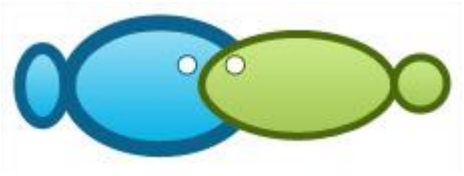

The effect of washing seed cells on the growth patterns and quantity of *Spirulina platensis* cell culture

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Abstract. *Spirulina platensis* is a microalga that is a promising sustainable food source. The most important aspect in cultivating *S. platensis* is the quality and quantity of the microalgae cells, which has a direct impact on economic viability and nutritional value. Washing seed cells is one method that can be applied to improve the quality of *S. platensis* cell seeds. This research focuses on exploring the impact of cell washing on *S. platensis* seedlings. This research used liquid and agar of Zarrouk media for *S. platensis* culture. All media was sterilized using an autoclave and *S. platensis* seeds were inoculated on Zarrouk agar medium in a Petri dish. Seed observations were carried out for 10-12 days under an inverted microscope. The seeds which are uniform and solid are then transferred to liquid Zarrouk medium in a 50 ml flask, then 100 ml. To see optimal growth, cell density measurements were carried out. The results show that the cell density of *S. platensis* has the same density, that is 3.6 X 10^5 cells/ml. The growth curve shows that washed cells can survive and continue increasing until day 12, meanwhile the unwashed cell go through to the death phase after day 6. Furthermore, the washed cells have shorter sinuses ranging from 6 to 9 sinuses, but with a denser spiral shape, and the cells are homogeneous. It can be concluded that washed cells have better growth and are able to survive longer than cells that are not washed.
Key Words: microalgae, washed cells, Zarrouk media.

Introduction. *Spirulina platensis* is a type of microscopic blue-green algae that belongs to the phylum Cyanobacteria. These microalgae are rich in essential amino acids, vitamins, minerals, and other bioactive compounds, making them a very promising sustainable food source (Hachicha et al 2022). An important aspect of *Spirulina platensis* cultivation lies in the quality and quantity of microalgae cells, which directly impacts economic viability and nutritional value (Kapoore et al 2021).

Lifespan of *Spirulina platensis* culture is supposed to last 12 to 14 days, but based on several studies it was found that Spirulina can only survive up to the 6th day (Ravelonandro et al 2011; Mahardika et al 2022). It is suspected that this could happen because the *S. platensis* culture is not pure and has been heavily contaminated by other microalgae or other microorganisms, causing cell quality to decrease and is affecting the growth of the *S. platensis* cells (Sow & Ranjan 2021; Chilmawati & Suminto 2012). The

initial cultivation stage, which involves inoculation of microalgae seed cells into the growth medium, is a very important stage in determining the quality of subsequent *S. platensis* biomass. Therefore, improving the quality of seed cells at the beginning of cultivation is very important.

Cell washing is one method that can be used to improve the quality of *S. platensis* seed cells. The aim of this process is to obtain an inoculum that is pure and clean from contaminants so that it can increase the quality and quantity of *S. platensis*. Apart from that, it is known that pure culture can also extend the life of microalgae cell cultures (Andreas et al 2014). Therefore, the effect of cell washing on the growth and quality of microalgae cells remains to be fully addressed.

This study focuses on exploring the impact of cell washing on *S. platensis* germ cells. It is hoped that the results of this research will help in identifying differences in the quality and quantity of *S. platensis* cells from washed and unwashed seed cells, based on analysis of growth patterns and microscopic differences in cells.

Material and Method

Description of the study sites. This study was conducted at Natural Feed Laboratory, Faculty of Fisheries and Marine Science, Diponegoro University, Indonesia. Research took place between 1st of July and 30th of October 2023.

Preparation of tools and materials. Tools were sterilized in two ways, glass tools such as Erlenmeyer flasks were sterilized with the hot steam method in an autoclave at a temperature of 121°C, 1 atm pressure for 20 minutes and other tools such as Petri dishes and dropper pipettes were sterilized with the dry heat method in an oven at a temperature of 180°C, for 1 hour.

Preparation of media. Zarrouk media was used for microalgae culture with the following composition per liter: 1 g NaCl, 0.04 g $CaCl_2.2H_2O$, 2.5 g $NaNO_3$, 0.01 g $FeSO_4.7H_2O$, 0.08 g EDTA (Na), 1 g K_2SO_4, 0.2 g $MgSO_4.7H_2O$, 16.8 g sodium bicarbonate $NaHCO_3$, 0.5 g K_2HPO_4, and A5 micronutrient (H_3BO_3 2.86 g, $MnCl_2.4H_2O$ 1.81 g, $ZnSO_4.4H_2O$ 0.222 g, Na_2MoO_4 0.0015 g, $CuSO_4.5H_2O$ 0.079 g, per L) (Rajasekaran et al 2016). Liquid Zarrouk media was made by mixing the composition of Zarrouk media and A5 micronutrients into 1 L of distilled water, then the media was sterilized using an autoclave at 121°C, 1 atm pressure for 20 minutes. The Zarrouk agar medium was made in the same way, but there is an addition of 7 g/L of natural stem agar and after the sterilization stage it was poured into a sterile Petri dish in an amount of approximately 2-3 ml.

