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Salicylic Acid-Induced Astaxanthin Accumulation in Mixed Culture of *Nannochloropsis oculata* and *Arthrospira platensis* Cultivated in Tofu Wastewater

(Pengumpulan Astaxantin Teraruh Asid Salisilik dalam Kultur Campuran *Nannochloropsis oculata* dan *Arthrospira platensis* yang Dikultur dalam Air Sisa Tauhu)

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ABSTRACT

Astaxanthin is a significant ketocarotenoid with health benefits, and its use is widely celebrated in nutraceuticals, cosmetics, and aquaculture. Increasing customer demand is driven by concerns over synthetic alternatives and naturally occurring astaxanthins have been greatly marketed, prompting attempts to improve their biological production. In addition to the literature that has been previously explored, we developed a culture of the *Nannochloropsis oculata* and *Arthrospira platensis* (*Spirulina*) under open raceway pond (55 L working volume). We used tofu wastewater as growth medium and evaluated salicylic acid (SA) reaction effect as the elicitor. Three concentrations of SA (0, 20, and 200 µM) were tested. Growth rate (0.66 d^{-1}), biomass yield (1.33 g L^{-1}), biomass productivity (0.14 g L^{-1} d^{-1}), and chlorophyll-*a* content (7.03 mg L^{-1}) was highest after adding 20 µM of SA. This treatment also obtained the highest astaxanthin content (0.11 mg g^{-1} dry biomass), with a 1.88-fold increase over the control. A higher SA concentration (200 µM) did not further improve biomass or astaxanthin accumulation. The results indicate that moderate SA will increase astaxanthin production in mixed microalgal cultures. Moreover, the application of tofu wastewater forms a sustainable mode of biowaste valorization and is cost-effective to carry out large-scale cultivation. Overall, this strategy is a prospective approach for developing natural astaxanthin under open cultivation.

Keywords: Mixed culture; open raceway pond; salicylic acid; *Spirulina*; tofu wastewater

ABSTRAK

Astaxantin adalah ketokarotenoid yang penting dengan manfaat kesihatan dan penggunaannya diraikan secara meluas dalam nutraseutik, kosmetik dan akuakultur. Permintaan pelanggan yang semakin meningkat didorong oleh kebimbangan terhadap sintetik alternatif dan astaxantin yang berlaku secara semula jadi telah dipasarkan dengan meluas, mendorong usaha untuk meningkatkan pengeluaran biologi mereka. Selain daripada kepustakaan yang telah diterokai sebelum ini, kami telah membangunkan kultur *Nannochloropsis oculata* dan *Arthrospira platensis* (*Spirulina*) di bawah kolam palung terbuka (55 L isi padu kerja). Kami menggunakan air sisa tauhu sebagai medium pertumbuhan dan menilai kesan reaksi asid salisilik (SA) sebagai pengelisit. Tiga kepekatan SA (0, 20 dan 200 µM) telah diuji. Kadar pertumbuhan (0.66 d^{-1}), hasil biojisim (1.33 g L^{-1}), produktiviti biojisim (0.14 g L^{-1} d^{-1}) dan kandungan klorofil-*a* (7.03 mg L^{-1}) adalah tertinggi selepas menambah 20 µM SA. Rawatan ini juga memperoleh kandungan astaxantin tertinggi (0.11 mg g^{-1} biojisim kering) dengan peningkatan 1.88 kali ganda berbanding kawalan. Kepekatan SA yang lebih tinggi (200 µM) tidak meningkatkan lagi pengumpulan biojisim atau astaxantin. Keputusan menunjukkan bahawa SA sederhana akan meningkatkan pengeluaran astaxantin dalam kultur mikroalga campuran. Selain itu, penggunaan air sisa tauhu membentuk satu cara lestari untuk memanfaatkan sisa bio dan adalah kos efektif untuk menjalankan penanaman berskala besar. Secara keseluruhan, strategi ini adalah pendekatan yang berpotensi untuk membangunkan astaxantin semula jadi di bawah penanaman terbuka.

Kata kunci: Air sisa tauhu; asid salisilik; kultur campuran; kolam palung terbuka; *Spirulina*

INTRODUCTION

The global demand for natural astaxanthin has increased rapidly due to its well-documented health benefits and broad applications in nutraceuticals, cosmetics, and aquaculture. In 2020, the global market for natural astaxanthin was valued at approximately US$1.94 billion

and is projected to reach US$2.57 billion by 2025 (Chen et al. 2021; Zohir, Kapase & Kumar 2022). This growth is largely driven by increasing consumer preference for natural products over synthetic alternatives, particularly in the health and wellness sector. Astaxanthin is recognized for its strong antioxidant capacity and associated health benefits, including anti-inflammatory effects and skin protection (Oslan et al. 2021). Consequently, advancements in bioprocessing and cultivation technologies are required to meet this rising demand. Sustainable production strategies are therefore essential to ensure the efficient and environmentally responsible supply of natural astaxanthin (Abd El-Ghany et al. 2023).

Natural astaxanthin is primarily obtained from marine organisms such as salmon, trout, shrimp, and lobster (Ambati et al. 2014; Aneesh et al. 2022). However, reliance on these sources is limited due to competition with food supply chains and sustainability concerns. As a result, the current market is largely dominated by synthetic astaxanthin, which has raised concerns regarding its safety for direct human consumption (Stachowiak & Szulc 2021). In contrast, natural astaxanthin exhibits higher oxidative stability, likely due to its occurrence as mono- and di-esters of fatty acids. Given these advantages, the development of sustainable and natural sources of astaxanthin is increasingly important to meet growing market demand (Nishida et al. 2023).

