

INCREASING GROWTH RATE OF *Botryococcus braunii* USING ULTRASONIC WAVES

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Abstract

Microalgae have emerged as a promising alternative biomass source, offering distinct advantages over conventional crops in terms of oil content per mass. Maximizing their reproductive capacity throughout their life cycle is essential for optimizing biomass production. This study investigates the influence of ultrasonic waves on the reproductive rate of *Botryococcus braunii* microalgae. Various ultrasonic parameters, including power (56.2, 86.3, and 132.2 W for 37 kHz; 46.3, 67.2, and 101.2 W for 80 kHz), exposure times (3 and 5 min), and time intervals (2 and 4 days) were examined. The best outcomes were obtained with a 37 kHz ultrasonic frequency, 83.6 W power, 3-min exposure, and a 2-day interval, resulting in a maximum relative specific growth rate (16.67%) and relative biomass productivity (34.62%). Beyond these parameters, adjustments in ultrasonic treatment conditions resulted in decreased growth rates and biomass productivity. It is crucial to consider multiple ultrasonic factors, including frequency, power, exposure time, and time intervals, as each parameter significantly impacts microalgae growth.

Keywords: Biomass productivity; *Botryococcus braunii*; specific growth rate; ultrasonic parameters

Introduction

Renewable and sustainable energy sources harnessed from photosynthetic microalgae have garnered significant attention. Among these microalgae, *Botryococcus braunii* stands out for its ability to utilize sunlight and CO₂, converting them into valuable biomass and products through photosynthesis (Wang *et al.*, 2008). This unique capability not only aids in reducing atmospheric carbon dioxide levels but also supports microalgae growth and carbon dioxide sequestration (Onyeaka *et al.*, 2021).

Nevertheless, the relatively slow growth rates observed in *B. braunii*, possibly due to the production of energy-intensive compounds like hydrocarbons and polymers (Blifernez-Klassen *et al.*, 2018), pose challenges for industry. Previous studies focusing on optimal culture conditions have underscored the significant influence of factors such as culturing conditions and nutrient availability on *B. braunii*'s growth and productivity (Fu *et al.*, 2019; Saatovich *et al.*, 2021). To address these challenges, researchers have exposed microalgae to

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various environmental stress conditions, aiming to stimulate biomass growth and enhance the biosynthesis of specific compounds within microalgae (Xiao-Man *et al.*, 2018; Pereira *et al.*, 2023). Ultrasonic waves have been employed in several investigations to induce cellular stress and improve cell proliferation (Shi-Kai *et al.*, 2014; Sivaramakrishnan and Incharoensakdi, 2019; Vintila *et al.*, 2023).

Utilizing ultrasonic waves, researchers disrupted individual cells, facilitating the release of valuable products contained within them (Xiao and Ju, 2018). This process enhances cell permeability, enabling better transport of substances across cell membranes and the release of compounds like lipids (Pereira *et al.*, 2023). Numerous studies have associated ultrasound treatment with the stimulation of *B. braunii* microalgae growth under specific ultrasonic parameters (Shi-Kai *et al.*, 2014; Xu *et al.*, 2014). This research investigates the impact of ultrasonic waves on improving growth rates and biomass productivity by optimizing critical parameters, including frequency, power, exposure time, and time intervals.

Materials and Methods

B. braunii was isolated from the reservoir at Walailak University, Thasala, Nakorn Si Thammarat, Thailand. The microalgal sample was cultivated in BG-11 medium (Dayananda *et al.*, 2007) within a sterile chamber under constant conditions at 25°C, with illumination of 3,000 lux during a 16-hour light and 8-hour dark cycle. The culture's purity was regularly assessed through microscopic observation using an optical microscope (OLYMPUS CH20i).

We investigated the influence of various ultrasonic parameters, namely frequency, power, exposure time, and time interval, on the reproductive rate of *B. braunii* microalgae to determine optimal conditions. The microalgae sample was exposed to ultrasonic waves for a period of 30 days under controlled conditions.

