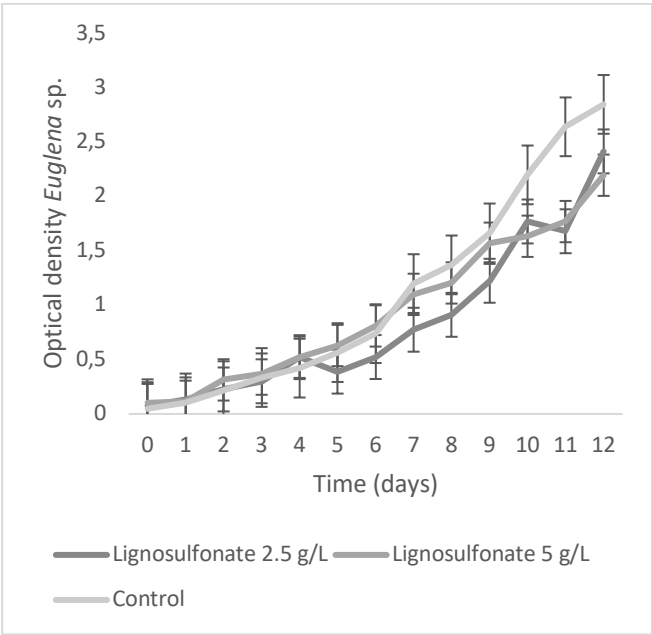


Figure 1. The growth rate of *Euglena* sp.

Based on Figure 1, it can be seen that the growth phase of the treatment with the addition of a lignosulfonate chemical stimulator showed a lag phase that occurred from day 0 to day 2. Day 3 to the last day of observation is the log phase and still shows growth. The stationary phase and death phase were not seen in the results.

Based on statistical tests using SPSS, the growth curve data were normally distributed, but the ANOVA results showed that the overall treatment was not significantly different. Furthermore, the ANOVA results have a significance value of 0.108 indicating that they are not significantly different (see Table 3).

Table 3. Average absolute growth curve

Treatment	Average
A1	2.339 ^a
A2	2.092 ^a
A3	2.848 ^a

Based on the three treatments, namely A1 (lignosulfonate concentration of 2.5 g/L), A1 (lignosulfonate concentration of 5 g/L), and A3 (control), the results showed no difference.

Cell density of *Euglena* sp.

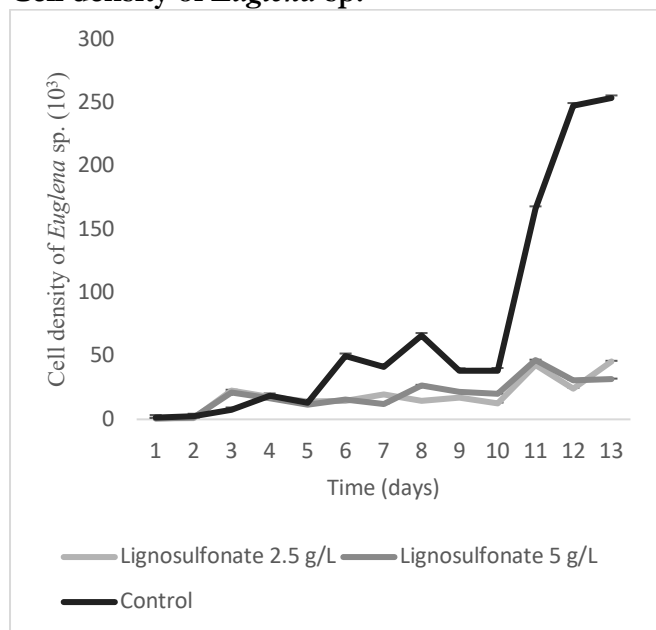


Figure 2. Cell density measurement graph

Figure 2 shows that the growth phase of the treatment with the addition of a lignosulfonate chemical stimulator had a lag phase from day 0 to day 2. Day 3 to the last day of observation is the log phase. It also shown that stationary phase and death phase are not present after 12 days observation.

The cell density data showed a normal distribution based on statistical tests using SPSS with a 95% confidence level, but the ANOVA results revealed a significant difference in the overall treatment. Furthermore, ANOVA results show a significance value of $0.001 < 0.05$, which is significantly different.