Astaxanthin, which is a high value ketocarotenoid, is mainly manufactured by selected microalgae; mainly *Haematococcus pluvialis, Dunaliella salina*, and *Chlorella zofingiensis* (Fabris et al. 2020; Liu et al. 2014; Mulders et al. 2014). This pigment exhibits antioxidant activity and has already been extensively researched by diverse industries, such as food, feed, nutraceutical, and cosmetics (Amelia, Akmal & Suyono 2023; Villaró et al. 2021). Under stress conditions such as nutrient depletion, high light intensity or salinity, astaxanthin biosynthesis in microalgae, which leads to carotenoid accumulation, can be triggered as a protective mechanism (Debnath et al. 2024). Cultivation conditions play a crucial important role in the astaxanthin yields of the microalgae. Biomass productivity and pigment accumulation may also be influenced by light quality, nutrient availability, and cultivation methods (phototrophic, mixotrophic, or heterotrophic) (Khazi et al. 2021). However, despite these promising attributes, the potential of mixed microalgal cultures for astaxanthin production remains insufficiently explored. Therefore, further investigation is required to evaluate their effectiveness and underlying mechanisms.

Given that the influencing factors of astaxanthin production need to be investigated, ways must also be identified to maximize its production of it, as well as increase its economic value (Ajijah et al. 2020; Ugya & Meguellati 2022). Specific strains of microalgae are characterized by different degrees of astaxanthin synthesis capacity. *H. pluvialis* is one of its major producers owing to its high capacity for accruing high accumulation of this carotenoid under stress factors (Radice et al. 2023). Recently, mixed microalgal cultures have gained increasing attention as a strategy to optimize resource utilization and improve resilience to environmental stress. For instance, co-cultivation of *Tisochrysis lutea* and *Microchloropsis salina* enabled the simultaneous production of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), demonstrating the potential of mixed systems to enhance metabolite productivity (Thurn et al. 2022). Similarly, previous studies have shown that mixed cultures, such as *Chlorella vulgaris* and *N. oculata* can achieve comparable astaxanthin yields (Taufikurahman et al. 2024). The advantage of multiple species, as they will share certain light-harvesting capabilities, has been mentioned to assist nutrient utilization and light absorption which can help in the photobioreactors (Thurn, Schobel & Weuster-Botz 2024). However, despite these promising attributes, the potential of mixed microalgal cultures for astaxanthin production remains insufficiently explored. Therefore, further investigation is required to evaluate their effectiveness and underlying mechanisms.

Although *N. oculata* and *A. platensis* are not recognized as primary astaxanthin producers, both species are well-established, cultivable, and widely utilized in large-scale applications. Importantly, they are classified as food-grade microorganisms and are known for producing high-value bioactive compounds, including phycocyanin from *Arthrospira* and long-chain polyunsaturated fatty acids (EPA and DHA) from *Nannochloropsis* (Chaiklahan et al. 2022; Piyatilleke et al. 2024). However, information regarding their potential to produce astaxanthin, particularly under induced stress conditions, remains extremely limited. Therefore, this study aims to explore the potential of these two species in a mixed-culture system and to evaluate whether salicylic acid (SA) can enhance astaxanthin accumulation under wastewater-based cultivation.

SA a plant hormone involved in stress response and regulating growth, has been shown to elevate astaxanthin accumulation within microalgae (Fu et al. 2021). SA has the capacity to stimulate the biochemical pathways in microalgae that lead to biomass accumulation and carotenoid synthesis (Pan et al. 2020; Tharek et al. 2020). A can modulate the physiological responses of microalgae at certain concentration, leading to enhanced astaxanthin synthesis under specific stress conditions (Pan et al. 2020). This interaction indicates optimization of SA concentration may also be a feasible approach to optimize astaxanthin yields in microalgae strains (Nishshanka et al. 2022).

As the human population increased, the production of tofu increased, with China, Japan, South Korea, and Indonesia as major producers. As a consequence, the amount of tofu-waste rose significantly, with 15 liters of wastewater per kilogram of tofu producing 300 billion liters annually (Ginting et al. 2024). This amounts to over 300 million cubic meters of tofu wastewater annually.

Tofu wastewater is also an alternative and promising source of nutrients for microalgae cultivation as it is rich in organic matter (proteins and carbohydrates) which helps in the growth of microalgal species (Wang et al. 2018). Tofu wastewater, which contains organic nutrients in a significant proportion, can make the cultivation of microalgae environmentally sustainable, reducing waste whilst providing essential nutrients to the growth of the species (Wang et al. 2019). Utilizing tofu wastewater addresses environmental pollution associated with its disposal and provides a cost-effective medium for cultivating microalgae, thereby enhancing astaxanthin production (Wang et al. 2018). For instance, studies have shown that microalgae like *Chlorella* sp., which grew well in tofu wastewater (Widayat, Philia & Wibisono 2018) achieve higher growth rates and levels of astaxanthin accumulation compared with the conventional growth medium.

The further application of SA under tofu wastewater medium remains unexplored. More comprehensive studies are required to clarify the optimal conditions to produce astaxanthin, so that the synergistic effects of SA and tofu wastewater on a chosen number of microalgae strains, even mixed culture, can be understood. The absence of data on specific responses of various microalgae strains to either SA or tofu wastewater means that further optimization of astaxanthin production may be limited in some cases. Moreover, growing microalgal cultures out of wastewater (the wastewater used as a growth medium) is a promising sustainable strategy to increase the production of astaxanthins, thus alleviating local environment concerns (Zhang & Lu 2024). Although the potentials of microalgae for production of astaxanthin are promising, scale-up production of microalgae and cost-cutting practices still exist as hindrances. Low biomass yields and the complications for cultivation of microalgae significantly hinder commercial development (Oslan et al. 2022). Development of a cultivation system to help maximize astaxanthin production should therefore progress the bioprocess. Market demand for naturally produced astaxanthin (as part of a general trend toward health benefits and applications in aquaculture) will continue to evolve and grow and further investigation and innovation of microalgal biotechnology must make up for this by developing methods and procedures to produce astaxanthin in a sustainable manner (Zhang et al. 2024).