Ultrasonic excitation was carried out using an ultrasonic bath (Elmasonic P 60 H). Multiple combinations of ultrasonic parameters were tested, including ultrasonic powers of 56.2, 86.3, and 132.2 W for a frequency of 37 kHz, as well as 46.3, 67.2, and 101.2 W for an 80 kHz frequency. Exposure times of 3 and 5 min, along with time intervals of 2 and 4 days, were systematically varied to identify the optimal conditions for ultrasonic excitation. Temperature was continuously monitored during ultrasonic bath operation to gauge ultrasound

power dissipation. The power of the ultrasonic bath can be determined using the equation for thermal energy transfer (Liu *et al.*, 2022).

The microalgae population was quantified through daily image capture using a hemocytometer under a microscope, and cell counts were determined via image processing. The growth curve and specific growth rate were calculated using the logistic growth model, consistent with previous research findings (Zhang *et al.*, 2016; Hanief *et al.*, 2020).

At three-day intervals, 12 ml of the samples were subjected to centrifugation at 1,725 rpm for 20 min using a Labconco CentriVap Benchtop Vacuum Concentrator to facilitate filtration. Subsequently, these samples were transferred to a 47-mm glass fiber filter (Glass Microfiber GF/C, Whatman) that had been pre-dried and pre-weighed in a forced convection oven (FD240). The biomass on the filter was then dried at 103°C for 13 h, followed by equilibration in a desiccator for 30 min before the final weighing. Biomass productivity was subsequently calculated following the method outlined by Chen *et al.* (2016).

Results and Discussion

Effect of Ultrasonic Wave with Microalgae

Ultrasonic waves were employed to culture and stimulate *B. braunii* microalgae over a 30-day period, varying ultrasonic parameters such as frequency, power, exposure time, and time intervals. Figure 1 depicts the cell count of *B. braunii* microalgae exposed to a 37 kHz ultrasonic frequency at different ultrasonic powers and during various culture days. Similarly, Figure 2 presents the cell count of *B. braunii* microalgae subjected to an 80 kHz ultrasonic frequency. Upon observation, the number of cells initially remained consistent for both frequencies. However, starting on the 5th day, the cell count for those exposed to a 37 kHz ultrasonic wave with a 2-day interval (Figures 1(a) and 1(b)) was higher compared to those with a 4-day interval (Figures 1(c) and 1(b)) across all ultrasonic power conditions. Conversely, when observing cells stimulated with an 80 kHz frequency, no significant difference in cell abundance was observed between the 2-day (Figures 2(a) and 2(b)) and 4-day intervals (Figures 2(c) and 2(d)). Therefore, the data indicate that, after the 5th day, the 37 kHz excitation led to a higher cell count with a 2-day interval compared to a 4-day interval. However, it remains inconclusive regarding which condition resulted in a higher cell count for the 80 kHz frequency.