Further tests (post hoc) revealed that treatment control was significantly different from lignosulfonate treatments. However, the lignosulfonate treatment with a concentration of 2.5 g/L was not significantly different from that of 5 g/L (Table 4).

Table 4. Specific growth rate and doubling time of *Euglena* sp.

Treatment	Specific Growth Rate	Doubling Time
A1	0.356 ± 0.086^a	2.017 ± 0.429^a
A2	0.327 ± 0.032^a	2.136 ± 0.218^a
A3	0.400 ± 0.062^b	1.766 ± 0.300^b

At cell density, calculations were also made for the specific growth rate and doubling time which showed the highest value for the specific growth rate was in the control treatment, which was equal to 0.400 ± 0.062 with a doubling time of 1.766 ± 0.300 . In comparison, the lowest specific growth rate was

found in the lignosulfonate treatment with a concentration of 2.5 g/L, 0.356 ± 0.086 , and a doubling time of 2.017 ± 0.429 .

Parameters of lipid

The highest lipid productivity was found in the control treatment, which was 129.292 ± 13.439 mg/day, while the lowest productivity results were found in the lignosulfonate treatment with concentrations of five g/L equal to 18.807 ± 4.941 mg/day (Table 5).

Table 5. Lipid productivity

Treatment	Lipids (mg/day)
A1	32.570 ± 15.745^a
A2	18.807 ± 4.941^a
A3	129.292 ± 13.439^a

Lipid data showed a normal distribution on statistical tests using SPSS, but the ANOVA results revealed no significant difference across the entire treatment. Furthermore, the ANOVA results show a significant number of 0.264, which is similar.

Parameters of carbohydrates

The highest carbohydrate productivity was found in the control treatment, which was 0.176 ± 0.004 µg/day. In contrast, the lowest productivity results were found in the lignosulfonate treatment with a concentration of 2.5 g/L equal to 0.020 ± 0.006 µg/day (Table 6).

Table 6. Carbohydrate productivity

Treatment	Carbohydrate (µg/day)
A1	0.020 ± 0.006^a
A2	0.028 ± 0.003^a
A3	0.176 ± 0.004^b

On statistical tests using SPSS, the carbohydrate data were normally distributed, but the ANOVA results showed that the overall treatment was not significantly different. Furthermore, the ANOVA results show a significance value of 0.102, which is similar.

Parameters of protein

The highest protein productivity was found in the control treatment, namely 0.026 ± 0.014 µg/day, while the lowest productivity results were found in the lignosulfonate treatment with a concentration of 2.5 g/L, 0.009 ± 0.002 µg/day (Table 7).

Table 7. Protein productivity

Treatment	Proteins (µg/day)
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A1	0.009 ± 0.002^a
A2	0.010 ± 0.000^a
A3	0.026 ± 0.014^b

Statistical tests using SPSS revealed a normal distribution of protein data with ANOVA results, indicating significant differences across all treatments. Furthermore, the ANOVA results showed that the $F_{\text{count}} > F_{\text{table}}$ was 5.142, indicating that the results obtained were significantly different from the control treatment.

Parameters of paramylon

The highest paramylon productivity was found in the control treatment, which was 0.041 ± 0.012 µg/day, while the lowest productivity results were found in the lignosulfonate treatment with concentrations of 5 g/L which is equal to 0.024 ± 0.011 µg/day (Table 8).

Table 8. Paramylon productivity

Treatment	Paramylon (µg/day)
A1	0.029 ± 0.014^a
A2	0.024 ± 0.011^a
A3	0.041 ± 0.012^a

Statistical tests using SPSS typically distributed the parametric data, but the ANOVA results showed that all treatments were not significantly different. Furthermore, the ANOVA results have a significance value of 0.251, indicating that they are not significantly different.

Parameters of biomass productivity

The highest biomass productivity was found in lignosulfonate treatment with concentrations 5 g/L, which is equal to 15.438 ± 2.833 mg/day, while the lowest productivity results were found in the control treatment 5 g/L, which is 13.321 ± 4.659 mg/day (Table 9).

Table 9. Biomass productivity

Treatment	Biomass Productivity (mg/day)
A1	14.992 ± 5.986^a
A2	15.438 ± 2.833^a
A3	13.321 ± 4.659^a

The growth curve data, when subjected to statistical tests using SPSS, displayed a normal distribution. However, the ANOVA results revealed no significant differences among all treatments. Furthermore, the ANOVA results show that the

significant number is 0.86, meaning it is not significantly different.