This study examined the strategies to improve astaxanthin production by cultivating mixed cultures of *A. platensis* and *N. oculata* in tofu wastewater supplemented with SA using an open raceway pond (ORP) system. This work sheds new light on the interaction between SA elicitation and tofu wastewater–based culture media during open cultivation, particularly the dynamics of microalgal growth and astaxanthin accumulation.

MATERIALS AND METHODS

MICROALGAE CULTURE AND MEDIA PREPARATION

The microalgal strains *N. oculata* and *A. platensis* were extracted from the Centre for Brackish Water Aquaculture (BBPBAP) in Jepara, Indonesia. The culture was initially done in 1 L vessels with 800 mL for the growth medium which was made of 0.3 % Walne synthetic medium (Ali 2021) and 8 g L^{-1} sodium bicarbonate (Figure 2). Culture specimens were incubated at 25 °C for 7 days under continuous aeration conditions through a bubbling system. Illumination was maintained at 70 µmol photons m^{-2} s^{-1} under a 16:8 light/dark photoperiod. Tofu wastewater was sourced from local home industries tofu production plants in Sumedang, West Java. Before use, the waste was pre-treated by filtering through cloth to remove solids, followed by sterilization in an autoclave to eliminate microbial contamination.

SCALE-UP OF MICROALGAE CULTURES

For cultivation, a mixed microalgal culture consisting of *N. oculata* and *A. platensis* (optical density, OD = 2 ± 0.1; inoculum ratio 1:1, v/v) was prepared. A total volume of 500 mL of the mixed culture was then transferred into a 25 L vertical tubular reactor (VTR) (diameter: 25 cm; height: 50 cm). Microalgae were collected after 7 days of growth in 1 L bottles and made into the inoculum. Cultures in the VTR culture medium consisted of 5 L of 0.3 % Walne synthetic medium and 8 g L^{-1} of sodium bicarbonate. Then, incubation was performed for 7 days at 25 °C with continuous aeration. The light intensity of the cultures was 70 µmol photons m^{-2} s^{-1} and was subjected to a 16:8 light/dark photoperiod. Subsequently, after 3 days of cultivation, 960 mL of tofu wastewater, previously sterilized by autoclaving, was gradually added to the medium to initiate acclimatization. No physicochemical characterization (COD, total nitrogen, total phosphorus) of tofu wastewater was conducted in this study. A further 5,040 mL of distilled water was then added to the medium, yielding 11 L of total working volume (Figure 3). Time of acclimatization phase was 7 days enabling the microalgae to adapt for these new conditions.

LARGER-SCALE CULTIVATION IN OPEN RACEWAY POND (ORP)

Larger-scale cultivation was carried out in a 65 L Open Raceway Pond (ORP). The ORP had dimensions of 81 cm × 49 cm × 40 cm (length × width × height) and was equipped with a paddle wheel agitator to maintain circulation. The paddle wheel operated at 50 rpm, with each paddle blade measuring 5 cm × 25 cm (length × height). Seven-day-old microalgal cultures from the VTR were harvested, and

5.5 L of this culture was transferred into the ORP. To this, 8.8 L of tofu wastewater (16% of the working volume) was added, along with sodium bicarbonate, at a concentration of 8 g/L. The volume was then adjusted to 55 L with distilled water. The cultivation was maintained at 25 °C under continuous illumination at 60 µmol photons m^{-2} s^{-1}, with a 16:8 light/dark photoperiod. On the fifth day, at stationary, salicylic acid (SA) was introduced into the culture at two different concentrations (20 µM and 200 µM), and free SA was used as control. The selected SA concentrations (0, 20, and 200 µM) were chosen to represent control, moderate elicitation, and high-stress conditions, based on ranges reported in previous studies (Régnier et al. 2015). The cultivation continued for an additional 7 days to assess the effects of SA on the microalgae.

GROWTH MEASUREMENT

The daily growth of microalgal cultures was monitored by measuring cell density and chlorophyll-a content every 24 h. Cell density was determined using a hemocytometer. The presence and stability of both species were monitored throughout the cultivation period using microscopy and cell counting. Due to differences in size and morphology between *N. oculata* and *A. platensis*, species-specific counting approaches were applied. For *N. oculata*, cells were counted in five small squares within the central medium square of the hemocytometer grid, following standard counting procedures. The chosen squares were the upper left, upper right, lower left, lower right corners, and the center square. *A. platensis* cells were counted in nine medium squares of the large grid that corresponded to its filamentous morphology. Automated cell counting with the NIS-Elements D 4.00.06 software through the 'count' function was also enabled. The microalgal cell numbers per species were calculated from the following Equations (1) and (2):

$$S_{N.\ oculata} = \frac{\sum n}{5} \times 0.25 \times 10^6 \quad (1)$$

$$S_{S.\ platensis} = \frac{\sum n}{9} \times 10^4 \quad (2)$$

where S is the number of cells; and $\sum n$ is the number of microalgae cells in the nth box. Furthermore, a growth curve was plotted based on the calculated cell numbers to monitor the overall growth trend of the microalgal cultures. The specific growth rate (μ) was determined using the following Equation (3).

$$\mu = \frac{\ln X_m - \ln X_i}{t} \quad (3)$$

where µ is the microalgae-specific growth rate (/day); X_m is the maximum biomass concentration (cells/mL); X_j is the initial biomass concentration (cells/mL); and t is the cultivation time between X_j and X_m (days).

