JOURNAL OF PURE AND APPLIED MICROBIOLOGY, May 2014. Vol. **8**(Spl. Edn. 1), p. 519-524

Influence of Ultrasonic Intensity on *Microcystis* sp. Viability: A Flow Cytometric Analysis

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(Received: 12 April 2014; accepted: 09 May 2014)

Cyanobacteria belong to the photosynthetic bacteria by some characteristics of algae. It has long been found that some cyanobacteria may produce toxic hazardous, thus posing a threat to both animals and human health. The green ultrasound technique has been a promising tool for removing cyanobacteria from water while research on the influence of ultrasound on *Microcystis* sp. viability has attracted wide attention. The effect of ultrasonic intensity on *Microcystis* sp. viability in this study was investigated while the quantitative analysis of the *Microcystis* sp. viability during ultrasound was carried out with flow cytometry (FCM). Results indicated that with a lower ultrasonic intensity below the cavitation threshold, 62.23% of the algal cells were damaged but the death rate of algal cells was 0.82%. However, higher ultrasonic intensity above the cavitation threshold led to a death rate up to 41.51% and damage to algal cells of 58.16%.

Key words: Flow cytometry (FCM), Ultrasound; Cell viability; *Microcystis* sp., Ultrasonic intensity.

The *Microcystis* sp. is most common harmful algae seen in water blooms. It belongs to the light autotrophic cyanobacteria that have unique physiological mechanisms and evolutionary mechanisms, which enable it to have competitive advantage of ecology in eutrophic water[1]. One of the problems that need to be solved quickly in the process of eutrophication governance is how to control the growth of algae[2]. At present, people has carried out some researches on different methods such as physical, chemical and biological methods. The study found that sonication can inhibit the growth of algae, and has the advantages of relatively easy to operate and control, no introduction of other chemical substances, mild reactive conditions, fast reaction[3], which is economical. Thus, the method is called environment-friendly and "green" technology[4,5] and has a broad prospect in application[6,7]. After ultrasonic irradiation, some algal cells were dead directly; some were damaged; and a small number of cells were not affected[8-13]. The Effect of ultrasound of different intensity on the algal cells is also different[14,15]. The focus of this study is using specific cell identification method to characterize the content of dead, injury and normal cells intuitively[16].

Flow Cytometry (FCM) can do quantitative analysis of sub-cellular structures in the suspension of single-cell quickly[17,18]. It can distinguish living algal cells from dead cells quickly[19], particulate matters and mixed algae, and can also do multi-parameter measuring including size, shape, and fluorescence intensity of *chlorophyll a*[20]. In addition, FCM can reflect the integrity and activity of cell membrane, the content of RNA and DNA and other physiological and biochemical characteristics[21]. Currently, some

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scholars at home and abroad have used the FCM to study Nano phytoplankton[18]. Olson R.J. *et al.* measured the auto fluorescence of phytoplankton chlorophyll to determine its photosynthetic capacity by FCM[22]. Guo utilized FCM to measure the content of photosynthetic pigments and light scattering signals of freshwater Nano phytoplankton in order to distinguish the cells fast and instantly and draw the cell counts. Wang and Yingying et al. made a summary and outlook about FCM in the application of aquatic microorganisms, demonstrating that the method has a broad application prospect in the analysis of single-cell and group microorganisms[23].

This study analyzed the effect of different ultrasonic intensity on the growth, integrity of membrane and activity of esterase, characterized condition of algal cells quantitatively after sonication, and discussed the physiological and biochemical mechanisms about suppressing the growth of *Microcystis* sp. under different ultrasonic intensity, providing theoretical basis for the more effective use of ultrasound to control *Microcystis* sp. and the development of the technology.

MATERIALS AND METHODS

Algae species and culture

Microcystis sp. was provided by fresh water algae culture collection of the Institute of Hydrobiology of Chinese Academy of Sciences (FACHB-1027). We placed the algae in a constant-light incubator to inactivate the culture. The temperature was set to 25 °C, light intensity to 2000 lx, and the light–dark cycle to 14 h: 10 h. The algae were initially cultivated in the prepared monoculture BG11, which was provided by the Freshwater Algae Culture Collection of the Institute of Hydrobiology. The pH value of the BG11 medium was 7.1, which was regulated using NaOH or HCl. The algae were cultivating until their density reached 10^7 cells/mL. At this concentration, the algae liquid can be used for the ultrasonic experiment.

