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Full Length Article

Effect of pH and Neem Extract on Metabolite Content and Bacterial Contaminant of *Arthrospira platensis* Cultures

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Abstract

Arthrospira platensis is a type of microalgae known as a superfood because it has many benefits in the health and renewable energy sectors. Cultivation of *A. platensis* as a renewable energy source is carried out in an open pond system. Bacteria, fungi, grazers and other microalgae frequently contaminated the mass culture of *A. platensis*. Bacteria were the most dominant contaminant in microalgae culture, reaching up to 60%. Botanical pesticides are known to reduce contaminants without damaging microalgae cells. This study presents the combined effect of pH and neem (*Azadirachta indica*) extract on the growth, biomass, pigment, secondary metabolite and bacterial contaminants diversity of *A. platensis*. The results showed that the optimal conditions for growth and metabolite production in *A. platensis* were in the pH range of 9–10 with the addition of neem extract. pH 9 + neem extract produced the highest SGR (specific growth rate), density and cell biomass, namely 2.02×10^6 cells/mL, 0.8 g L^{-1} d^{-1} and 6.22 g L^{-1}, respectively. The pH range of 9–10 is optimal for the production of chlorophyll and metabolites (carbohydrates, proteins and lipids). On the other hand, pH 13 + neem extract produced the highest phycocyanin (0.007 mg L^{-1}) and reduced the number of bacteria by up to 98%. The next-generation sequencing (NGS) analysis involved three samples specifically control treatment on day 0 (A), control on day 7 (B) and pH 13 + neem extract (C) because it had the lowest CFU. The NGS results showed that the bacteria were dominated by the phylum proteobacteria, firmicutes and bacteroidota. Each sample had a different abundance of OTUs (operational taxonomic units). The bacterial community in sample B (227 OTUs) was similar to sample C (202 OTUs) but not similar to sample A (518 OTUs). However, the bacteria in *A. platensis* culture were not completely harmful because they did not significantly suppress the growth and production of metabolites in *A. platensis*. More research is required to investigate the bacteria's relationship pattern with *A. platensis*.

Keywords: Bacterial abundance; High alkali tolerance; Microalga contaminants; Neem extract; Next-generation sequencing

Introduction

Microalgae are living things that are naturally abundant in nature and can play a role in regulating greenhouse gases (CO_2) (Shokravi *et al*. 2019). One of the potential microalgae is *A. platensis*. It is a cyanobacterium known to produce a variety of products including cosmetics, medicines, and feed, as well as renewable fuels. It is capable of producing 65% biomass in the presence of nitrogen depletion and high light intensity stressors (Bautista and Laroche 2021). The success of biomass accumulation depends on the cultivation process applied in the culture process. The cultivation of *A. platensis* in an open pond is the best method for producing biofuel biomass because it does not require expensive equipment or maintenance, lowering production costs (low cost). In addition, the open pond method allows access to the scaling-up and harvesting processes. However, there is a significant impediment, and that is contamination (Shokravi *et al*. 2019).

Contamination continues to be a crucial problem in the cultivation process of *A. platensis*. Bacteria are the most common types of contaminants (Caprio 2020). *Pannonibacter phragmitetus*, a type of bacteria known to be a contaminant in *A. platensis* culture, invades by interfering with the accumulation of products (ethanol) produced by cyanobacteria (Zhu *et al*. 2017). Based on Zhu *et al.* (2020) findings, the impact of contaminants is the destruction of microalgae cells, which inhibits growth and causes the metabolite products produced to be deficient because they become a source of nutrition for contaminant organisms. However, efforts to optimize *A. platensis* mass culture are still needed. Efforts to eliminate contamination using the proper technique and at a low cost because the methods used in previous studies still have various limitations, including the requirement for chemical compounds that are relatively expensive to test in mass cultivation, therefore a solution is required.

In a previous study, Huang *et al.* (2013) applied biological control methods to remove contaminants in microalgae culture. Biological controls used are botanical pesticides, including celangulin, toosedanin, matrine and azadirachtin. The results show that botanical pesticides can be toxic to contaminants while having no effect on photosynthesis or microalgae cell density. In addition, botanical pesticides are known to have the ability to protect the photosynthetic performance of microalgae and help repair cell damage (Zhang *et al.* 2020). One of the potential local botanical pesticides used for the biological control of contaminants in microalgae is neem extract. It has antibacterial, insecticidal, antifungal (Kanwal *et al.* 2011; Khan *et al.* 2020) and larvicidal properties (Sianipar 2020). Neem extract is usually known by the trade name neem oil, an extract from the seeds of the neem plant. This extract contains active compounds, namely azadirachtin, nimbidin, nimbin, salanin, gedunin, margolon, nimbolide, various antioxidant compounds (alkaloids, flavonoids, carotenoids, triterpenoids, steroids) and phenolic compounds (Khan and Javaid 2021). These active components kill pathogenic bacteria, both gram-negative and gram-positive bacteria (Kanwal *et al.* 2011; Gosh *et al.* 2016). In another experiment, it is stated that the compound Azadirachtin, the main component of neem, is used to kill rotifers in microalgae culture along with celangulin, toosendanin, and matrine. Observations were made gradually for seven days. The results showed that azadirachtin can act as a biological control on microalgae cultures, is environmentally friendly, and has an affordable price. In addition, the wide distribution of its habitat makes it easy to find anywhere (Huang *et al.* 2013). Neem plants have an even habitat distribution, especially in Asia, Indonesia, Malaysia, and Thailand. For example, it thrives and is abundant in East Lombok, Indonesia), it can also grow in extreme environments, in this case, in low-nutrient environments and it is resistant to physical disturbances and drought (Susila *et al.* 2014). Therefore, this study aimed to obtain the right strategy to deal with contamination in *A. platensis* cultures by combining pH and botanical pesticides and analyzing metabolite content, growth patterns and bacterial abundance. They are combining environmental and biological controls to optimize methods of dealing with contamination in *A. platensis* cultures.