CHLOROPHYLL-*a* CONTENT MEASUREMENT

The chlorophyll-*a* (chl-*a*) content was measured following the protocol described by Šimat et al. (2022). In brief, 10 mL of microalgal culture was collected and centrifuged at 5,000 × g for 10 min at room temperature. After centrifugation, the supernatant was discarded, and the pellet was resuspended in methanol. The sample was incubated in a water bath at 70 °C for 10 min, followed by vertexing and another centrifugation at 5,000 × g for 10 min at room temperature. The resulting clear supernatant was measured at a wavelength of 665 nm using a UV-Vis spectrophotometer. Each measurement was performed in triplicate. The amount of chl-*a* was calculated using the following Equation (4):

$$\text{Chl} - a \left(\frac{\text{mg}}{\text{L}}\right) = 12.9447 \times A_{665} \quad (4)$$

MICROALGAE BIOMASS YIELD AND PRODUCTIVITY MEASUREMENT

The microalgae were harvested after the seventh day of cultivation. Microalgae harvesting was performed through two stages: flocculation and centrifugation. In the first stage, the microalgae cultures were precipitated by adding sodium hydroxide (NaOH) in a 1:1 ratio, with 2 g of NaOH added for every 2 L of culture. The mixture was allowed to settle for 24 h to ensure complete biomass separation from the clear liquid. Following flocculation, the precipitated biomass was separated by centrifugation at 5,000 rpm for 10 min. The resulting pellet was carefully removed from the supernatant and dried in an oven at 40 ºC for 13 h. Once dried, the biomass was ready for subsequent extraction processes. To determine the dry biomass yield of microalgae, 10 mL of the culture was dried in a cabinet dryer at 60 ºC for 24 h. The biomass yield was calculated using Equation (5):

$$\text{Biomass yield} \left(\frac{\text{g}}{\text{L}}\right) = \frac{\text{Dry weight biomass}}{\text{initial culture volume}} \quad (5)$$

Biomass productivity was calculated according to Equation (6)

$$\text{Biomass productivity} \left(\frac{\text{g}}{\text{L. d}}\right) = \frac{B_t - B_0}{t} \quad (6)$$

where B_t is biomass yield at the end of cultivation (g L^{-1}); B_0 is biomass yield at the beginning of cultivation (g L^{-1}); and t is cultivation period (d).

ASTAXANTHIN EXTRACTION AND ANALYSIS

Astaxanthin extraction was done using acetone. Briefly 0.1 g of dried microalgal powder was dissolved into 10 mL of acetone and incubated at 45 ºC on an incubator shaker for 15 min. After extraction, the solution was filtered with a syringe filled with fat cotton. All residual biomass were rinsed with 5 mL acetone for maximum extraction. The combined extract was dried using a vapor cup for 24 h in a dark room to prevent degradation. The dried extract was re-dissolved in 5 mL of HPLC-grade methanol, filtered through a 0.22 µm PTFE syringe filter, and transferred to an HPLC vial for analysis. HPLC analysis was conducted (95:5, v/v) with a reverse-phase column (C18) consisting of the mobile phase methanol and distilled water. A column temperature of 25 ºC and a 1 mL min^{-1} flow rate was maintained. The absorbance at 482 nm was used to derive astaxanthin concentration (Dianursanti et al. 2014). A standard curve was produced using a 97% astaxanthin standard available from *H. pluvialis*. The astaxanthin content in the samples was calculated using the following equations:

$$[\text{astaxanthin}]\left(\frac{\text{mg}}{\text{L}}\right) = \text{A} \times \text{b} - \text{c} \quad (9)$$

$$\text{Astaxanthin content}\left(\frac{\text{mg}}{\text{g}}\right) = \frac{[\text{Astaxanthin}] \times \text{V}}{\text{m}_{\text{sample}}} \quad (10)$$

where [astaxanthin] is the astaxanthin concentration present in the sample (in ppm or mg/L); A is the peak area on the chromatogram; V is the volume of sample solvent (L); m_{sample} is the mass of extracted *N. oculata* and *A. platensis* microalgae biomass; and b and c are constants of the linear regression.

STATISTICAL ANALYSIS

Analysis of experimental data was conducted through one-way ANOVA for the effect of SA concentrations on the measured parameters. Post-hoc comparisons were conducted by Duncan's multiple range test (DMRT) to detect statistically significant differences between treatment groups. The significance level of $p < 0.05$ was considered statistically significant. To analyze the relationships among key parameters, Pearson's correlation coefficient (r) was calculated. Statistical analyses were performed using SPSS Statistics 20 software.

RESULTS AND DISCUSSION

GROWTH OF MIXED MICROALGAE *N. oculata* AND *A. platensis*

Based on the cell number, the microalgae exhibited a lag phase from days 0 to 2, followed by a logarithmic growth phase from days 2 to 5. By day 5, the culture entered the early stationary phase (Figure 1). However, according to the Chl-*a*, the lag phase was from day 0 to day 1, and the log phase was from day 2 to day 4. Then, from day 4, the mixed culture entered the stationary phase.