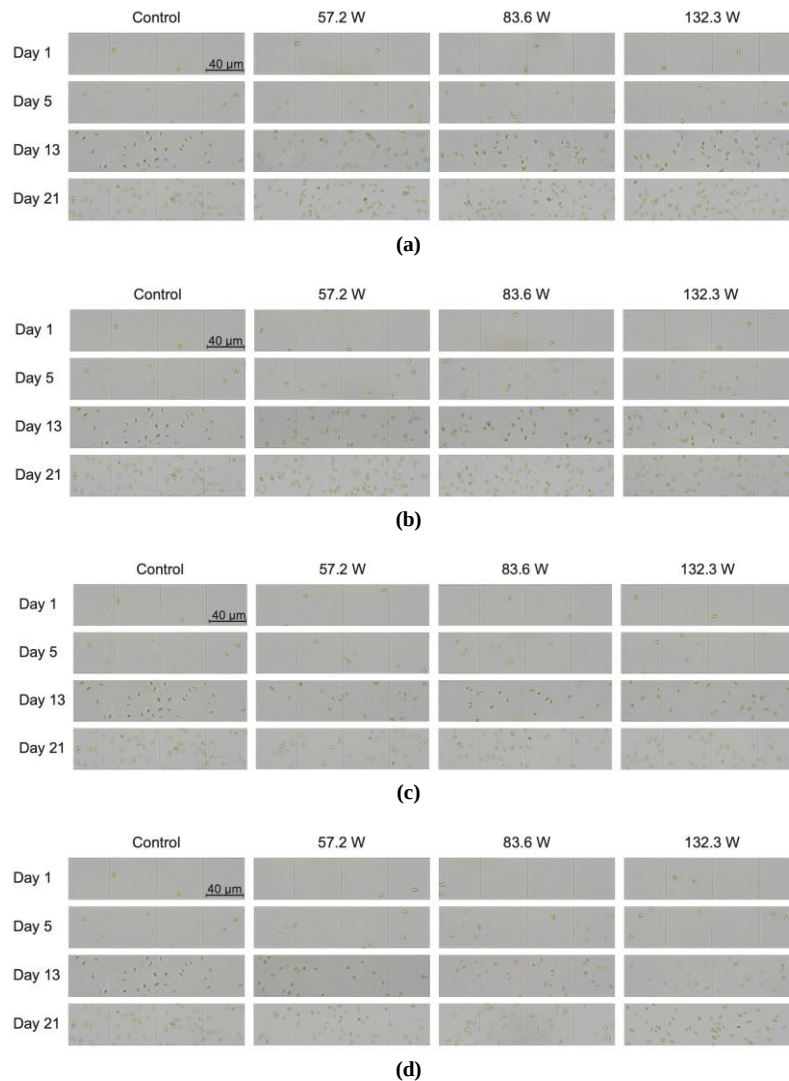


Figure 1. The *B. braunii* algae was excited by ultrasonic wave at frequency of 37 kHz

- (a) Exposure time of three minutes, two-day intervals.
- (b) Exposure time of five minutes, two-day intervals.
- (c) Exposure time of three minutes, four-day intervals.
- (d) Exposure time of five minutes, four-day intervals.

Influence of Ultrasonic Time Intervals

Figure 3 demonstrates the impact of varying ultrasonic intervals on the growth of *B. braunii* microalgae. The growth curves depict the microalgae's response to specific experimental conditions, including (Figure 3(a)) a 37 kHz ultrasonic frequency with 83.6 W ultrasonic power and (Figure 3(b)) an 80 kHz ultrasonic frequency with 67.2 W ultrasonic power, each exposed for 3 min. The results unmistakably indicate that stimulating the microalgae with a 2-day interval significantly enhances their growth. In contrast, employing ultrasonic waves at a 4-day interval inhibits the growth of *B. braunii* microalgae.

Notably, in the case of the 37 kHz growth curves, when the microalgae were initially exposed to ultrasonic waves on day 4, their growth initially matched that of the control condition but subsequently declined on day 7. These findings support the conclusion that subjecting *B. braunii* microalgae to ultrasonic waves with a 4-day interval hinders their growth.

When examining the specific growth rate and biomass productivity for both the 37 kHz and 80 kHz ultrasonic frequencies, certain data become evident. For the 37 kHz frequency, employing a 4-day interval resulted in a reduction of approximately 10.60% in the specific growth rate