Materials and Methods

Experimental details and treatments

Experimental material: The culture of *A. platensis* was obtained from the Jepara Brackish Water Aquaculture Center in Central Java, Indonesia. *A. platensis* was grown to a density of 5×10^4 cells mL^{-1}.

Treatments

A total of 370 mL of *A. platensis* was put into 1.85 liters of the medium. The culture tanks used were sterilized using soap and rinsed with clean water. A total of 1.85 L of water was put into the culture bath, and salt (NaCl) was added. Water sterilization was using chlorine as much as 0.03 g L^{-1} for 24 h. Sterilization was carried out again by adding 0.02 g L^{-1} alum. After 24 h, *A. platensis* was put into the medium, followed by the addition of nutrients according to the composition in Table 1. The culture was carried out in a 5 L tank in an open pond and aerated all the time until harvesting. The pH treatment was performed following the administration of nutrients. The pH included pH 9, 10, 11, 12 and 13, according to the preliminary research that had been done before. In addition, there were negative control (-) and positive control (+). Negative control (-) samples were without pH adjustment and without adding neem extract, while the positive control (+) samples were without pH adjustment and with the given neem extract. The pH was adjusted using sodium hydroxide (NaOH) and hydrochloric acid (HCl). pH was measured using a pH meter and conditioning was observed for seven days. The neem extract concentration used was 1 mg L^{-1} (Chia *et al.* 2016). Neem extract was introduced into the culture on the first and third day of culture as much as 0.5 mL L^{-1} (v/v) based on references from previous studies (Huang *et al.* 2013) and preliminary data. The experiments were conducted in triplicate.

Growth, biomass and pigment analyses

Daily measurement of cell density was carried out using the Improved Neubauer hemocytometer. Andersen's method (Andersen *et al.* 2005) was used to determine cell density (N) and specific growth rate (SGR). Daily biomass measurements were performed by passing 15 mL of *A. platensis* culture through a Whatman filter paper (11 µm). Filters were dried at 30ºC in an incubator until constant dry cell weight (DCW) was obtained (Mello and Chemburkar 2018). The following is the biomass calculation formula:

$$DCW = \frac{(\text{Weight of filter + Dried residue – Weight of filter})}{\text{Sample volume}} \times 1000$$

Analysis of pigment content includes chlorophyll *a*, chlorophyll *b*, carotenoids, and phycocyanins. Pigment extraction refers to the method developed by Ilvarasi *et al.* (2012), Lichtenthaler and Buschmann (2001) and Bennet *et al.* (1973). The calculation formula was as follows:

$$\text{Chlorophyll } a \text{ (mg L}^{-1}) = (16.72\ A_{665.2}) – (9.16\ A_{652.4})$$
$$\text{Chlorophyll } b \text{ (mg L}^{-1}) = (34.09\ A_{652.4}) – (15.28\ A_{665.2})$$
$$\text{Carotenoids (mg L}^{-1}) = (1000\ A_{470} – 1.63 \text{ chlorophyll } a – 104.96 \text{ chlorophyll } b) / 221$$
$$\text{Phycocyanins (mg L}^{-1}) = (A_{620} – 0.474A_{652}) / 5.34$$

Where A is absorbance.

Measurement of growth rate and characterization of bacterial contamination

The Total Plate Count (TPC) and spread plate methods were used to count the bacteria in *A. platensis* culture samples.

The stratified dilution was carried out up to 6 times. The TPC procedure follows the method of Boczek *et al.* (2014). After an incubation period of 48 h, count spreader-free plates were filled with 30–300 colonies. The final unit is colony forming units per mL (CFU mL^{-1}) (Soesetyaningsih and Azizah 2020).

The Next Generation Sequencing (NGS) method analyzes the abundance of contaminant bacteria. The samples used were *A. platensis* liquid culture on day 0 (control), day 7 (control) and samples with the lowest number of CFU. The sample with the lowest CFU count is assumed to have the best treatment because it can reduce the number of contaminant bacteria as low as possible compared to other treatments. The selection of the 3 NGS samples was used to see whether or not there were differences in the effect of pH and NE on the diversity of bacterial contaminants in *A. platensis* cultures. The DNA extraction process used the FavorPrep™ Tissue Genomic DNA Extraction Mini Kit. The results of the DNA extraction were sent to the Genetics Sciences laboratory for molecular analysis using NGS.

Metabolite characterization

Carbohydrate characterization followed a modified and optimized procedure developed by Dubois *et al.* (1956). The basic idea behind measuring carbohydrates with the Phenol-Sulfuric Acid method is that carbohydrates produce furfural derivatives when hydrated by sulfuric acid. Colors can be detected colorimetrically as a result of the furfural derivatives and phenol reaction. The procedure of this method was as follows. First, 30 mg of *A. platensis* powder was diluted with 10 mL of distilled water and homogenized. Then, 1 mL was taken, diluted again with 9 mL of distilled water, and homogenized. Next, 1 mL was taken, 0.5 mL of 5% phenol was added and 2.5 mL of sulfuric acid was added rapidly. After allowing the test tubes to stand for 10 min, they were incubated in a water bath at 60ºC for 30 min for color development. After that, light absorption at 490 nm was recorded on a spectrophotometer.

Lipids were measured following the Bligh and Dyer (1959) method. The basic principle of this method was to add chloroform, methanol and water to the sample with an initial ratio of 1:2:0.8 and a final ratio of 2:2:1.8. The total lipid calculation formula was the weight of the lipid in the aliquot multiplied by the volume of the chloroform layer, then divided by the volume of the aliquot.