The mixed culture of *N. oculata* and *A. platensis* experienced a lag phase from day 0 to day 2. This lag phase could happen due to the microalgae using simple organic compounds in the tofu wastewater as a source of organic nutrients (Prayitno 2016; Syaichurrozi & Jayanudin 2016). Unlike previous evidence, the lag phase identified during the first cultivated day led to exponential growth after that (Heneash, Ashour & Matar 2015). These differences could be due to the cultivation media used, as in this study tofu wastewater was used but the study

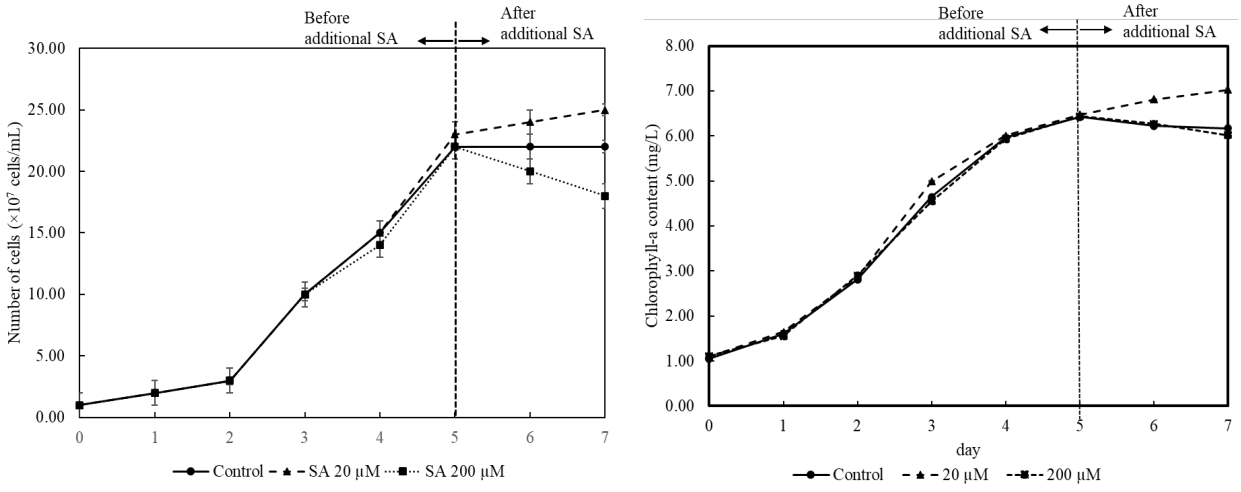

FIGURE 1. Growth of mix culture *N. oculata* and *S. platensis* under the present of SA; error bar indicated standard deviation

referred to using Walne medium. In a similar manner, other literature described a lag phase from day 0 to day 3 in the growth medium of anaerobically digested dairy manure wastewater (ADDMW). The long duration of adaptation shown in the present study indicates that the mixed culture needed more time to acclimate to the nutrient composition of tofu wastewater. Variations in lag phase duration can also be attributed to differences in nutrient availability and the physiological state or the age of the inoculum used (Istirokhatun, Aulia & Utomo 2017).

Upon addition of SA on day five, microalgae exhibited exponential growth under 20 µM SA until the end of the cultivation period. This sustained growth may be associated with the presence of low SA concentrations, which could induce moderate levels of reactive oxygen species (ROS). At low levels, ROS have been reported to act as signaling molecules that can enhance photosynthetic activity and maintain cellular redox balance (Mittler 2017; Sachdev et al. 2021).

Although ROS were not directly measured in this study, previous reports suggest that SA-induced ROS, such as hydrogen peroxide (H_2O_2) and superoxide anions (O_2^-), may be involved in stress signaling and cellular regulation. These species are also known to be cytotoxic at elevated levels, potentially causing damage to cellular components including DNA, lipids, and proteins (Sun et al. 2018). As described by Mittler (2017) the detoxification of ROS typically occurs via enzymatic antioxidant systems, as summarized in Equations (11) and (12):

$$2H^+ + O_2^- \xrightarrow{superoxide\ dismutase} H_2O_2 + O_2 \qquad (11)$$

$$2H_2O_2 \xrightarrow{Catalase} 2H_2O + O_2 \qquad (12)$$

The water produced is used in the light reactions of photosynthesis, mainly in the photolysis of water, to generate ATP and NADPH energy sources for the Calvin cycle (Zhang & Wang 2023). Additionally, previous studies suggest that SA may influence carbon fixation processes, potentially enhancing ribulose-1,5-bisphosphate (RuBP) carboxylation and glucose biosynthesis. Glucose serves as a fundamental building block for microalgal biomass and provides energy for various metabolic processes (Czerpak et al. 2002).

Therefore, the addition of 20 µM SA successfully boosts the number of microalgal cells, indicating its possible activity as an elicitor. The current results are in accordance with previous reports of *A. platensis* in which a total of 20 µM SA increased cell counts until the 10th day of cultivation (Hadizadeh et al. 2019). Similarly, *Nannochloropsis* sp. cultures showed improved growth until the 21st day under the same conditions (Udayan, Sabapathy & Arumugam 2020).

Conversely, microalgae mix culture did not grow under 200 µM SA. High SA content inhibited the activity of RuBP carboxylase and affected the photosynthesis dark reaction (Zhang & Wang 2023). It is assumed that the production of sugars in microalgal cells is less, thus decreasing energy for the establishment of cell division (Czerpak et al. 2002). Moreover, high SA concentrations increase ROS levels beyond reach of the cellular scavenging system. Oxidative stress, cellular damage, and finally cell death follow (Sachdev et al. 2021).

These findings are consistent with previous literature, which reported that SA concentrations exceeding 100 µM significantly reduce microalgal cell numbers. For instance, (Hadizadeh et al. 2019) observed a 5-65% reduction in *Nannochloropsis* sp. cell numbers over 21 days under 100 µM SA. Similarly, Udayan, Sabapathy and Arumugam (2020) reported a 3-26% reduction in *A. platensis* cell counts over 20 days when exposed to SA concentrations above 100 µM. These studies demonstrate that higher concentrations of SA act as inhibitors, potentially limiting the growth and viability of microalgae.

