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EXPEDITING GROWTH RATE OF BOTRYOCOCCUS BRAUNII USING 37- AND 80-KHZ ULTRASONIC WAVES

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Energy from natural sources are widely investigated nowadays. Biomass is an alternative source of energy extracted from biological wastes or materials as fuel. Various plants are the sources of bio fuel; however, the downfall of plants is that they require large area to grow for proper product. On the other hand, microalgae are possibly a better source of energy since they require less area for farming and produce greater amount of oil as compared to that derived plants. *Botryococcus braunii* is a green microalga and is found in natural freshwater reservoirs in colony form. The algae can provide oil at a maximum of 75 percent of their dry weight. In this work, we use ultrasonic waves with the frequencies of 37 and 80 kHz to excite mix culture of algae samples in an ultrasonic bath. Ultrasonic power and exposure time are parameters in this study. The ultrasonic-exposed algae are sampled for photo-taking purpose under a microscope. The photos are then processed using particle analysis to count the number of algae colonies in comparison to the control algae sample. The result shows that the algae display significant growth with the exposure time of 3 and 5 minutes for 37 kHz waves with the ultrasonic power of 54 and 72 W. For 80 kHz, the power of 60, 90, and 120 W also provide significant multiplication of the algae. With exposure period at a 2-day interval we find that the multiplication is twice of that of the control.
Keywords: Ultrasonic wave, growth rate, *B. braunii*

1. Introduction

Currently, energy has become indispensable for both industries and everyday life. The main source of energy is derived from fossil fuels in the form of crude oil. Due to dramatic need of energy because of rapid industrial growth, raw materials for energy have significantly reduced causing oil price crisis. These problems have led research to find alternative sources of energy.

Alternative sources of energy are, for example, nuclear power, wind power, water power, solar energy, and biofuels. Biofuels play an important role in Thailand due to its abundant agrarian sources. Various plants are currently extracted for bio fuel. However, the planting area is limited [1]. Microalgae are possibly a better source of energy since they require less area for farming and produce greater amount of oil as compared to that derived plants [2].

Microalgae is unicellular lifeform typically found in water reservoirs, for example, freshwater, saltwater or brackish water etc. [3, 5]. In addition, it has the ability of photosynthesis for the production of biofuels and the conversion of carbon dioxide into biomass supporting algal growth. *Botryococcus braunii (B. braunii)* is interesting since it provides fuel oil at a maximum of 75 percent of its dry weight [4]. However, *B. braunii* shows slow growth but for practical use certain amount of extracted oil is required. Therefore, accelerating its growth is essential. In this work, ultrasonic waves are used to expedite the growth in terms of frequency and effective acoustic power.

Ultrasound has been widely used for stimulating biological tissues in which the wave causes change of membrane permeability leading to increases of protein synthesis and cell growth [6-8]. Increasing the growth rate of Staphylococcus epidermidis, Pseudomonas aeruginosa and Escherichia coli cells was performed using ultrasonic waves at 70 kHz with the power of < 2 W/m^2. The result shows that ultrasonically-stimulated cells display the faster growth than those without ultrasonic exposure. Ultrasonic waves help increasing the transport of oxygen and nutrients to the cells and transport biological waste from cells leading to the proliferation of bacterial cells [9]. Outdoor cultivation of *B.braunii* were stimulated by ultrasonic waves at 40 kHz with the power of 300 W for 5 minutes for 4-day interval. As a result, the ultrasonically-treated algae yielded the growth rate of 0.089 per day as contrast to those untreated yielding 0.077 per day [10]. Furthermore, ultrasound was also employed to stimulate Cyanobacteria using different frequencies (20, 40, 580, 864, and 1146 kHz) and different powers. The bacteria were ultrasonically-exposed for 0-, 5-, 10-, 20-and 30 minutes. The outcome indicated that the 40-kHz ultrasonic wave displayed the maximum increase of cell population [11].