Extraction and analysis of protein content were determined using the methods of Kadam *et al.* (2017) and Bradford (1976). The *A. platensis* powder was dissolved first using 40 mL of distilled water. The solution was then stored in a room at 4ºC for 16 h. Then, it was centrifuged for 20 min at 9000 rpm at 4ºC. A supernatant (soluble protein) was used for protein analysis. The comparison of the sample extract and Bradford solution is 1: 50 (μL). The standard curve used bovine serum albumin (BSA) with a range of 15.625–2000 g mL^{-1}. A microplate reader was used to measure protein content (standards and samples) at 595 nm. A control blank containing *A. platensis* extract was prepared to remove the effects of extraction solutions and possible reagent incompatibilities.

Statistical Analysis

ANOVA was used for statistical analysis, and the Duncan Multiple Range Test was used for follow-up tests with a significance level of $P<0.05$.

Results

Growth rate, biomass and pigment productivity

Overall, the growth of *A. platensis* in this study was concise. This study's initial cell of *A. platensis* was 5×10^4 cells mL^{-1}. During seven days, cell density and biomass tended to decrease significantly (Fig. 1 and 3). Density, SGR and cell biomass (Fig. 1–3) were highest in the log phase with pH 9 + neem extract treatment of 2.02×10^6 cells mL^{-1}, 0.8 g L^{-1} day^{-1} and 6.22 g L^{-1}, respectively. This result indicated that pH 9 was the optimum pH for *A. platensis* growth, possibly because pH 9 was close to the natural pH of *A. platensis* grown in nature.

Pigment content (chlorophyll *a*, chlorophyll *b*, carotenoids) and phycocyanin on *A. platensis* were presented in Fig. 4–7. Chlorophyll *a* was the highest at pH 10 + neem extract (12.745 mg L^{-1}), chlorophyll *b* was highest in the negative control (7.677 mg L^{-1}), and carotenoid was at pH 9 + neem extract (2.130 mg L^{-1}). Each treatment was significantly different at the 5% significance level. These results indicated that the pH requirement for pigment and phycocyanin production was directly proportional to the pH requirement for optimal cell density and biomass production, which was in the range of pH 8–10. The positive control had a high pigment and phycocyanin content. These results indicated that neem extract did not inhibit pigment and phycocyanin production in *A. platensis*. Phycocyanin levels were low but continue to increase with increasing pH. The highest phycocyanin content was at pH 13 + neem extract (0.007 mg L^{-1}), followed by positive control (0.006 mg L^{-1}). Phycocyanin levels were higher when treated with a combination of neem extract with a high pH.

Combination effect of pH and neem extract on the production of metabolites

The metabolite content and productivity of *A. platensis* were presented in Table 1. The highest carbohydrate and lipid content at pH 9 + neem extract were 0.524 g L^{-1} and 0.132 g L^{-1}, respectively. These results indicate that neem extract has optimum production of carbohydrates and lipids at pH 9–10. The concentration of carbohydrates fluctuated, while the concentration of lipids decreased as the pH of the medium increased.

Table 1: Carbohydrate content (CC, g L^{-1}); carbohydrate productivity (CP, g L^{-1} day^{-1}); protein content (PC, g L^{-1}), protein productivity (PP, g L^{-1} day^{-1}); lipid content (LC, g L^{-1}); lipid productivity (LP, g L^{-1} day^{-1}) of *A. platensis* at different pH and neem extract combinations

Treatment	CC	CP	PC	PP	LC	LP
Control (-)	0.250±0.019^{c}	0.03±0.015	1.883±0.090^{b}	0.25± 0.094^{b}	0.095±0.746^{c}	0.013±0.005
Control (+)	0.469±0.154^{ab}	0.09±0.064	2.382±0.189^{c}	0.37± 0.181^{a}	0.108±0.573^{bc}	0.016±0.008
pH 9NE	0.524±0.108^{a}	0.05±0.043	1.844±0.102^{b}	0.16± 0.093^{b}	0.132±1.066^{a}	0.012±0.007
pH 10NE	0.519±0.092^{a}	0.06±0.032	2.223±0.244^{a}	0.28± 0.137^{a}	0.123±0.168^{ab}	0.015±0.007
pH 11NE	0.310±0.026^{bc}	0.02±0.010	0.676±0.177^{a}	0.04 ± 0.029^{c}	0.121±0.969^{ab}	0.007±0.004
pH 12NE	0.247±0.014^{c}	0.03±0.013	0.519±0.046^{a}	0.06 ± 0.030^{c}	0.119±1.209^{ab}	0.014±0.005
pH 13NE	0.347±0.079^{abc}	0.03±0.015	0.504±0.109^{a}	0.04 ± 0.015^{c}	0.104±0.905^{bc}	0.008±0.004

All tests were performed in triplicates (n = 3) with standard deviation (mean ± standard deviation). Symbols a, b, and c indicate that treatments were significant (P<0.05)

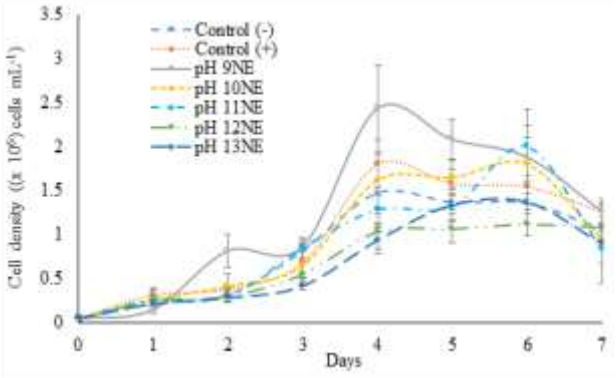

Fig. 1: The cell density of *A. platensis* in combination with treatment of pH and neem extract for seven days of culture process. Cell density indicated significant difference between treatments and were calculated by one–way ANOVA followed by Duncan Multiple Range Test (P<0.05)