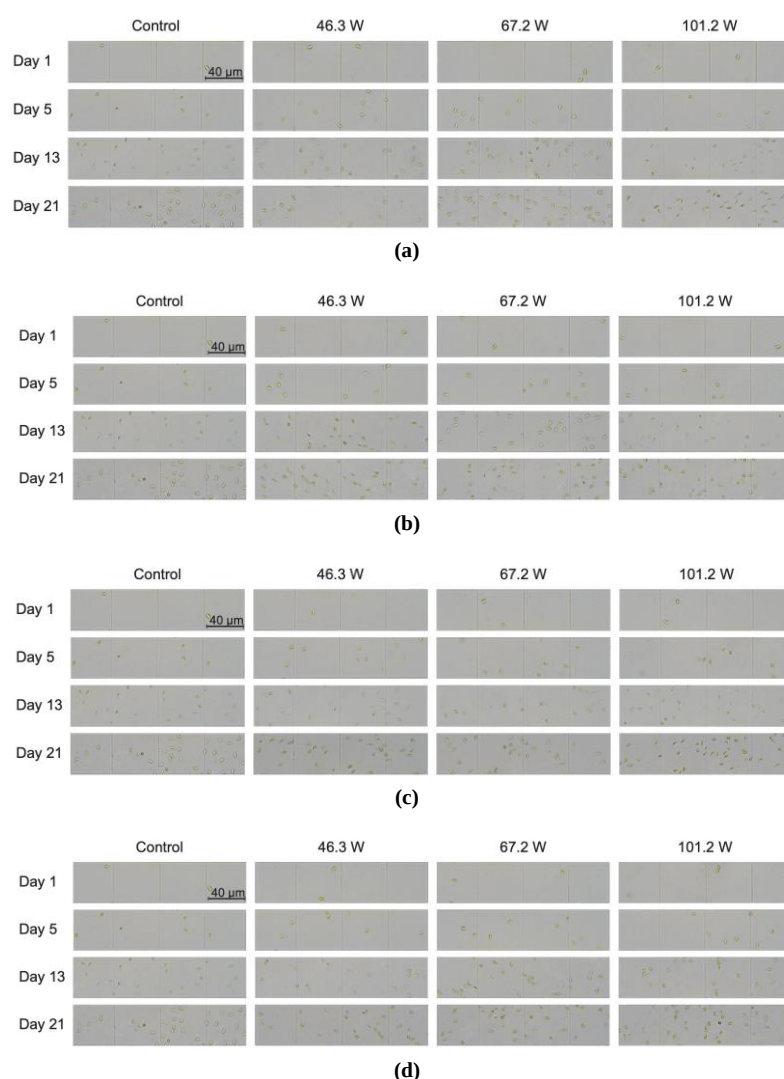


Figure 2. The *B. braunii* algae was excited by ultrasonic wave at frequency of 80 kHz

- (a) Exposure time of three minutes, two-day intervals.
- (b) Exposure time of five minutes, two-day intervals.
- (c) Exposure time of three minutes, four-day intervals.
- (d) Exposure time of five minutes, four-day intervals.

and a 29.49% decrease in biomass productivity compared to the control condition. Conversely, adopting a 2-day interval led to an increase of approximately 16.96% in the specific growth rate and a 34.62% enhancement in biomass productivity when compared to the control. Similarly, for the 80 kHz frequency, the 4-day interval condition caused a decline of approximately 7.93% in the specific growth rate relative to the control, while the 2-day interval condition yielded a 14.83% improvement in the specific growth rate. Intriguingly, biomass productivity increased in both interval conditions. Ultrasonic waves induce environmental stress changes in microalgae cultures. Exposure to ultrasonic waves leads to

stress in microalgae, referred to as stress memory (Vintila *et al.*, 2023). In the present study, exciting microalgae with ultrasonic wave every 2-day interval resulted in a better growth than the stimulation for every 4-day, attributed to being stressed frequently throughout the experiment. Consequently, ultrasonic stimulation at a 2-day interval significantly enhanced both the growth rate and biomass productivity of microalgae. Sivaramakrishnan and Incharoensakdi (2019) studied ultrasonic wave effects on microalgae at varying intervals (0-5 sec). Optimal biomass occurred at 2-sec intervals, declining with longer exposures. This highlights the importance of selecting suitable intervals to maximize biomass.