2. Material and methods

2.1 Culture System

Algae samples (*Botryococcus braunii – B.braunii* – algae) were collected from water reservoirs in Walailak University, Nakhon Si Thammarat, Thailand (latitude 8°38′32.25″N, longitude 99°54′26.52″E). This reservoir is brackish water and slow flow with the pH of 7.2. Dissolved oxygen is 6.47 mg/L [5]. A plankton net of 67-micron size was used to collect algal samples. The collected samples are divided to culture in 200-ml flask at 25°C with light intensity of 5,000 lux for 16:8 hour of light : dark cycle period. The algal density is then determined by using Sedgewick-Rafter slide under a microscope.

Figure 1: Samples from Walailak University reservoirs.

2.2 Ultrasonic treatment and experiments

Algae culture were stimulated in an ultrasonic bath (Elmasonic P60H). The effective ultrasonic powers used to excite the algae are 54, 72, 90, 108, 126, 144, 162 and 180 W for 37 kHz and 45, 60, 75, 90, 105, 120, 135, and 150 W for 80 kHz. Two flasks or test tubes are placed in the bath as shown in Figure 2. The flasks are held stable using a 1-cm thick acrylic plate. The bath is operated using a sweep mode.

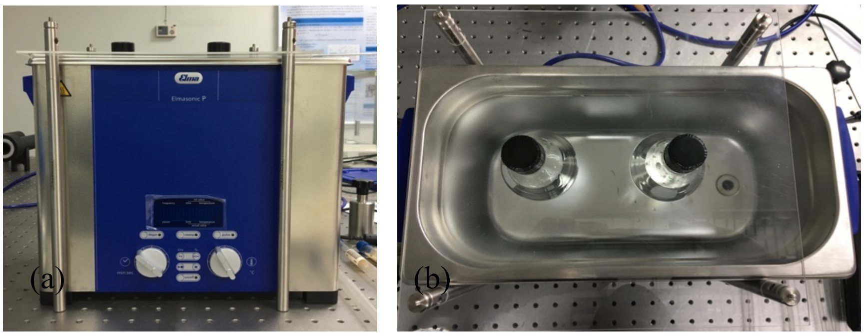

Figure 2: (a) Ultrasonic bath and (b) Flask position in the bath.

Initially, the tests are performed to find appropriate ultrasonic frequencies, powers, and exposure time that accelerate the growth of algae. The experiments are undergone by using 5-ml of algal sample in each test tube (80 tubes overall). Two tubes are exposed to ultrasonic waves at a time. Each time the experimental conditions are different, e.g., ultrasonic frequency of 37 kHz with the power of 54 W, and 1-min exposure time. Five exposure times are used—1, 3, 5, 10 and 15 minutes.

The later experiments are to search for proper intervals—1, 2, 3, and 4 days—of ultrasonic treatment. These experiments are done in 200-ml flasks.

2.3 Cell counting

Colonies of algae are counted using image processing which is performed on images taken under a microscope (Nikon eclipse ME600 microscope) using a smartphone. The images are then processed using particle analysis for counting the number of algae colonies. The data images are converted to grayscale and background is removed using threshold colour value. The number of pixels representing the smallest colony is given for the particle analysis in order to properly counting algal samples. The number of ultrasonically-treated algal colonies is then compared to those of the control sample.