Discussion

Lignosulfonate have the potential to increase the growth curve and cell number in *Englena* sp. cultures. However, it is possible that lignosulfonate can also cause fluctuations in the increase in the cell content of *Englena* sp. so that the quantity of lipids, carbohydrates, proteins, paramylon, and biomass productivity is unstable towards the next day. This is reasonable because lignosulfonates are complex phenolic polymers that are less susceptible to degradation caused by many aryl-aryl bonds and high redox potential (Guo et al., 2017).

In other cases, this lignosulfonate compound can temporarily increase the induction of endogenous indole-3-acetic acid (IAA) in polar shoots. In addition, research by Zhu & Wakisaka, (2021) shows that the growth results of *Englena gracilis* are related to the effects of phytohormones like those of higher plants because they have similarities to microalgae in terms of cellular response.

The growth curve test for microalgae conducted by Tan et al. (2021) using the treatment with the addition of vanillic acid obtained results that were not significantly different either. This is because vanillic acid can inhibit microalgae's growth by inducing the formation of superoxide anion radicals.

However, the research conducted with the addition of the lignosulfonate chemical stimulator did not reveal the presence of inhibitory compounds in the lignosulfonate that could inhibit the growth of *Englena* sp. Based on research conducted by Tan et al. (2021) on *Englena gracilis* using low concentrations of vanillic acid (10 mg/L and 100 mg/L) there was no increase in the growth of *Englena gracilis* cells, so the results were not significantly different. The addition of vanillic acid treatment with high concentrations (500 mg/L and 800 mg/L) was found to stimulate *Englena gracilis* cell growth. It is known that treatment at high concentrations experienced an increase, as indicated by the stimulation effect starting after the sixth day, so significantly different results were obtained.

This statement is inversely proportional to the treatment of adding lignosulfonate to *Englena* sp. cells that was carried out in the present study. The addition of lignosulfonate in high concentrations (2.5 g/L and 5 g/L) has an unfavorable impact on the growth of *Englena* sp cells due to interference in the absorption process of light absorption. This causes the growth of *Englena* sp. cells to also decrease. Due to this decrease, the results obtained were similar

because the lignosulfonate addition treatment and the control obtained had similar results (Table 3). Therefore, future research can be carried out using the addition of a lignosulfonate chemical stimulator at optimal concentrations, namely in the low concentration range in the lignosulfonate treatment, to produce significantly different biomass productivity (Zhu & Wakisaka, 2021). However, in future research, it is necessary to do trial and error first so that we can find out and detect any mistreatment used and at the same time, anticipate failures in research so that maximum results can be obtained through an optimal research process.

Based on research conducted by Zhu & Wakisaka, (2021) shows that the addition of a lignosulfonate chemical stimulator with the highest concentration reaching 5000 mg/L can increase the specific growth rate and reduce cell doubling time in *Englena gracilis* cells. The cell density reached its maximum value on day 23, which indicated that the cell growth stabilized at the end of the logarithmic phase. Adding lignosulfonate at a concentration of 5000 mg/L can increase the growth rate to 1.95 times. This proves that the addition of lignosulfonates can have a significant effect on increasing the number of *Englena* sp. cells.

In this study, the addition of lignosulfonate concentrations of 2.5 g/L and 5 g/L significantly increased cell density for 12 days. The addition of the number of cells occurs slowly and only takes place moderately, increasing in large numbers. Lignosulfonate has a high glucose content, so the carbon source of *Englena* sp. is fulfilled and biomass productivity also increases because there are abundant carbon sources. The suitability of the conditions in the culture medium in the presence of a lignosulfonate chemical stimulator indicated that *Englena* sp. can survive and develop properly and optimally.

In a study by Zhu & Wakisaka (2021), adding lignosulfonate at a concentration of 5000 mg/L could increase the relative lipid content by 6.3%. The yield of this lipid increased by about 2.07 times. Then there was lipid test with the addition of 150 mg/L of the chemical stimulator phenol to *Dunaliella salina* cells which also increased by 26% (Tan et al., 2021). It is possible that this effect is due to the emergence of antioxidant stress through the induction of phenolic acids which leads to the activation of the antioxidant system and also increases the production of metabolites (Zhou et al., 2017). Based on research conducted by Kong et al. (2013), it was found that the addition of glucose and glycerol to *Chlorella vulgaris* under mixotrophic conditions was significant.