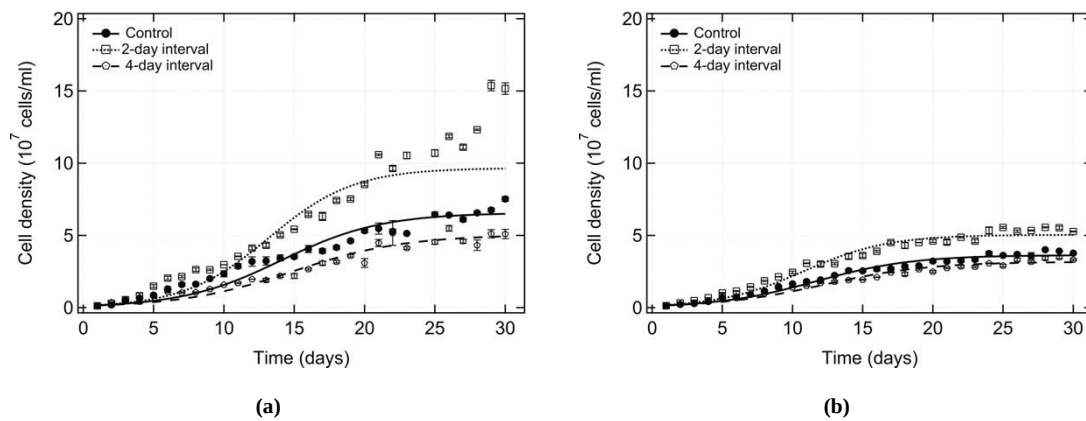


Figure 3. Effect of ultrasonic intervals (2-day and 4-day) on *B. braunii* algae. Symbols: $\square$ (2-day), $\diamond$ (4-day), $\bullet$ (control). Ultrasonic conditions: (a) 37 kHz frequency, 83.6 W power, 3-min exposure. (b) 80 kHz frequency, 67.2 W power, 3-min exposure. Data points show average values from triplicate measurements, with error bars indicating standard deviations

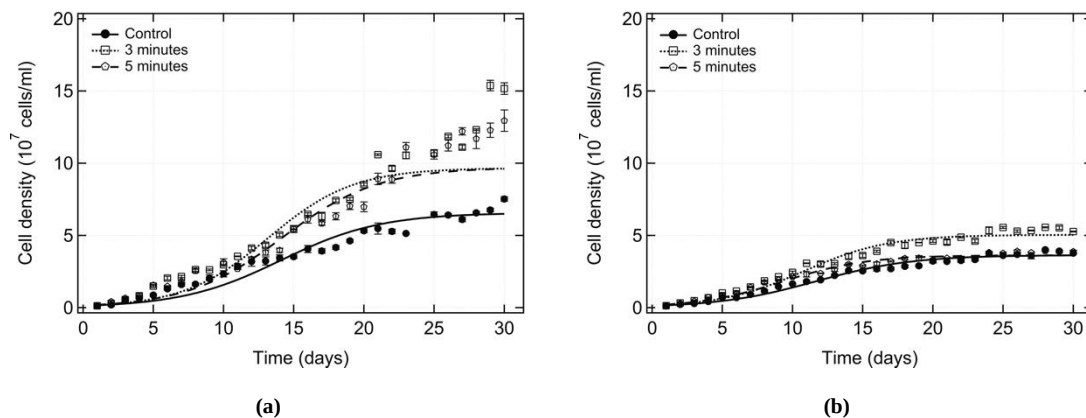


Figure 4. Impact of ultrasonic exposure times on *B. braunii* microalgae. Symbols: $\square$ (3 min), $\diamond$ (5 min), $\bullet$ (control). Ultrasonic conditions: (a) 37 kHz frequency, 83.6 W power. (b) 80 kHz frequency, 67.2 W power. 2-day interval between exposures

Additionally, daily ultrasound exposure enhanced *Nannochloris* sp. growth and biomass productivity, suggesting initial exposure stress alone is inadequate (Vintila *et al.*, 2023).

Influence of Ultrasonic Exposure Time

The impact of various ultrasonic exposure times (3 and 5 min) on the growth curve and biomass productivity of *B. braunii* microalgae was investigated. As depicted in Figure 4, the growth curves illustrate how the microalgae responded to specific conditions: Figure 4(a) a 37 kHz ultrasonic frequency with 83.6 W ultrasonic power and Figure 4(b) an 80 kHz ultrasonic frequency with 67.2 W ultrasonic power, each applied at 2-day intervals. The results demonstrate a significant increase in

the cell density of *B. braunii* microalgae when exposed to ultrasonic waves for both 3 and 5 min, in comparison to the control condition without ultrasonic waves, for both ultrasonic frequencies. Supporting these results, specific growth rate and biomass productivity data further substantiate the higher reproductive rates for *B. braunii* microalgae stimulated with ultrasonic waves for 3 min, with an increase of approximately 11.53% compared to the 5-min stimulation for the 37 kHz frequency. Similarly, for the 80 kHz frequency, microalgae exposed to ultrasonic waves for 3 min exhibited a reproductive rate increase of approximately 2.46% compared to the 5-min stimulation. Sivaramakrishnan and Incharoensakdi (2019) examined the effect of ultrasonic wave exposure on