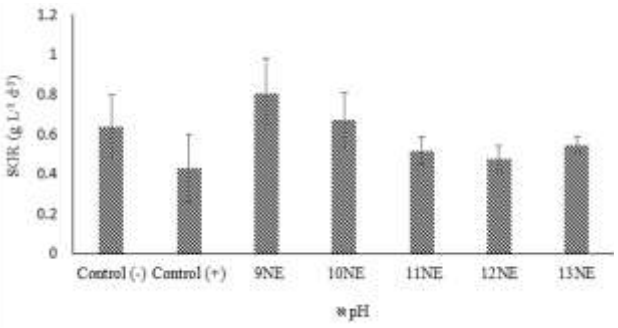

Fig. 2: SGR (Specific Growth Rate) of *A. platensis* survived seven days of the culture process with the treatment of pH and neem extract. SGR indicated unsignificant difference between treatments and were calculated by one–way ANOVA (P>0.05)

The highest protein was in positive control 2.382 g L^{-1}. The highest protein production was in the positive control. These results indicate that neem extract can restore abiotic stress. On the other hand, the highest productivity of carbohydrates, proteins and lipids was in the positive control. These results indicate that neem extract can trigger an increase in the production of carbohydrates, proteins and lipids in *A. platensis* and increase its productivity.

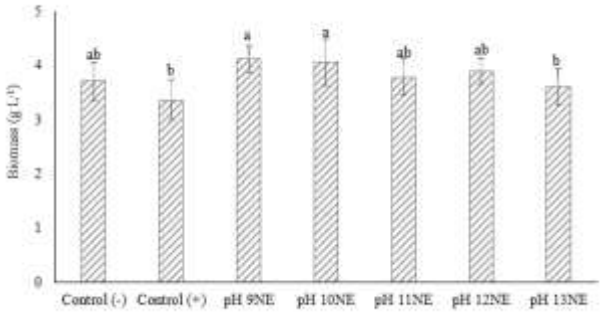

Fig. 3: The biomass of *A. platensis* in combination with the treatment of pH and neem extract for seven days of culture process. Symbols a and b represent the significance between treatments by one–way ANOVA followed by Duncan Multiple Range Test (P<0.05)

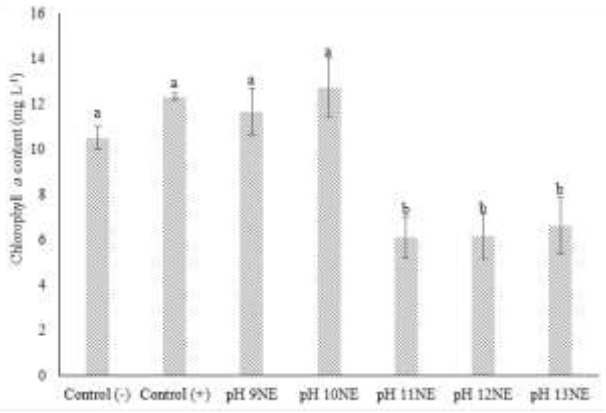

Fig. 4: Chlorophyll *a* content in *A. platensis* after treatment with NE (neem extract) and varied pH values. Symbols a and b represent the significance between treatments by one–way ANOVA followed by Duncan Multiple Range Test (P<0.05)

Analysis of bacterial contaminants

Total plate count on day 0 (beginning of culture) and day 7 (end of culture) during the combination treatment of pH and neem extract is shown in Fig. 8. The initial number of bacteria on day 0 differed in each treatment, so the data were presented in percentage form. There had been a decrease in the number of bacteria at pH 13 + neem extract (98%), positive control (75%), pH 10 + neem extract (74%) and

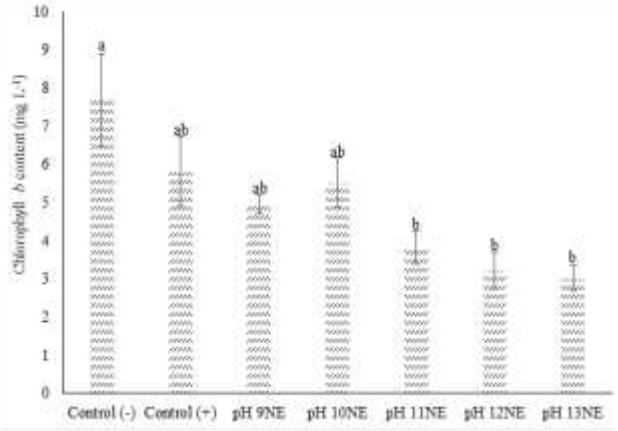

Fig. 5: Chlorophyll *b* content in *A. platensis* after treatment with neem extract and varied pH values. Symbols a and b represent the significance between treatments by one–way ANOVA followed by Duncan Multiple Range Test (P<0.05)

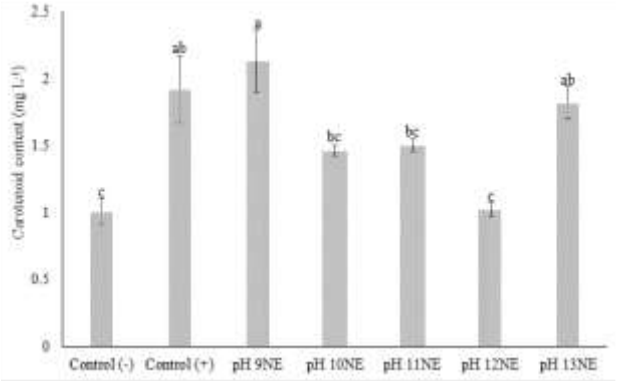

Fig. 6: Carotenoids content in *A. platensis* after treatment with neem extract and varied pH values. Symbols a, b and c represent the significance between treatments by one–way ANOVA followed by Duncan Multiple Range Test (P<0.05)

negative control (17%). By contrast, other treatments experienced an increase in the number of bacteria, namely the treatment of pH 9 + neem extract (35%), pH 11 + neem extract (23%) and pH 12 + neem extract (2%).