The lipid produced from the addition of 10 g/L glycerol and 2 g/L glucose reached 10.64%. These glycerol and glucose compounds are a source of organic carbon, increasing the metabolite content of microalgae (Kong et al., 2013).

However, the case of adding a lignosulfonate chemical stimulator to *Englena* sp. carried out in this study did not differ significantly in the increase on lipid levels in *Englena* sp. due to the use of too high lignosulfonate concentrations. Medium culture of *Englena* sp. changed the color to black-brown, so it greatly affected the survival of *Englena* sp.. There is a disturbance in the process of light absorption. This causes the productivity of the resulting lipids to decrease. Therefore, based on previous studies, a decrease in metabolite productivity will cause the results obtained to be similar (Zhu & Wakisaka, 2021).

Based on research conducted by Kong et al. (2013) on *Chlorella vulgaris*, treatment with the addition of glycerol at a concentration of 1 g/L showed results that were not significantly different from those of carbohydrates. However, if the glycerol concentration is increased to 10 g/L, it can show significantly different results (Kong et al., 2013). This is inversely proportional to the treatment with the addition of lignosulfonate, which at high concentrations disrupts the metabolic processes of *Englena* sp. cells. It is because if the culture medium is filled with color pigments that are too concentrated from the added lignosulfonate compound and cause decrease in the light absorption process for survival *Englena* sp. (Zhu & Wakisaka, 2021). that is possible to produce values that are not significantly different in the treatment of adding 2.5 g/L and 5 g/L lignosulfonate. This is evidenced in Table 6, where treatments A1, A2, and A3 have similar results.

In addition, glycerol compounds do not have a strong color compared to lignosulfonates, which can easily change the color of water in culture media. This causes no disturbance in the absorption of light on the *Chlorella vulgaris* culture medium so the results can differ significantly. However, in conditions of minimal lighting due to the addition of too high a concentration of lignosulfonate, *Englena* sp. can still live and grow even though it has not reached its optimal point. In the case of research on *C. pyrenoidosa*, there was a process of decreasing the cell's carbohydrate content while the resulting protein content continued to increase. These differences in changes indicate that in the absence of light, carbohydrates can still be stored intracellularly in metabolic processes to serve as an energy source

in cell maintenance and protein synthesis (Kong et al., 2013).

Based on protein tests through research conducted by Zhu & Wakisaka, (2020), there was treatment with the addition of mannitol at a concentration of 4 g/L and xylitol with a concentration of 4 g/L against *Euglena gracilis* showing that the results obtained were significantly different because they could be appropriately identified. A study conducted by Kong et al. (2013) on the *Chlorella vulgaris* protein test showed that the treatment of glycerol supplementation with a concentration of 10 g/L and glucose with a concentration of 2 g/L added to the SEM showed significantly different results from the results of the protein test. The two studies that tested protein productivity showed similarities with the results of the protein productivity test conducted in this research.

The addition of the lignosulfonate chemical stimulator at concentrations of 2.5 g/L and 5 g/L used in this study showed significantly different results in increasing protein productivity levels in *Euglena* sp. This is because lignosulfonates contain various compounds such as macromolecular polyelectrolytes, polymer anionic surfactants, and phenolic complexes that are less susceptible to degradation due to a large number of aryl-aryl bonds and high redox potential (Guo et al., 2017). In addition, this lignosulfonate can act as an emulsifier, dispersing agent, and binder of metabolites including proteins in *Euglena* sp cells. This can improve the quality of physical pellets in *Euglena* sp cells. (Catargiu, 2015). Therefore, research on protein productivity in *Euglena* sp. can increase too.

In a study conducted by Ivus'ic & S'antek, (2015), the chemical stimulator DAP (*Diammonium Hydrogen Phosphate*) was added with concentrations above 2 g/L to *Euglena gracilis*. This research shows that the results obtained are not significantly different with paramylon productivity on *Euglena gracilis*. This research follows the literature that adding a lignosulfonate chemical stimulator at concentrations of 2.5 g/L and 5 g/L in the paramylon productivity test obtained results that were not significantly different from *Euglena* sp. cells. This is evidenced in Table 8 where treatments A1, A2, and A3 have similar results.