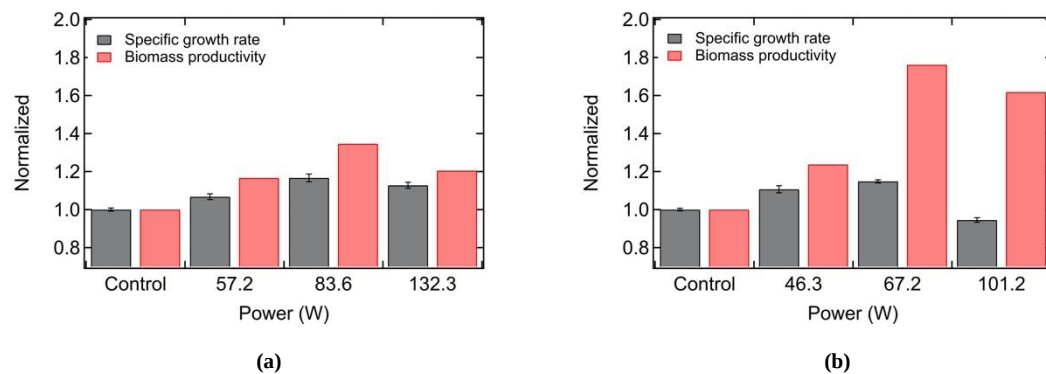


Figure 5. Impact of ultrasonic power and frequency on the relative specific growth rate and biomass productivity of *B. braunii* microalgae. Ultrasonic conditions : (a) 37 kHz frequency, (b) 80 kHz frequency, both with 3-min exposures at 2-day intervals

microalgae using various durations, ranging from 0 to 10 min. Results consistently showed that a 4-min treatment yielded the highest biomass. Beyond this optimal duration, biomass declined noticeably. Notably, ultrasonic waves play a crucial role in promoting pore formation on cell walls, enhancing biomass by facilitating better nutrient and light absorption (Han *et al.*, 2016; Pereira *et al.*, 2023). The use of a short-duration ultrasound process is a common practice due to the direct relationship and proportionality between ultrasonic time and energy consumption (Duan *et al.*, 2017). When subjected to increasing levels of ultrasonic energy and prolonged treatment times, microalgae are exposed to heightened ultrasonic wave energy, leading to adverse effects due to elevated environmental stress.

Influence of Ultrasonic Power

B. braunii microalgae reproduction rate under different ultrasonic powers was investigated. The microalgae samples were subjected to ultrasonic waves with an exposure time of 3 min at 2-day intervals. Figure 5 presents the normalized data for specific growth rate and biomass productivity under three distinct ultrasonic power conditions: 57.2, 83.6, and 132.3 W for 37 kHz (Figure 5(a)), and 46.2, 67.2, and 101.3 W for 80 kHz (Figure 5(b)). The results reveal that all ultrasonic power conditions are more effective than the control for the 37 kHz ultrasonic frequency. The most significant increase in specific growth rate and biomass productivity, approximately 16.67% and 34.62%, respectively, was achieved with an ultrasonic power of 83.6 W when exposed to a 37 kHz ultrasonic frequency. For the 80 kHz ultrasonic frequency, the use of ultrasonic power at 67.2 W resulted in a positive increase in specific growth rate and biomass productivity of approximately 14.83%