EFFECT OF SA ON PHOTOSYNTHETIC PIGMENTS

The chlorophyll content increased gradually in the course of cultivation (Figure 2), characteristic of a microalgal population. A mixed culture showed much higher chlorophyll than *A. platensis* grown on tofu wastewater by more than seven times, and from bicarbonate-enriched *N. oculata* by approximately five times (Ajijah et al 2020; Salbitani et al. 2021). This means that mixed-culture conditions have a markedly effective effect on the photosynthetic capacity of the consortium. This increased pigment accumulation results from a synergy between nutrient use to complement and metabolic interaction between the two species. *A. platensis* was able to efficiently uptake organic nitrogen from tofu wastewater, whilst *N. oculata* can be enriched with bicarbonate enhancing inorganic carbon fixation and chlorophyll biosynthesis. Metabolic cross-feeding might balance intracellular carbon and redox availability further, preventing chlorophyll degradation under conditions of stress. Similar findings have been reported in znutrient-enriched mixed cultures (Taufikurahman et al. 2024), suggesting that differences in pigment composition between cyanobacteria and microalgae can amplify light-harvesting efficiency. Evidence for species-specific characteristics agrees with this observation: *N. oculata* has mostly high chlorophyll-*a* levels (~7-14% dry weight), while the cyanobacterium *A. platensis* has less chlorophyll-*a* content (~0.8% dry weight) yet has a large fraction of phycocyanin (~6.7-11.7% dry weight) (Hadizadeh et al. 2019; Udayan, Sabapathy & Arumugam 2020). As a result, the mixed cultures showed higher chlorophyll-*a* contents compared with the *A. platensis* monocultures, which indicated an improved pigment profile.

SA also modulated chlorophyll accumulation in the mixed culture. The highest chlorophyll-*a* content and cell density was observed during the addition of 20 µM SA, while the chlorophyll-*a* decreased significantly after adding 200 µM SA probably due to excessive ROS generation that inhibited photosystem I activity (Okoro et al. 2019; Sachdev et al. 2021).

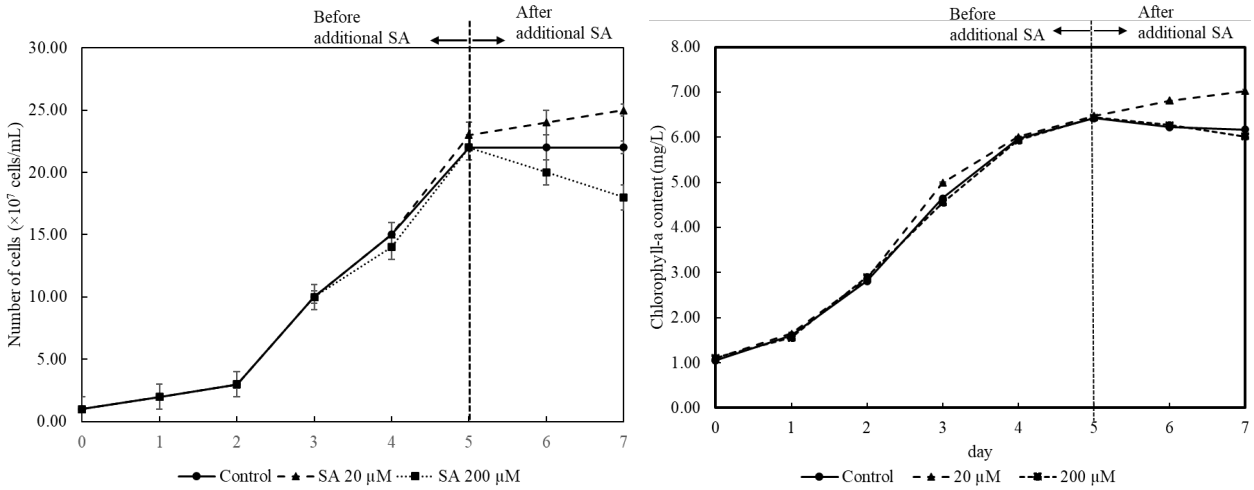

FIGURE 2. Growth of mix culture *N. oculata* and *S. platensis* under the present of SA; error bar indicated standard deviation

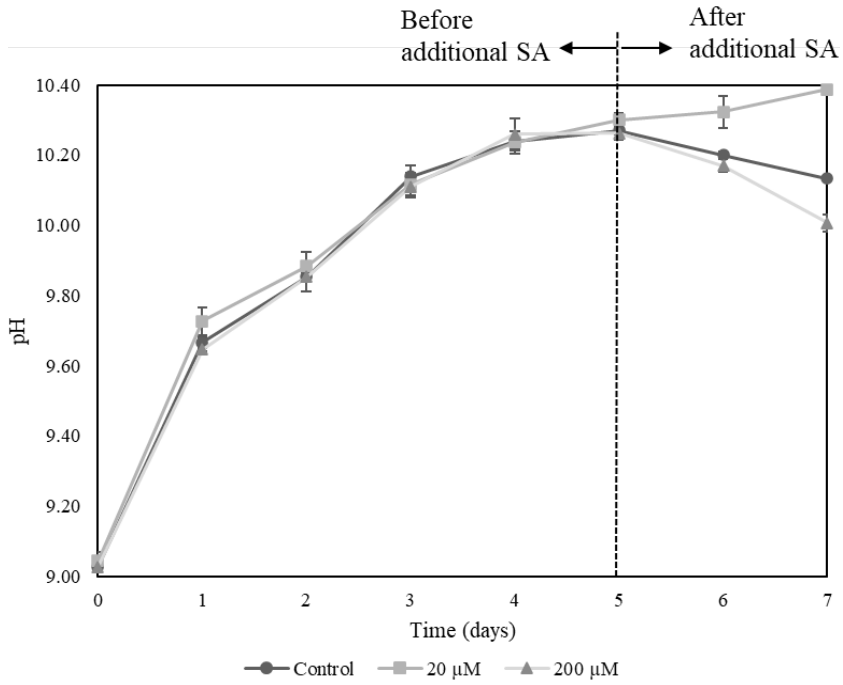

FIGURE 3. pH profile of *N. oculata* and *S. platensis* mixed cultures at each salicylic acid concentration