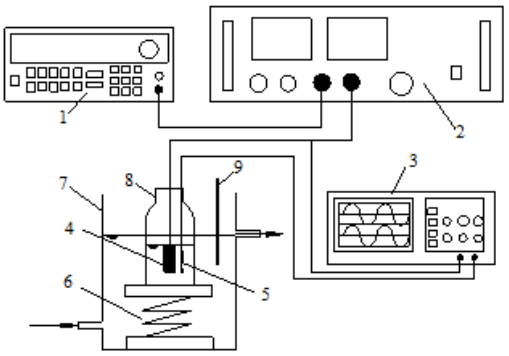


Fig. 1. Experimental ultrasonic apparatus (1. Signal Generator; 2. Power Amplifier; 3. Oscilloscope; 4. Transducer; 5. Hydrophone; 6. Telescopic stand; 7. Cooling device; 8. Glass flask; 9. Thermometer)

Ultrasonic algae removal device

The device used in this study was developed by the Institute of Acoustics of the Chinese Academy of Sciences. The other parts of reactors were homemade. The experimental setup is shown in Fig. 1. The ultrasound source was an ATANA AT3020 ultrasonic signal generator. The power amplifier with model number HFVA-62 was from Nanjing Foneng Co., Ltd. The oscilloscope with model number was UT2025C produced by Hong Kong Unit Co.

Preparation of dye and dye

According to Franklin[24], PI (Propidium Iodide, Sigma-Aldrich, P-4170) was diluted to the 100 µmol/L by DPBS (Dulbecco Phosphate Buffered Saline, Hyclone);then they were saved in a brown bottle at 4°C in a refrigerator. The final concentration of dye was 60 µmol/L when observed. Dissolving FDA (Fluorescein Diacetate, Sigma-Aldrich, F-7378) in acetone to obtain the solution of 1 mmol/L, and then save them in a brown bottle at -18°C. The final concentration of dye was 100 µmol/L observed under fluorescence microscopy.

Determine integrity of cell membrane and activity of esterase by FCM

The following are the steps of determine integrity of cell membrane and activity of esterase by FCM. Researchers took 10 mL of algae solution into centrifuge tube, used vortex mixer to oscillate for 10 min which allowed the cluster state of *Microcystis* sp. cells to be sufficiently spread. After filtering with a 300 mesh filter, researchers used pipette to take 1 mL into 1.5 mL PE centrifuge tube. Then , 2 µL of FDA fluorescent dye was taken to stain cell. The concentration of FDA was 2 µmol/L after staining, then incubated for 8 minutes in the dark place under 25°C to make cells fully stained. The 2 µL of PI was put to dye stain again.

The concentration of PI was 2 µmol/L after staining, then incubation for 15 min under the same conditions to stain fully. FACSCalibur (Becton Dickinson Biosciences, America) was used to determine integrity of cell membrane and activity of esterase at the same time. The flow rate in the determination of them was 1 µL/s. Each sample costed 20 s and the number of tested cells was 30000.

Data processing and analysis

In this experiment, statistical and analytical software are Microsoft Excel, Origin8.0 and SPSS Statistics 19. The data tested by FCM was analyzed by FCS Express 4 Flow Research Edition..

RESULTS AND DISCUSSION

The viability determination of *Microcystis* sp. cells by double staining with FDA and PI

In order to prove FDA-PI double staining with fluorescence Microscope can effectively characterize the dead and live cells, the pre-experiment with live and dead cells was needed to do. First, *Microcystis* sp. cells in logarithmic phase were mixed, and then they were divided into two parts. One was control group with no treatment, and the other was boiled for 30 minutes to make all cells dead. After staining, cells were tested and analyzed in FCM. Fig. 2 is the forward scatter histogram plot of live and dead cells subgroup.