and 76.18%, respectively, compared to the control. Notably, the biomass productivity exhibited a similar trend to the specific growth rate for both ultrasonic frequencies. Sivaramakrishnan and Incharoensakdi (2019) investigated the impact of ultrasonic power on *Scenedesmus* sp. They found that the highest biomass was observed at an optimal ultrasonic power of 20 W. However, increasing the power beyond this optimal level resulted in a decrease in biomass, likely due to cell damage caused by the higher ultrasonic power (Sivaramakrishnan and Incharoensakdi, 2019). Cell damage may occur as a result of using high-intensity ultrasonic radiation. Furthermore, the cavitation phenomenon, which is caused by ultrasonic waves, might negatively impact intracellular enzymes that are essential for important metabolic processes linked to growth rate and biomass productivity of microalgae. (Wu *et al.*, 2012; Joyce *et al.*, 2014; Sivaramakrishnan and Incharoensakdi, 2019). Ultrasonic waves may harm crucial intracellular enzymes, disrupting cell membranes or creating pores via cavitation near cell surfaces. This may increase membrane permeability, facilitating the release of intracellular components or solvent penetration. Ultrasonic waves may also influence microalgae hydrocarbon accumulation, altering metabolic processes within cell membranes due to environmental stress (Gani *et al.*, 2016; Liu *et al.*, 2022; Pereira *et al.*, 2023).

Influence of Ultrasonic Frequency

Under the influence of ultrasonic frequency, the specific growth rate and biomass productivity of *B. braunii* microalgae were considered from the optimal conditions in previous section. In the previous section, the maximum specific growth rates for two specific ultrasonic frequencies included 37 kHz with an ultrasonic power of 83.6 W

and 80 kHz with an ultrasonic power of 67.2 W. These conditions led to a rise in specific growth rate relative to the control condition of approximately 16.67% for 37 kHz and 14.83% for 80 kHz. According to these findings, the *B. braunii* microalgae reproduced more quickly under the 37 kHz ultrasonic frequency. This difference can be attributed to the number of acoustic cycles and the behavior of cavitation bubbles. As the frequency increases, the size of the bubble decreases, requiring fewer acoustic cycles to reach the desired resonant size (Gogate, 2002). Consequently, the collapse of the bubble is accelerated at higher frequencies, resulting in a greater magnitude of pressure collapse (Gogate, 2002; Zhang *et al.*, 2006). Additionally, higher frequencies influence radical production and attack, leading to the disruption of cell walls and subsequent release of cell contents due to free radical interactions with the cell surface (Wu and Mason, 2017). If the resonance radii align with the size of microalgae, the oscillation of these bubbles near microalgae cells can serve as a source of excitation for their mechanical resonance, thereby inducing shape vibrations in the cells (Yamamoto *et al.*, 2015). For instance, *S. dimorphus*, with a length of 10-20 μm and a clustered appearance, undergoes the separation of cell clusters when exposed to ultrasonic wave, increasing the overall number of cells. Conversely, *N. oculata*, characterized by small (2-4 μm), spherical, and dispersed cells, is susceptible to disruption by ultrasonic wave, potentially leading to cell breakage or complete destruction (Wang and Yuan, 2016).

After cultivating *B. braunii* for 30 days with ultrasonic waves, a total electric energy consumption of 19.4 kJ was used for each stimulation. Throughout 15 stimulations, a cumulative energy usage of 291.6 kJ was used. Consequently, the final biomass productivity exhibited a notable increase of 34.62% compared to cultures without ultrasonic waves.

Conclusions

The impact of ultrasonic wave on *B. braunii* microalgae was studied, with a focus on growth rate and biomass productivity. Various combinations of ultrasonic parameters, including ultrasonic power (56.2, 86.3, and 132.2 W for 37 kHz; 46.3, 67.2, and 101.2 W for 80 kHz), exposure times (3 and 5 min), and time intervals (2 and 4 days), were explored to determine the optimal conditions for ultrasonic excitation. The findings clearly demonstrate that the best results were achieved with a 37 kHz ultrasonic frequency, 83.6 W power, 3-min exposure, and a 2-day interval, leading to

a maximum relative specific growth rate (16.67%) and relative biomass productivity (34.62%). It is evident that altering ultrasonic treatment conditions beyond these parameters led to a decrease in both growth rate and biomass productivity. This underscores the importance of considering multiple ultrasonic parameters, including frequency, power, exposure time, and interval, to optimize microalgae cultivation using ultrasonic waves, as neglecting any of these factors can hinder reproduction.

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