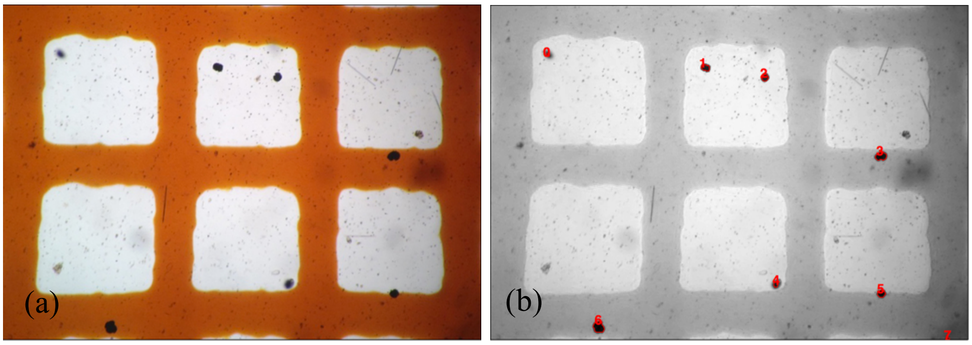

Figure 3: Processed images for colony counting (a) The micrograph has been taken using magnification of 50x. (b) Photo was taken image processing by analyzing the number of colonies.

3. Results and discussion

3.1 The effects of ultrasonic frequency and power on microalgae at various exposure times

From Tables 1 and 2, it is clear that the exposure time of 3 and 5 minutes displays increases in colony ratio—colony number of ultrasonically-treated per that of control sample—for both 37 and 80 kHz ultrasonic waves. The increase is insignificant for 1-min exposure. On the other hand, for longer exposure time, 10 and 15 minutes, the ultrasonic waves damage the algal colonies and cells. When considering effective ultrasonic powers at 37 kHz, 54 and 72 W show consistent rise in the number of colonies. The difference in colony ratio (DCR) between that of exposure time, 3 and 5 minutes, is used to determine the temporal growth of the algae sample. The DCRs are 0.0318 and 0.0056 for 54 and 72 W, respectively. For intense ultrasonic powers, 126, 162, and 180 W, the DCRs are decreased significantly due to cell destruction. The DCRs for 90, 108, and 144 W are 0.3501, 0.414, and 0.2195, respectively. Even though, the values of DCR are quite high but they are not consistent when considering the temporal development.

For 80 kHz, the colony ratios smoothly increase for all powers, except for 75 and 105 W due to decrease of the colony ratio or negative DCR. The DCR is maximum, 0.1552, at 120 W and minimum, 0.0431 at 90 W. At 60-W power, the DCR is 0.0947. The DCRs are 0.0503, 0.0459, and 0.0503 for 45, 135, and 150 W, respectively.

The above results provide crucial information on how the algal sample should be treated in terms of frequency, power, and exposure time. They allow us to select appropriate conditions for ultrasonic radiation. For 37 kHz, two power levels are chosen, 54 and 72 W with the exposure time of 3 and 5 minutes. For 80 kHz, three power levels are chosen, 60, 90, 120 W with the exposure time of 3 and 5 minutes. The selection criterion includes the consistency rise in colony ratio as time progresses.

Table 1: The effects ultrasonic waves on microalgae representing in colony ratio (Colony per millilitre of ultrasonically-treated per that of control) at ultrasonic frequency 37 kHz for various exposure duration

Power (W)	Exposure time (minute)					
	0	1	3	5	10	15
54	1	0.9256	0.9821	1.0139	0.9464	0.9940
72	1	1.0380	1.0342	1.0398	1.0474	0.9488
90	1	0.9696	0.9193	1.2694	1.0930	1.1233
108	1	1.0152	0.8782	1.2922	0.9810	0.6888
126	1	1.0765	0.9272	0.9053	0.9927	1.0128
144	1	1.1020	0.9699	1.1894	1.1275	1.0765
162	1	0.8244	0.9843	0.9580	0.9924	0.6084
180	1	1.0612	0.9808	0.9213	0.8427	0.7640

Table 2: The effects ultrasonic waves on microalgae representing in colony ratio (Colony per millilitre of ultrasonically-treated per that of control) at ultrasonic frequency 80 kHz for various exposure duration