TABLE 1. The Pearson correlation analysis results for the microalgae cell number parameter in relation to Astaxanthin and Chlorophyll-*a*

	Pearson correlation coefficient (r)		
	Cell number	Chlorophyll-*a*	Astaxanthin
Cell number	-	0.99	0.79
Chlorophyll-*a*	0.99	-	0.87
Astaxanthin	0.79	0.87	-

Pearson correlation analysis showed a very positive relationship between the number of microalgal cells and chlorophyll-*a* content (r = 0.990) (Table 1), confirming that higher biomass directly increased pigment deposit levels. Good pH dynamics also supported microalgal growth. Although tofu wastewater is acidic (pH ~3.93), bicarbonate addition buffered the medium and provided inorganic carbon, thereby increased Calvin cycle activity and generated biomass (Phua et al. 2021; Sathinathan et al. 2025).

As for enhanced photosynthetic activity and nutrient absorption observed in the 20 µM SA treatment, cell growth was sustained until day 7, and the growth stagnated, followed by pH drop owing to depleted nutrients (Utomo, Julyantoro & Putu Wiweka Krisna Dewi 2020).

EFFECT OF TOFU WASTEWATER ON BIOMASS PRODUCTIVITY

The concentration of salicylic acid (SA) was closely associated with biomass yield and productivity (Table 2). The highest yield (1.33 g L^{-1}) and productivity (0.14 g L^{-1} day^{-1}) were observed after treatment with 20 µM of salicylic acid (SA), corroborating the observed increase in specific growth rate. This means moderate SA concentrations efficiently stimulate metabolic processes and biomass build-up, while too much SA (200 µM) inhibits growth, indicating a dose-dependent response. At 20 µM SA, growth is promoted via regulated ROS signaling, that may affect photosynthetic efficiency, redox balance and nutrient uptake. In this regard, mild SA-induced oxidative stress has been reported to activate MAPK signaling, increase PSII turnover, and ATP and NADPH production which are found to foster biomass formation. The organic-rich composition of tofu wastewater can enhance this response by supplying easily assimilable carbon and nitrogen sources. The biomass yield recorded is considered to be in-between the reported production achieved by mixed cultures grown in nutrient-bearing wastewaters (though still less than results with high-strength substrates like ADDMW). However, productivity under 20 µM SA is similar to SA-stimulated monocultures grown under standard laboratory conditions, indicating that SA application in wastewater-based production is still very strong. The optimum SA concentrations can serve as effective metabolic elicitors in mixed cultures while SA that's too high can lead to oxidative stress, which is a negative effect for biomass production.

EFFECT OF SA CONCENTRATION ON ASTAXANTHIN CONTENT

Organic elicitors, SA, have been reported to enhance secondary metabolite synthesis in microalgae by modulating metabolic pathways and stress signaling (de Faria Ferreira Carraro, Almeida Loures & de Castro 2022; Sahabudin et al. 2022). SA had a marked effect on the accumulation of astaxanthin in mixed microalgal cultures in this study. Its content increased from 0.06 mg g^{-1} in the control to 0.11 mg g^{-1} under 20 µM SA, a 1.83-fold augment (Table 3). For example, astaxanthin was undetectable at 200 µM SA, showing clearly concentration-dependent stimulation. The stimulant nature of 20 µM SA is in harmony with earlier researches which found improvement of secondary metabolite formation at very low concentrations of SA like increased alkaloid concentrations in the presence of *Spirulina platensis* (Hadizadeh et al. 2019) and increasing lipid contents in *Nannochloropsis* sp. (Udayan, Sabapathy & Arumugam 2020). In mixed cultures, astaxanthin concentration was most closely controlled by *Nannochloropsis* sp., which was found to have a higher nitrogen and phosphate assimilation capacity, which are important for chlorophyll and carotenoid biosynthesis (Qu, Liu & Qin 2015).

TABLE 2. A comparison of the growth parameters of microalgae for each variation in salicylic acid concentration

Species	Medium	Elicitor (SA)	Bioreactor	Specific growth rate (/day)	Biomass yield (g/L)	Biomass productivity (g/L/day)	Reference
N. oculata and *S. platensis*	Tofu wastewater	-	ORP	0.65 ± 0.004^{a}	1.12 ± 0.07^{b}	0.11 ± 0.007^{b}	This study
N. oculata and *S. platensis*	Tofu wastewater	20 µM	ORP	0.66 ± 0.006^{a}	1.33 ± 0.02^{a}	0.14 ± 0.005^{a}	This study
S. platensis	Zarrouk	20 µM	Culture bottle	0.40	3.70	0.31	(Hadizadeh et al. 2019)
Nannochloropsis sp.	Walne	18 µM	Culture bottle	0.14	0.64	0.03	(Zhang et al. 2020)
N. oculata and *S. platensis*	Tofu wastewater	200 µM	ORP	0.64 ± 0.01^{a}	1.10 ± 0.03^{b}	0.10 ± 0.006^{b}	This study
S. platensis	Zarrouk	100 µM	Culture bottle	-	2.5	0.14	(Kapouri et al. 2025)