Cell washing method. 1-2 ml of original *Spirulina platensis* seeds were inoculated on Zarrouk agar media in a Petri dish and grown for 2-3 days, then observed under a microscope. After finding *S. platensis* filaments, which were far from contaminants, 3 filaments were taken using a long dropper pipette and then isolated into 3 different test tubes containing Zarrouk's medium by blowing until there were air bubbles in the liquid medium. Then the test tube was shaken and placed on a test tube rack and cultivated under lighting of 3000 lux (L:D = 24:0) and a temperature of 26°C (Andreas et al 2014). For pH, measurements were carried out using a pH meter, the desired pH being of 9 and for salinity a refractometer was used, for a desired salinity of 13 ppt. Observations were carried out for 10-14 days and then observed again under a microscope to see whether the microalgae in the test tube had a uniform cell/filament shape. When they are uniform and dense enough, 10-15 ml of washed seeds can be transferred to liquid Zarrouk media in 3 flasks of 50 ml, then to a larger scale of 100 ml. Cell seeds that were not washed were immediately cultured in 3 flasks of 50 ml flasks, amounting to 10-15 ml, then to a larger scale of 100 ml.

Cell density measurement. Microalgae cell density during observations was calculated using the following formula (Bagun et al 2015):

$$N = \frac{1000}{3{,}14 \times \left(\frac{d}{2}\right)^2 \times p} \times n$$

Where:
N = density of *Spirulina* sp. (unit/ml)
n = number of *Spirulina* sp. per field of view
p = number of visual fields
d = diameter of the field of view (mm)

Observing the quality of S. platensis cell culture. A microscope was used to see the quality of cell culture. Observations were made with 100x magnification.

Results. The growth pattern of *Spirulina platensis* can be seen in Figure 1. The cell density of *S. platensis* was observed every 2 days for 12 days to see the effect of the cell washing method on cell growth. Then, to see the effect of cell washing on the shape and quality of *S. platensis* cells, the cells were observed under a microscope with 100x magnification.

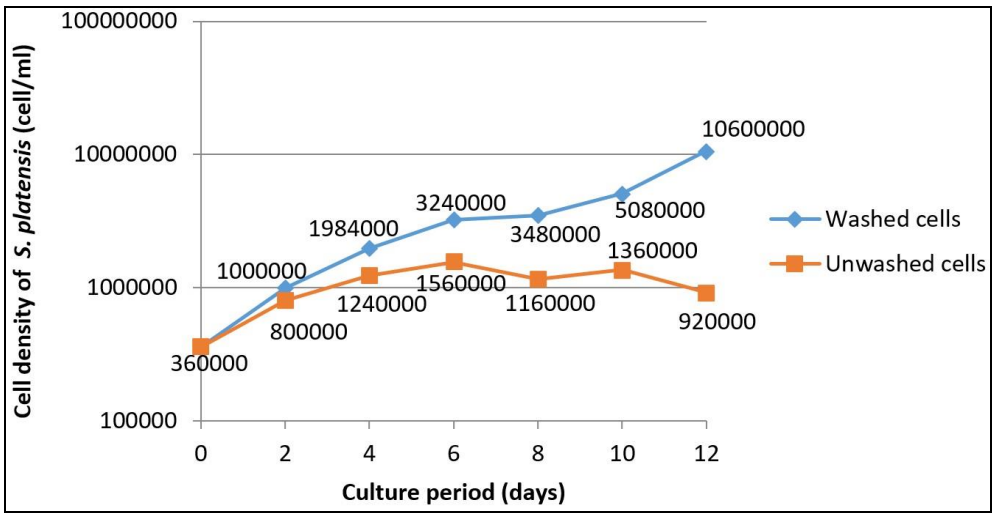

Figure 1. Growth curve of washed and unwashed *S. platensis* for 12 days.

Growth pattern of S. platensis. The results depicted in Figure 1 show that the cell density of *S. platensis* at the beginning of stocking for all treatments had the same density at 3.6 X 10^5 cells/mL. Differences in growth patterns can be seen in cells that have gone through the washing stage. In the culture that had been washed, the cells density increased from day 2 to day 6, then the stationary phase occurred until day 8. However, *S. platensis* cells density then experienced an increase again and continued to increase until day 12. The difference was seen in the culture that had not been washed, *S. platensis* cells density increased from day 2 to a peak on day 6, then decreased on the following day. This shows the washed cells have better growth and are able to survive longer compared to cells that are not washed.

Quality of S. platensis cell culture. There were differences in microscopic observations of washed and unwashed cells (Figure 2). Microscopic images were taken at 12 days old cells.