Power (W)	Exposure time (minute)					
	0	1	3	5	10	15
45	1	0.9737	0.8671	0.9174	0.8048	0.9006
60	1	0.8515	0.8263	0.9210	0.8120	0.7868
75	1	0.8970	0.9246	0.8240	0.8263	0.7808
90	1	0.7988	0.7377	0.7808	0.8754	0.7737
105	1	0.9784	0.9987	0.9285	0.9217	0.9069
120	1	1.0472	0.8920	1.0472	0.9325	1.0337
135	1	0.8448	0.9339	0.9798	0.8151	1.0297
150	1	0.9737	0.8671	0.9174	0.8048	0.9006

3.2 The cultivation of *Botryococcus braunii* algae with ultrasonic waves

In this part, results from previous experiment are employed for algal cultivation in order to find growth rate of *B.braunii* algae at various temporal exposure interval. Algae sample in 200-ml flask are cultured and stimulated with ultrasonic wave for different intervals (1, 2, 3 and 4 days, respectively) within 10 days. The excitation conditions are followed from previous section, that is, for 37 kHz, ultrasonic power of 54 and 72 W are used with 3- and 5-min exposure time and for 80 kHz, ultrasonic power 60, 90 and 120 W are employed with 3- and 5-min exposure time.

Table 3: Colony ratio of *B. braunii algae* of with ultrasound stimulation in 3 minute

Treatment	Control	Frequency 37 kHz		Frequency 80 kHz		
		Power 54 W	Power 72 W	Power 60 W	Power 90 W	Power120 W
1-day interval	1.20	1.22	1.09	1.43	1.22	1.19
2-day interval	1.20	2.04	2.04	1.83	2.50	1.96
3-day interval	1.20	1.48	1.68	1.82	1.79	1.79
4-day interval	1.20	1.78	1.75	1.46	1.54	2.05

Table 3 displays the results of ultrasonic radiation for designated conditions. The most increase of colony ratio shows in every-two-day interval for all conditions, excluding the power of 120 W at 80 kHz. The outstanding rise, twice the control, occurs at the frequency of 80 kHz with 90-W power. At one-day interval the increase is insignificant and erroneous. For 80 kHz, other intervals show slight increase; however, the increase is rather strong at 37 kHz for every other day exposure.

Meanwhile, for 5-min exposure, the increase is maximum at 37 kHz with 72 W for every other day interval identical to those of 3-min exposure as shown in Table 4. All other conditions display the increase in colony ratio as well.

The cultivation of *B. braunii* algae with ultrasonic waves are stimulated at each ultrasonic frequency and power with 3- and 5-min exposure for 1-, 2-, 3- , and 4- day intervals for 10 days. As a result, the colony ratio for 2-day interval with both exposure times is *twice* of that the control.

Table 4: Colony ratio of *B. braunii algae* of with ultrasound stimulation in 5 minute

Treatment	Control	Frequency 37 kHz		Frequency 80 kHz		
		Power 54 W	Power 72 W	Power 60 W	Power 90 W	Power120 W
1-day interval	1.20	1.34	1.30	1.24	1.29	1.39
2-day interval	1.20	2.33	2.52	1.91	1.80	1.77
3-day interval	1.20	1.71	2.00	1.45	1.48	1.58
4-day interval	1.20	1.76	1.75	1.96	1.53	1.73

4. Conclusion

B. braunii algae are collected from water reservoirs in Walailak University, Nakhon Si Thammarat, Thailand with a 67-micron size of plankton net. Colonies of algae are counted using image processing analysis. In the first experiment, the algae samples in 5 ml are tested for the effects of ultrasonic frequency, power, and exposure time. The results allow us to choose appropriate conditions. For 37 kHz, we use the power of 54 and 72 W. For 80 kHz, 60, 90, and 120 W are chosen. The exposure times are 3 and 5 minutes. These conditions play important roles when ultrasonic treatment is imposed on algal cultivation. The ultrasonic treatment for algal cultivation is carried on for four intervals—1- , 2-, 3- and 4-days—within 10 days. The outcome indicates that every-two-day stimulation yield the maximum growth in algal colony ratio of approximately twice as many for all frequencies and certain power level.

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