In Fig. 2, the FSC-H of live cells located in the right side of the histogram was higher than dead cells, indicating that boiling made cells deformed, broken and inactive.

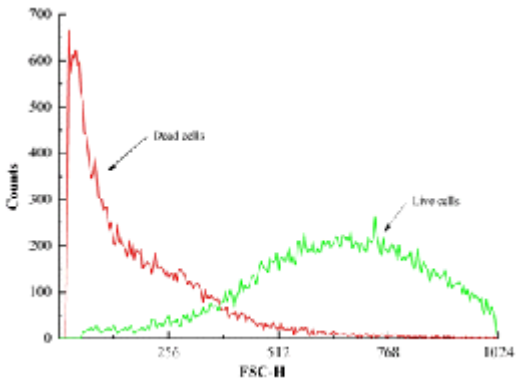


Fig. 2. Aglae cells control sub-populations analyzed by forward scatter histogram plot

FCM can characterize cellular viability in a scatter by identifying dead cells, live cells and injured cells. Each point in the figure represents a single event, and each event represents different histogram. The dates were divided into four parts namely four quadrants, and each quadrant had different meaning. FL1-H represented FDA fluorescence. FDA^+ was the characterization of live cells, and FDA^- was the characterization of dead cells. FL2-H was PI fluorescence. PI^+ characterized live cells, and PI^- characterized dead cells. Upper (UL) left area in FL1-H - FL2-H histogram was FDA^- - PI^+, representing dead cells, while the lower right (LR) area was FDA^+- PI^- that represented live cells. The lower left region (LL) and right upper area (UR) were FDA^-- PI^- and FDA^+- PI^+ respectively, both representing damaged cells, FDA^-- PI^- quadrant in these two areas represented that the damaged cells were in late apoptosis and were close to death. FDA^+- PI^+ quadrant represented the cells were in early apoptosis and they would go into the programmed cell death. Each point of each quadrant represented a cell or a fragment. The percentage of each quadrant was calculated to make a quantitative analysis of cell viability; the proportions of dead cells, live cells and damaged cells were obtained. Fig. 3 is live and dead cells control sub"populations analyzed by a "dot plot".

In Fig. 3, live cells accounted for 96.07% and the damaged accounted for 3.29% in figure A. The appearance of damaged cells was partly because of their metabolism that is programmed cell death, and partly due to the damage caused by pretreatment experiment. There were 0.01% of the dead in live group, indicating that *Microcystis* sp. cell activity in live cells group was higher. After boiling for 30 min, points moved from the lower right to the upper left significantly. Dead cells accounted for 95.30%, damaged cells accounted for 4.69%, and the live cells was only 0.01% in this diagram. In summary, FCM is available for quantitative analysis of the changes of algal cell viability.

Effect of sonication on the viability of *Microcystis* sp. Cells

The *Microcystis* sp. solution whose initial density was (207.4±6) $\times 10^4$ cells/mL was divided into three groups. The first was control group that had a normal growth process. The second group of 50 mL solution was subjected to sonication at

580 kHz of frequency, 5 min of reaction time, 10 W of power density that was lower than cavitation threshold. The second group was "no cavitation group" because there was no occurrence of cavitation in the group. The third group was 50 mL algal cell solution after sonication at 580 kHz, 5