Bacterial contaminants abundance

There were three samples in the next-generation sequencing (NGS) analysis, namely the control treatment on day 0 (A), the control treatment on day 7 (B), and the pH 13NE on day 7 (C). Sample C has the lowest CFU value and was considered the best treatment because it could reduce the lowest number of bacteria in the TPC (Total Plate Count) test. Samples A and B were used as comparisons to C. Data between samples was described qualitatively.

The diversity of bacterial contaminants was analyzed comprehensively; 16s rDNA sequences of bacteria were amplified using several primers in several amplified regions,

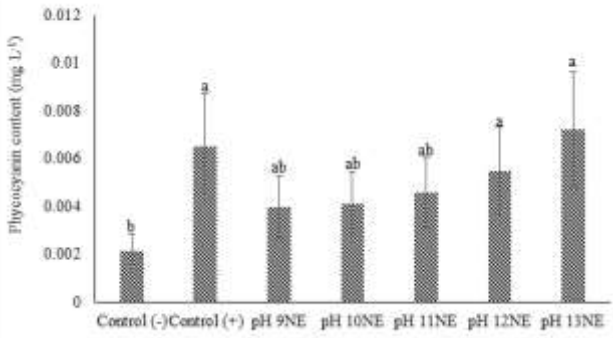

Fig. 7: Effect of pH and neem extract on phycocyanin content in *A. platensis*. Symbols a and b represent the significance between treatments by one–way ANOVA followed by Duncan Multiple Range Test (P<0.05)

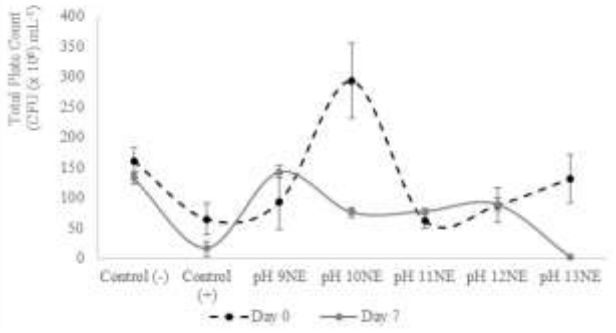

Fig. 8: Effect of pH and neem extract on bacterial count (CFU mL^{-1}) in *A. platensis* cultures at 0 and 7 days

namely primer pairs 341F (5'- CCTAYGGGRBGCASCAG -3')/806R (5'- GGACTACNNGGGTATCTAAT -3') (V3-V4, 470 bp). NGS results showed that there were 39 bacterial phyla in *A. platensis* culture. Fig. 9 shows a UPGMA diagram covering the 10 most unique phyla. The most dominant phyla in the three samples were Proteobacteriota, Firmicutes and Bacteroidota. Phylum Proteobacteria was more abundant than Firmicutes and Bacteroidota with different percentages in each sample. The percentages of Proteobacteria, Firmicutes, and Bacteroidota in sample A, B and C are shown in Table 2. Phylum Proteobacteria, Bacteroidota and Actinobacteriota decreased in sample B while increasing (by about 6%) in sample C. Proteobacteria and Actinobacteria are bacteria that help to remove nitrogen, ammonium and organic matter from the environment (Lee and Eom 2016; Ling *et al*. 2020). Phylum Firmicutes decreased in sample C (90% decrease). Phylum Chloroflexi, Acidobacteriota, Spirochaeta, Verrucomicrobiota completely disappeared in sample C.

The microbiota was dominated by three classes, namely Gammaproteobacteria, Alphaproteobacteria and Bacilli (Table 2) followed by Bacteroidia, Clostridia, Actinobacteria, Syntrophobacteria, Fusobacteriia, Acidimicrobiia, Polyangia, and others (Fig. 10 and 11). In this study, 311 genera of bacteria and 119 species of bacteria were found. Fig. 12 shows the top 35 bacterial genera from

Table 2: Percentage of abundance of phylum and class of dominant bacteria in *A. platensis* culture at different pH and neem extract combinations

Sample	Dominant Phyla (%)			Dominant Classes (%)		
	Proteobacteria	Firmicutes	Bacteroidota	Gamma-proteobacteria	Alpha-proteobacteria	Bacilli
A	72.65	8.32	15.9	49.65	68.02	40.82
B	72.35	24.86	1.41	22.99	4.32	36.18
C	77	2.35	19.33	8.16	23.4	2.05

A= control treatment day 0, B= control treatment day 7, C= pH 13+ neem extract day

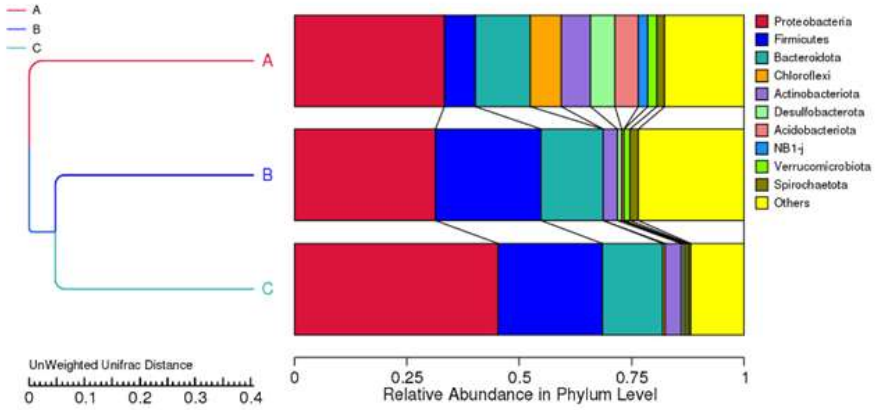

Fig. 9: Relative abundance at the Phylum level in unweighted pair-group method arithmetic (UPGMA) diagrams