Different notations (a and b) indicate significant differences ($p<0.05$) in each salicylic acid concentration variation

Astaxanthin accumulation was modest and not statistically significant ($p > 0.05$), but that might likely be due to short cell-cultivation time, since longer cultivation under nutrient stress further stimulates carotenoid synthesis (Bahador et al. 2019; Heldt et al. 1977; Hu et al. 2021). At low concentrations, SA is a non-stressful elicitor that manages intracellular reactive oxygen species (ROS) and drives secondary carotenogenesis. Scheduled ROS signaling triggers the biosynthesis of astaxanthin and regulates redox stability by increasing catalase and peroxidase activity (Abd El-Baky, El Baz & El-Baroty 2009; Mittler 2017; Udayan, Sabapathy & Arumugam 2020). SA also reinforces carbon flux to carotenoid substrates by fostering glycolysis and acetyl-CoA formation to generate isopentenyl pyrophosphate (IPP) for astaxanthin production (Cecchin et al. 2022; Zhang et al. 2018; Zhang et al. 2024).

In contrast, high SA (200 μM) probably induces potent oxidative stress which is evident by the increase in superoxide dismutase activity and downregulation of the catalase function which leads to H_2O_2 and hydroxyl radical deposition (Cecchin et al. 2022; Chen et al. 2009; Hirschberg et al. 1997). Under these conditions, the metabolic structure of the cells is damaged, and secondary metabolites are discouraged so astaxanthin cannot be detected, similar to reports of a metabolite inhibition at SA concentrations ≥100 μM (Hadizadeh et al. 2019; Yuan et al. 2022). Correlation analysis confirmed the relationship between astaxanthin accumulation and photosynthetic activity. The number of microalgal cells indicated an impressive positive correlation with the content of astaxanthin ($r = 0.786$), whereas those of chlorophyll-*a* had a highly significant correlation with astaxanthin concentration (Table 1).

These observations agree with previous reports suggesting that stronger photosynthetic activity and higher cell density may be associated with enhanced carotenoid biosynthesis (Xu et al. 2017). Taken together, 20 μM SA showed a trend toward increased astaxanthin accumulation; however, this effect was not statistically significant. The observed responses may be associated with changes in cellular redox balance and photosynthetic activity, as suggested in previous studies, although these mechanisms were not directly measured in this study.

While astaxanthin yield remains low, the system demonstrates potential as a cost-effective and sustainable platform, requiring further optimization. It should be noted that this study provides only a preliminary assessment, as the experimental design included three discrete SA concentrations (0, 20, and 200 μM), which does not allow the establishment of a comprehensive dose-response relationship. Therefore, future studies are recommended to include a finer concentration gradient (e.g., 10-100 μM) to better determine the optimal range and clarify dose-dependent responses.

In addition, the absence of physicochemical characterization of the tofu wastewater (COD, TN, TP) limits the interpretation of nutrient availability and reproducibility of the system. Accordingly, the results should be considered as a preliminary evaluation under semi-controlled conditions.

TABLE 3. The content of astaxanthin in microalgae increased as a result of the addition of various salicylic acids

Species	Medium	Elicitor	Bioreactor	Astaxanthin content (mg/g)	Reference
N. oculata and *S. platensis*	Tofu wastewater	-	ORP	0.06±0.001[a]	This study
Nannochloropsis sp.	F/2 media	-	250 mL flasks	0.38	Ahmed et al. 2014
Nannochloropsis sp.	Nitrogen depleted F/2 Media	-	250 mL flasks	1.78	Cecchin et al. 2022
S. platensis	Zarrouk	-	2 L Erlenmeyer flasks	0.007±0.16	Hanaa et al. 2003
S. platensis	Zarrouk	-	Erlenmeyer flasks	0.016±0.04	An et al. 2017.
S. platensis	Zarrouk	2 mM H2O2	4 L Erlenmeyer flasks	0.091	Abd El-Baky, El Baz & El-Baroty 2009.
N. oculata and *S. platensis*	Tofu wastewater	20 μM salicylic acid	ORP	0.11±0.08[a]	This study
N. oculata and *S. platensis*	Tofu wastewater	200 μM salicylic acid	ORP	Undetected	This study

The notation equation states that there is no significant difference ($p > 0.05$) in each variation of salicylic acid concentration

CONCLUSION

This study demonstrates that SA can influence growth and metabolite production in a mixed culture of *N. oculata* and *A. platensis* cultivated in tofu wastewater. Among the tested conditions, 20 µM SA showed a higher trend in specific growth rate (0.66 day^{-1}), biomass yield (1.33 g L^{-1}), biomass productivity (0.14 g L^{-1} day^{-1}), and chlorophyll-*a* content (7.03 mg L^{-1}) under alkaline conditions (pH 9.04-10.39). This concentration was also associated with an increase in astaxanthin content (0.11 mg g^{-1} dry biomass), corresponding to a 1.88-fold change compared to the control, although the difference was not statistically significant.

In contrast, a higher SA concentration (200 µM) suppressed microalgal growth and metabolite accumulation, indicating inhibitory effects under high-stress conditions. Overall, mixed microalgal cultivation combined with SA elicitation shows potential as a low-cost and sustainable strategy for biowaste valorization and carotenoid production, although further optimization is required. Future studies are required to improve system reliability, including detailed physicochemical characterization of tofu wastewater and microbial community profiling to ensure reproducibility under open cultivation conditions. In addition, optimization of SA concentration using a finer gradient is necessary to establish a clear dose-response relationship.

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