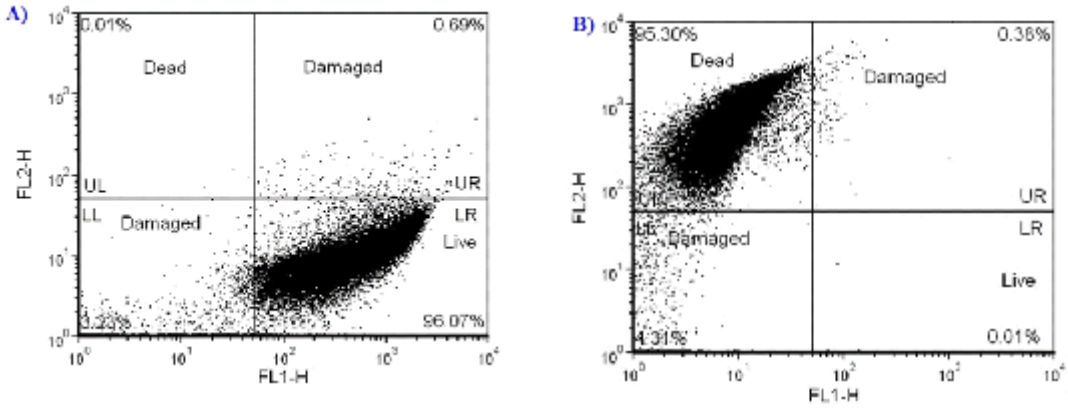


Fig. 3. Aglae cells control sub"populations analyzed by a "dot plot" A) live B) dead

min, 100 W that was higher than the ultrasonic cavitation threshold by testing. The second group was "cavitation group" because the occurrence of cavitation. Each group set three parallel samples. Did FDA-PI double staining immediately after sonication, and then detected in FCM. Fig. 4 depicts the forward scatter histogram plot of control sub"populations analyis of control, non-cavitation and cavitation group

As can be seen from Figure 4, compared with the control group, the number in FSC-H of no cavitation group on the right region reduced after sonication, and some of them moved to the left area, which revealed that ultrasound reduced the number of live cells of no cavitation group, and increased the number of damaged and cells dead

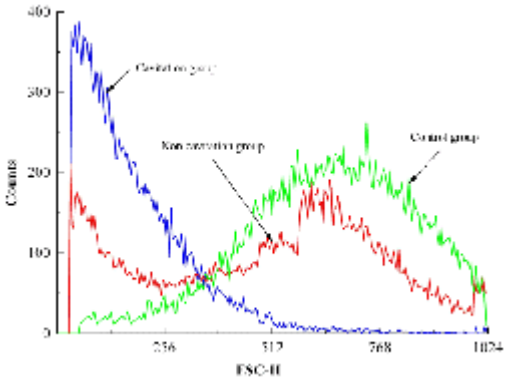


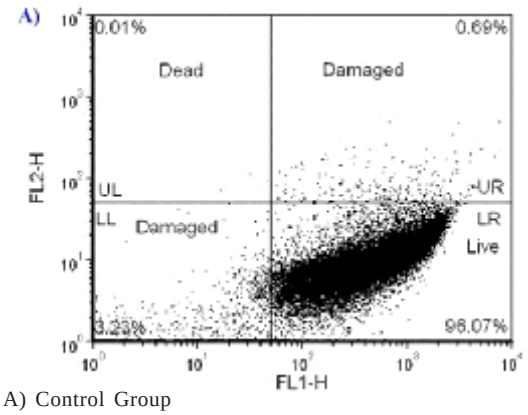
Fig. 4. Aglae cells control sub-populations analyzed by forward scatter histogram plot

cells. In cavitation group, live cells depleted after sonication, and most of them moved to the damaged or died area, indicating that the intensity of ultrasound made most of cell dead or damaged. Fig. 5 is forward scatter histogram plot of control sub"populations analysis of algae cells.

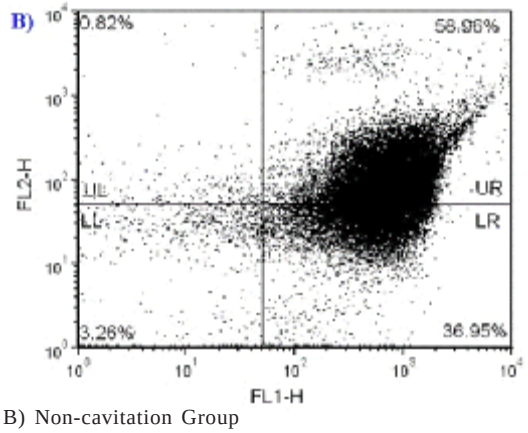
In the histogram of Fig. 5, compared with the control group, cells of non-cavitation group move from the lower right area to the upper right area, indicating that low-intensity ultrasound damaged *Microcystis* sp., and dead cells accounted for only 0.82% of the total cells. Contrast the histogram of control group and cavitation group, cells moved to the upper half area from the lower right corner, illustrating ultrasonic intensity above the cavitation threshold caused most of the algal cells get impaired or dead. After ultrasonic treatment, detecting *Microcystis* sp. cells immediately to get the statistical results of the cells viability of control group, cavitation group and non-cavitation group. The specific statistical values were shown in Figure 6.