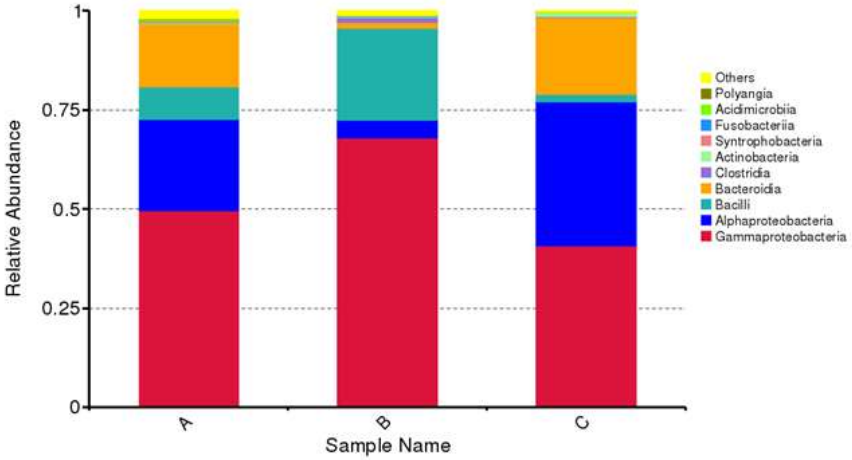

Fig. 10: Relative abundance of bacterial contaminant taxa at Class level in in an unweighted unique fraction (Unifrac) distance diagram of *A. platensis* culture

samples A, B and C. The dominating genera are *Pseudomonas*, *Halomonas* and *Exiguobacterium*. The dominant bacterial species is *Halomonas meridiana*. These bacteria are known to play a role in triggering growth in microalgae (Subasankari *et al*. 2020), with optimal growth in the pH range of 7–10 and optimum at pH 9.7 (Alquier *et al*. 2013).

Discussion

The results showed that the density is directly proportional to the cell biomass. High cell density allows microalgae cells to carry out photosynthesis better, resulting in higher biomass (Hu *et al*. 2013). Some studies reported that pH 9 is the optimal pH for *A. platensis*; the higher the alkalinity, the slower the growth (Capelli and Cysewski 2010; Ismaiel *et al*. 2016; Park *et al*. 2022). Reduced CO_2 concentration limits the process of photosynthesis and causes oxidative stress due to increased ROS (reactive oxygen species) (Pandey *et al*. 2010; Ismaiel *et al*. 2016; Park *et al*. 2022). ROS appears in response to environmental stress. High ROS concentrations cause oxidative damage, and can react with and modify biomolecules, resulting in mutagenesis and organelle dysfunction (Rezayian *et al*. 2019).

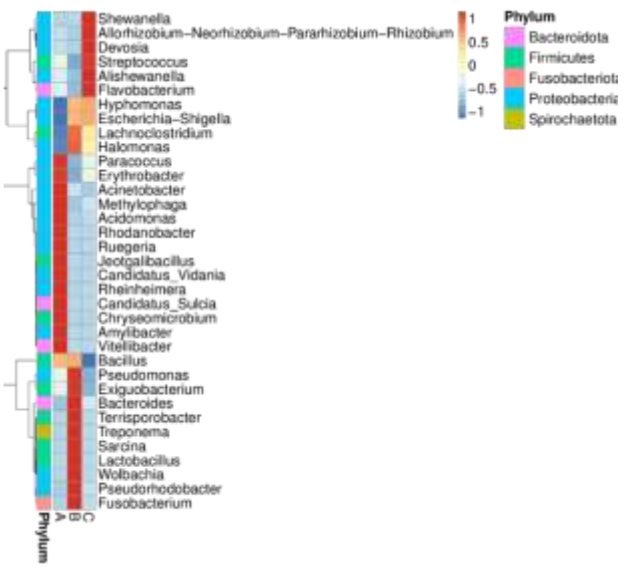

Fig. 11: The cluster heatmap showing the genus of various bacteria in *A. platensis* culture

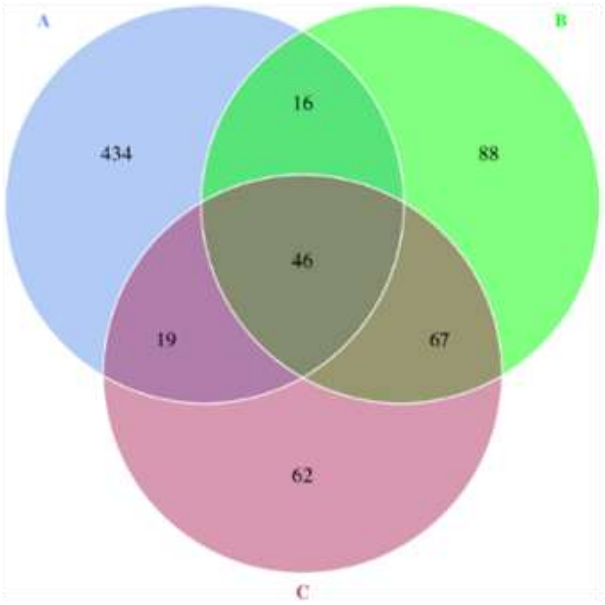

Fig. 12: Venn diagram showing the number of shared and unique species in the different libraries

Based on the data in Fig. 2 and 3, the positive control treatment had lower biomass and specific growth rate values compared to the other treatments. These results indicate that there is a possibility that neem extract has low killing power against contaminating bacteria. Previous research stated that azadirachtin from neem extract has low toxicity to contaminants (Huang *et al*. 2013). On the other hand, other studies state that neem extract can stimulate biomass production in cyanobacteria and increase the rate of photosynthesis (Prasad *et al*. 2007). However, the combination treatment of alkaline pH and neem extract did not kill *A. platensis* cells. This was due to the positive control treatment having the highest biomass productivity compared to the other treatments, namely 1.56 ± 0.831 mg L^{-1} day^{-1}.