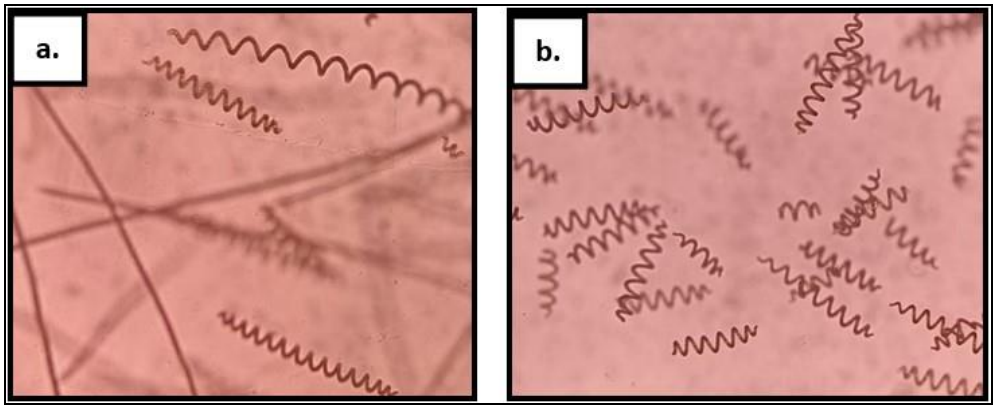

Figure 2. Microscopic view of (a) unwashed cells and (b) washed cells of *S. platensis*.

Based on Figure 2, it can be seen that cells that have not been washed (Figure 2a) there are still other cells in the form of long rods, *S. platensis* cells have longer sinuses ranging from 10 to 11 sinuses, and some of their shapes are spiral and dense, while other cells are not. In cells that have been washed (Figure 2b) the cells have shorter sinuses ranging from 6 to 9 sinuses, but with a denser spiral shape, and the cells are homogeneous.

Discussion. *Spirulina platensis* has 4 phases in its growth pattern, such as the lag phase (induction phase), log phase, stationary phase, and death phase (Caturwati & Setyati 2020; Kim & Lee 2018). The treatment used in this research was washing the cell seeds, and making the water quality parameters (temperature, pH, salinity) and light intensity between all treatments the same.

The washing treatment had a very good impact on the growth pattern of microalgae *S. platensis*, especially in the stationary phase. Cells that have been washed go through the stationary phase on day 6 and day 8, whereas in cells that have not been washed, the cells immediately run into a death phase. In addition, cells that have been washed experience a log phase again which is characterized by cell density increasing continuously and very significantly even after having the stationary phase. Where in general, after microalgae cells experience a stationary phase, the cells will go to the death phase (Suyoso et al 2022; Batac et al 2020), but the cells that have been washed in this study are not even entering the death phase. Similar results were also obtained by research done by Andreas et al (2014) who washed *Chlorella* sp. Cells, and cell expansion also gives positive results on the stationary phase and maximum cell density, which turns out to be able to extend the stationary phase with a high maximum cell density.

The increase in cell density after passing through the stationary phase can occur due to the influence of the treatment used. Cell washing can remove contaminants, both other types of microalgae and other microorganisms such as bacteria, so it can increase the growth of *S. platensis* because it can absorb nutrients in the culture media optimally. According to Chilmawati and Suminto (2012), this is related to competition between microalgae cells and other microorganisms in the culture media to obtain food which can cause the division and growth of microalgae cells to be disrupted. Many cells of microalgae are damaged due to existing contaminants or bacteria (Lian et al 2021). Negative effect of specific bacterial genera on microalgae growth was shown by Tait et al (2019) where the addition of *Pseudomonas* sp. to an axenic *Chlorella vulgaris* culture resulted in a decrease in optical density (OD) by 86%. Furthermore, it has been shown that specific bacteria and their secreted components can induce microalgae flocculation (Wang et al 2012; Lee et al 2013; Lian et al 2018).

Cell washing also affects the quality of the microalgae cells. The cells that have been washed are homogeneous, and there are no other contaminants, especially the other microalgae or other impurities. This can be seen in Figure 2, where the microalgae cells look cleaner and denser, which indicates that the quality of the cultured cells is better. According to Hindarti and Ayuningtyas (2020), the characteristics of good *S. platensis* cells are that they are cylindrical and septate, greenish blue in color, 6–8 μm in

diameter, and have cytoplasmic granules filled with gas. The cells form helical unbranched filaments, measuring 3–5 mm. The filament is motile, sliding along its axis. Apart from that, the most important thing that can be seen from a good *S. platensis* cell culture is that there are no heterocysts, where *Spirulina* spp. has various cell shapes such as larger and longer cells (Sankarapandian et al 2022).

Conclusions. This research was able to prove that the cell washing method affects the growth pattern, as well as the ability of *S. platensis* to grow for longer periods. Apart from that, the quality of the washed *S. platensis* cells meets the characteristics of good *S. platensis* cell quality.

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

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