It can be seen from Fig. 6 that live cells in control group, non-cavitation and cavitation group accounted for 96.07%, 36.95%, and 0.33% respectively, dead cells accounted for 0.01%, 0.82% and 41.51% respectively, and damaged cells accounted for 3.92%, 62.23% and 58.16% respectively. When the power was lower than the cavitation threshold, cells mainly got injured after sonication. A test for detection of *Microcystis* sp.

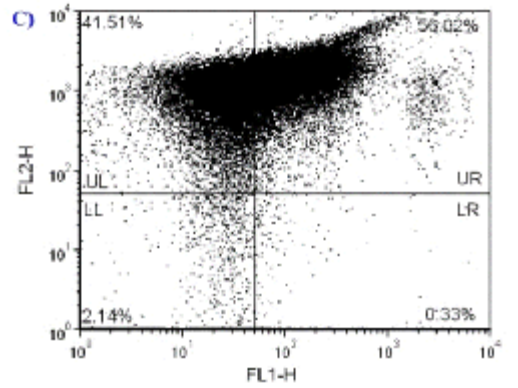
after being treated at 500 kHz, 0.00006 W/mL for 36 h showed that algal density decreased from 207.4×10^4 cells/mL to 172.1×10^4 cells/mL, and the removal efficiency was 25.25%; then cells entered the programmed death stage. However, ultrasound higher than the cavitation threshold made most of cells damaged or dead and live cells were depleted. After being treated at 500 kHz, 0.0101 W/mL for 36 h, algal density decreased by 97.35% from 201.5×10^4 cells/mL to 6.11×10^4 cells/mL, and damaged cells also underwent programmed cell death phase and died.



A) Control Group



B) Non-cavitation Group



C) cavitation Group

Fig. 5. Aglae cells control sub-populations analyzed by forward scatter histogram plot

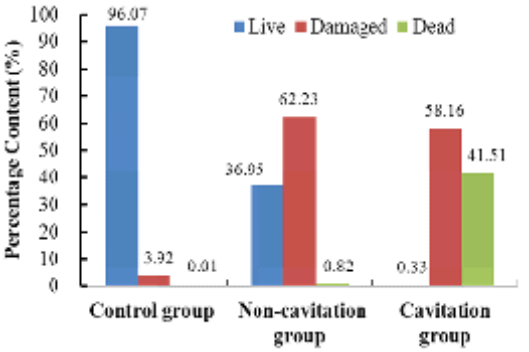


Fig. 6. The charts of algae cells activity of control group, non-cavitation and cavitation group of *Microcystis* sp

Integrated on, cellular viability decreased and a small number of cells died after sonication without the occurrences of cavitation, while sonication with cavitation caused the loss of the majority of viability and the death of most of cells. The ultrasound, whose intensity was lower than the cavitation threshold, mainly made cells injured. Higher intensity (higher than the cavitation threshold) made most of cells damaged or dead, and damaged cells would die in the end.

CONCLUSIONS

In this study, the influence of ultrasonic intensity on *Microcystis* sp. viability was analyzed by using the FCM as quantitative characterization of *Microcystis* sp. cell activity. For a *Microcystis* sp. solution of 50 mL with ultrasonic radiation of 10 W at 580 kHz for 5 min, 62.23% cells get injured and 0.82% cells died. However, with higher ultrasonic intensity at 100 W, 41.51% were dead and 58.16% were damaged. The damaged cells cannot be repaired, thus high-intensity ultrasound is available to destroy most of cells.

ACKNOWLEDGEMENTS

This work was financially supported by the National Natural Science Foundation of China

(No.51308123), the scientific research Foundation in Fuzhou University (2013-XY-25) and Fund for Fostering Talents of Fuzhou University (XRC-1266).

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