Based on Figs. 4–6, chlorophyll *a*, *b*, and carotenoid levels decreased at pH> 10. This is because the higher the pH, the lower the CO_2. At high pH, there is only a carbon source available in the form of carbonate and bicarbonate so the rate of photosynthesis decreases. Stress due to limited CO_2 allows increased levels of free radicals so that chlorophyll metabolism is also disrupted (Ismaiel *et al*. 2016). The concentration of chlorophyll *a* is higher compared to other pigments because heterotrophic conditions increase 13^2-hydroxy-chlorophyll *a*. Component 13^2-hydroxy-chlorophyll *a* is a building block of chlorophyll *a* (Maroneze *et al*. 2019). Phycocyanin pigments in this study ranged from 0.002–0.007 mg L^{-1}. Phycocyanin is a component in microalgae that plays a role in the photosynthesis of cyanobacteria, is non-toxic, dissolves in water, includes phycobiliproteins and has antioxidant activity. So far, most phycocyanin is extracted from *A. platensis*. The location of phycocyanin in the lamella or thylakoid on the cytoplasmic membrane. Phycocyanin conformation is affected by pH. pH stability for optimum phycocyanin production is in the pH range of 5.5–6.0. The optimum pH range for phycocyanin production increases to 5.0–7.5 when treated with an additional temperature of 9ºC (Morais *et al*. 2018). In this study, alkaline pH caused low phycocyanin production. However, when compared to other treatments, pH 13 + neem extract had the highest phycocyanin content. These results indicate that extreme pH stress (environmental stress) can trigger the overproduction of antioxidants such as phycocyanins (Ismaiel *et al*. 2016). The pigment content in each treatment fluctuated. However, the positive control tends to produce high pigment. These results indicated that neem extract did not inhibit pigment production in *A. platensis*. Previous research explained that neem extract does not affect the process of photosynthesis and can counteract oxidative stress (Pasquoto-Stigliani *et al*. 2017; Naz *et al*. 2022).

The optimal pH for the production of carbohydrates and lipids is in line with biomass, namely in the range of pH 9–10. Biomass in microalgae contains products from photosynthesis, including carbohydrates. Carbohydrates are related to biomass. When biomass is high, carbohydrates are also high. Low carbohydrates are possible at extremely high pH because *A. platensis* tends to produce lipids as a food reserve, resulting in a decrease in carbohydrates. Then, lipids are converted to carbohydrates via gluconeogenesis. The formed lipids, on the other hand, are oxidized. High pH increases the rate of lipid oxidation because the concentration of protons (H^+) is low. The availability of protons is limited so that none can be transferred to the radical groups (alkyl/R^-, alkoxy/RO^- and peroxyl/ROO^-). The formed radical groups will combine into one and form a stable non-radical product. Because free radicals lack protons at high pH, there is no merger between free radicals because not all of them receive

proton transfers. Furthermore, the high hydroxide ion (OH^-) increases the hydrolysis rate of triacylglycerol (TAG) into DAGs (Diacylglycerols), MAGs (Monoacylglycerols), FFA (Free Fatty Acids) through a saponification process. DAGs, MAGs, and FFAs are amphiphilic components and can increase the rate of lipid oxidation through the formation of associative colloids (Shahidi and Wanasundara 2008; Kim *et al*. 2016). In addition, the presence of ROS also causes lipid peroxidation. The mechanism of lipid peroxidation, namely ROS, causes the removal of hydrogen elements from fatty acid chains, resulting in the formation of cytotoxic products such as malondialdehyde (MDA) and aldehydes. The chloroplast of *A. platensis* is composed of membrane system rich in polyunsaturated fatty acids. These unsaturated fatty acids are the main targets of peroxidation (Rezayian *et al*. 2019).

Previous research stated that alkaline pH is an abiotic stress that can cause oxidative stress (Poonia and Priya 2013). There is an imbalance in the production of the active oxygen with the detoxification process of free radicals and peroxides. Therefore, high-energy electrons are transferred to molecular oxygen and ROS is formed. In addition, ROS will be formed due to the presence of pathogens (contaminants) (Liu *et al*. 2007). ROS refers to metabolites derived from molecular oxygen. The presence of ROS results in molecular damage such as lipids, DNA, and proteins (Nobuhiro and Mittler 2006; Sharma *et al*. 2014). The presence of molecular damage causes the protein concentration to decrease along with the increase in alkalinity. ROS causes amino acid oxidation, changes the charge (electrical charge) of proteins, breaks peptide chains, breaks protein cross-links, and makes proteins vulnerable to proteolysis and proteases. ROS binds to the sulfur groups of amino acids in proteins, forming disulfide bonds between amino acids with sulfur groups and destroying protein function and structure. If the dysfunctional protein accumulates, it will alter the function of microalgae cells (Rezayian *et al*. 2019).

Protein content at pH > 10 decreased significantly (Table 1). Protein had the highest concentration when compared to carbohydrates and lipids. The highest protein was in the positive control treatment which indicated that the addition of neem extract did not reduce the availability of carbon and nitrogen in the medium. Carbon and nitrogen are used by *A. platensis* as basic materials for protein synthesis. However, when a high pH was added, protein production decreased, indicating that protein synthesis was disrupted. The pH of the *A. platensis* environment had an effect on cell physiology and the production of metabolites such as protein. In addition, alkaline conditions reduced the activity of specific enzymes and caused protein deprotonation (Almutairi *et al*. 2020).