The similarity of the results in this study with the literature obtained lies in the number of chemical stimulator concentrations used in each treatment. The higher concentration of chemical stimulators disrupts metabolism. It has an impact on inhibiting the productivity of paramylon content in *Euglena* sp. cells. In this study, a lignosulfonate chemical

stimulator was used with the highest concentration reaching 5 g/L so that it could cause a black-brown color change in the culture medium. This condition's urgency significantly affects *Euglena* sp.'s survival due to interference in the absorption process of light. Metabolic processes in *Euglena* sp. cells. It will be hampered so that it can reduce the productivity of the paramylon. This also affects the productivity of the paramylon so that it is not significantly different (Zhu & Wakisaka, 2021).

This statement is supported by the literature, which states that an excess of the chemical stimulator DAP (*Diammonium Hydrogen Phosphate*) at a concentration of up to 10 g/L can stop the growth rate of *Euglena gracilis*. This will also decrease the productivity of metabolites, including paramylon (Ivus'ic & S'antek, 2015). In addition, the use of additional inorganic sources in the form of DAP (*Diammonium Hydrogen Phosphate*) causes inhibition of paramylon productivity. This is due to the process of releasing free ammonia and direct interaction with the *Euglena gracilis* plasma membrane, causing osmotic stress. Even though this free ammonia has a significant effect on the growth of *Euglena gracilis* (Ivus'ic & S'antek, 2015), the addition of glucose in high concentrations can lead to an increase in paramylon production because glucose acts as the main building block for paramylon the cells of *Euglena* sp. (Kim et al., 2021).

Euglena sp. cells. have a close correlation between paramylon and carbohydrates. *Euglena* sp. is capable of producing stored carbohydrates in the form of paramylon. Paramylon is a natural polysaccharide-derived compound that can be used as a food reserve for *Euglena* sp. cells. *Euglena* sp. synthesizes paramylon from excess carbon sources in the culture medium. *Euglena* sp. consumes carbohydrate polymers to increase the stability of metabolites and growth rates and maintain their live (Asiandu et al., 2023). In this study, the test on the productivity of carbohydrates and paramylon had similarities. In other words, the results were not significantly different. ANOVA results on carbohydrates show a significant number of 0.102, while ANOVA results on paramylon show a significant number of 0.251. This can be used as evaluation material for further research. One of them is paying attention to the use of optimal concentrations of chemical stimulators on the condition that they do not hinder the process of absorbing light and do not inhibit the metabolic processes of *Euglena* sp. so that they can be applied appropriately to culture media (Zhu & Wakisaka, 2021).

Based on research conducted by [Zhu & Wakisaka, \(2021\)](#) shows that the yield of metabolites in the form of biomass is affected by the lignosulfonate concentration which is closely related to the reception of light intensity in order to support the survival of *Euglena gracilis*. In this study, the highest lignosulfonate concentration used was only 5000 mg/L which caused the water in the culture medium to turn brownish yellow so that it did not become an obstacle in absorbing light for *Euglena gracilis* to use. So, this research resulted in high biomass productivity ([Zhu & Wakisaka, 2021](#)). While in this study, the highest lignosulfonate concentration used reached 5 g/L and caused the water in the culture medium to turn black-brown which greatly affected the survival of *Euglena* sp. due to interference in the process of absorption of light. This means that the productivity of the biomass produced could be much higher. So, the addition of lignosulfonate concentrations with too high concentrations can reduce biomass productivity, so the results also cause no significant difference. This is proven in Table 9 above demonstrates similar results, indicating minimal differences between treatments A1, A2, and A3. Future research can use optimal concentrations of a lignosulfonate chemical stimulator to produce significantly different biomass productivity ([Zhu & Wakisaka, 2021](#)).

Conclusion

The study's results indicate that the addition of a lignosulfonate chemical stimulator influences the significance of the increase in metabolite parameters and the growth rate of *Euglena* sp., as demonstrated by several parameter tests. The parameter test revealed that the results significantly differed in the number of cells and protein density tests. At optimal concentrations, adding lignosulfonates can increase cell growth by 2.5 times. In addition, it can significantly increase protein metabolites and *Euglena* sp. cell growth rate.

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