Based on the results of total plate count analysis (Fig. 8), pH and neem extract can reduce the number of bacteria in *A. platensis* culture. Neem extract has a main phytochemical component, namely azadirachtin which is an oxidized tetranortriterpenoids. In addition, neem extract contains antibacterial and antifungal chemical components. These components are nimbolid, margolone, gedunin and cyclic trisulphide. The concentration of these components varies depending on temperature conditions, light, humidity levels, and pH. The way neem extract works is influenced by pH (Baby *et al*. 2022). In this study, the treatment that reduced the total number of bacteria was the greatest and resulted in the most optimal growth at once, namely at pH 10 + neem extract. Previous studies explained that bacteria are the biggest contaminants in microalgae cultures, reaching 65%. The rest are contaminants in the form of other microalgae (14%), viruses (3%), fungi (6%) and grazers (12%) (Caprio 2020). The relationship between bacteria and microalgae can be in the form of mutualism, predation, competition, and parasitism (Yao *et al*. 2019; Caprio 2020). At pH 9 + neem extract there was an increase in the number of bacterial colonies so it is possible that the bacteria present at pH 9 + neem extract had a mutualistic symbiosis with *A. platensis* because pH 9 + neem extract had the highest density, biomass, pigment content, and metabolite content compared to other treatments. These bacteria play a role in breaking down complex components, carrying out nitrification and denitrification processes, synthesizing and transferring B vitamins (cobalamin, thiamine, biotin) into microalgae cells, and supporting the availability of growth factors for microalgae. Microalgae, on the other hand, provide oxygen (O_2) to bacteria, a habitat that protects bacteria's existence, and nutrients from the remains of dead microalgae cells (Suyono *et al*. 2018). Therefore, both of them benefit from each other and the growth of microalgae can be more optimal.

It is easier to reduce the number of bacterial contaminants in *A. platensis* culture because neem extract also has a hydrophobic component. This hydrophobic component of neem extract can combine with the lipid component of the bacterial cell wall, causing damage to the bacterial cell wall, resulting in the release of bacterial intracellular components and the bacteria dying. The combination of pH and neem extract can significantly reduce the number of bacteria in *A. platensis* culture. The antibacterial activity of neem extract was higher than artificial antibiotics such as ampicillin and tetracycline. The active compound components in this extract have been shown to be able to inhibit bacterial growth by forming an inhibition zone in laboratory experiments using agar media. In addition, neem extract also has high antioxidant activity so that it can assist cells in repairing cells damaged by free radicals (Ghosh *et al*. 2016; Heyman *et al*. 2017).

Proteobacteria is the most dominant phylum associated with microalgae, followed by the phyla Bacteroidota and Firmicutes (Lee and Eom 2016; Ling *et al*. 2020). Dominant bacteria usually have a role as competitors against microalgae or against other bacteria. These bacteria have the ability to quickly form micro-colonies, associate with microalgae and can inhibit other types of microorganisms from intervening in their colonies by producing special antibacterial proteins.

These bacteria can also form a microbiome together with microalgae (Astafyeva *et al*. 2022). In this study, 119 species of bacteria were found. One type of bacteria found is *Escherichia coli*. *E. coli* has been known as a pathogenic bacterium. *E. coli* is referred to as a bacterial contaminant in *A. platensis* culture (Navab-Daneshmand *et al.* 2018) and it was found in as much as 0% (sample A), 0.39% (sample B) and 0.41% (sample C). The highest number of *E. coli* in sample C indicated that this *E. coli* was able to adapt and grow at a pH with high alkalinity. On the other hand, *E. coli* usually grow in the pH range of 4.5–9. The adaptation of *E. coli* to high pH is most likely due to *E. coli*'s ability to restore the cytoplasmic pH to remain within its normal pH range even when the pH of the medium is extremely high (Wilks and Slonczewski 2007). Other research states that microalgae associated with *E. coli* increased biomass productivity by up to 592%. The relationship that develops is a symbiotic mutualism (Higgins *et al*. 2014). In this study, the phycocyanin content in sample C was the highest compared to the other samples. Therefore, the presence of *E. coli* helps increase phycocyanin in *A. platensis*.

The dominance of bacteria at the class, genus, and species levels in samples A, B and C was different. Fig. 12 shows that the addition of alkaline medium (pH 13) and the addition of neem extract succeeded in reducing the number of bacteria in *A. platensis* culture. The different number of OTUs in the three samples indicates that environmental factors affect and change the abundance of bacteria (Ge and Yu 2017). Bacterial adaptation to an alkaline environment is more complex than to an acidic pH because it requires many genes to be activated in the cytoplasm. An alkaline environment causes the pH of the bacterial cytoplasm to also increase to several levels which causes certain enzyme activities to become inactive so that growth is inhibited (Saito and Kobayashi 1933). Therefore, various bacteria that are able to survive at an alkaline pH are types of bacteria that have good cytoplasmic pH control regulation.

Conclusion

Overall, the results of this study indicated that pH 9–10 was the optimum pH for growth (cell density, biomass, pigment productivity) and *A. platensis* metabolite production. The combination of alkalinity and neem extract increased the concentration of phycocyanin and reduced the composition of bacteria in *A. platensis* cultures. However, it can be concluded that the pH 10 + neem extract treatment is the best treatment. This is due to the following benefits of the pH 10 + neem extract treatment: the high growth rate of *A. platensis*, which corresponds to the high production of biomass, pigments, and metabolites; and the bacterial population being reduced by up to 74%. The dominant bacterial communities were the phylum Proteobacteria, Firmicutes and Bacteroidota. Various bacteria species discovered in this study can be studied further to determine the type of symbiosis with *A. platensis*.

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Author Contributions

DPA planned the experiments, data analysis, and manuscript writing; DPA and IR participated in preparing tools and materials, and conducted the research; EAS participated in conceptual design experiment, drafting the manuscript and final approval.

Conflict of Interest

The authors have no conflicts of interest to declare.

Data Availability

All the related data reported in the manuscript will be available as requested.

Ethics Approval

The authors declare that the research was in accordance with all ethical standards.

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