An application of a Berthelot method using a metal tube to inactivation of bacillus subtilis

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Abstract: - Negative pressure of liquids is expected to inactivate bacteria by the pressure which is much lower in magnitude than positive pressure. Nevertheless, the pressure is difficult to be generated experimentally due to cavitation through heterogeneous nucleation. In order to check the expectation, a metal Berthelot tube was newly developed, solutions including bacillus subtilis experienced negative pressure repeatedly, and a number of colonies was counted by an agar dilute plate method. Results indicated that colonies which experienced negative pressures were less than those for non-treatment, and reduction ratios increased with numbers of repetition. The application of the Berthelot method will lead to a new means without any chemical compounds in future.

Key-Words: - Negative pressure of liquid, Berthelot method, Cavitation, Inactivation of bacteria
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1 Introduction

Negative pressure of liquids has been important basically in fields of science and technology. For example, it is said that the environmental stress cracking of the polymers is caused by increases in mutual solubilities between polymers and liquids as their non-solvents which undergo negative pressures [1]. However, studies on negative pressures have not been reported in comparison with those on positive pressures. The reason is that since liquids under negative pressures are thermodynamically metastable, the liquids tend to form cavities through heterogeneous nucleation. The phenomenon is called cavitation.

Of a few experimental methods, a Berthelot method using a metal tube seems to have potential as a means of studying liquid behavior under negative pressure though it may have some drawbacks [2]. The reason for the potential is that the method is capable to generate static negative pressure for not too small liquids in volume enclosed in a solid metal. Hence, metal Berthelot tube techniques for generating negative pressures up to ca. -20 MPa for water and some organics of ca. 1 cm3 have been developed [3] and has contributed to reveal properties such as phase diagrams including negative pressure regions of thermotropic liquid crystals [4].

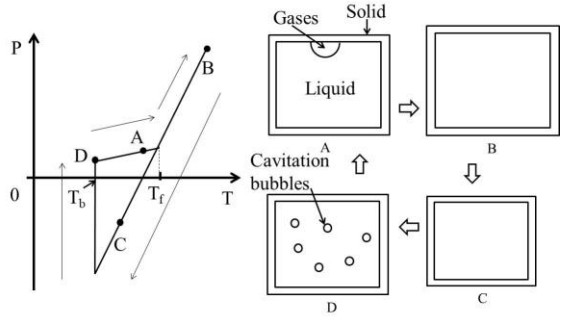
An interesting stability diagram for a kind of bacteria has been reported in a biological system [5]. The diagram of E. Coli. seems to be partly elliptical in a positive pressure-temperature plane for a lack of a negative pressure region of the plane [6]. The bacteria are inactive out of the elliptical region in a sense that a number of the bacteria decreases by two orders of magnitude within 5 min. The elliptical bottom seems to be located in a negative pressure region which is much more accessible. To authors' knowledge, there have been no studies on inactivation of bacteria in negative pressure regions.

Hence, in order to investigate inactivation effects of negative pressures, a solution including a kind of bacteria, that is Bacillus subtilis, was exposed to negative pressures as a sample liquid in a metal Berthelot tube, and a number of bacteria colonies were counted by an agar dilute plate method.

2 Experiment

A metal Berthelot method generates static negative pressure through an alternative heating and cooling procedure of a sample liquid sealed in a chamber of a solid metal tube over an appropriate temperature

range [7] as shown in Fig. 1 Since a coefficient of thermal expansion of the liquid is higher than that of the solid, the initial heating causes gases, which are both air and the liquid vapor remaining in the chamber, to be forced into the liquid so that it fills the chamber completely at a temperature T_f [8]. In the subsequent cooling, the liquid adhered to the chamber walls continues to fill it at temperatures below T_f. Thus, the liquid pressure becomes positive above the T_f, while negative below the T_f. At a lower temperature T_b, the liquid breaks, and cavitation bubbles appears with a sudden increase in pressure. The alternative procedure is called temperature cycle.



(a) (b)

Figure 1: Berthelot method; (a) a relation between pressure and temperature (b) a liquid in a chamber

Fig. 2 shows a newly developed metal Berthelot tube for this study. As shown in Fig. 2(a), the Berthelot tube consisted of main five parts, namely a screw, a socket, a nut, a ball and a pressure transducer having a specimen chamber of ca. 1500 mm3. The ball was made of brass, while the others were of type 630 stainless steel.

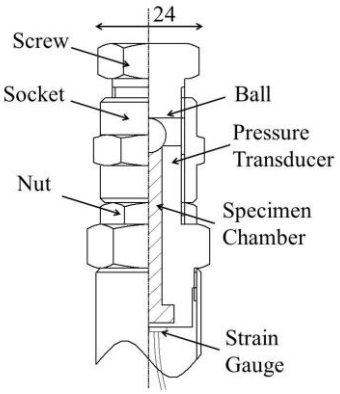


Figure 2: Metal Berthelot tube; left (overview), right (inside)

Sealing operation was carried out as follows: firstly, the five parts were pre-sterilized with ethanol and were dried sufficiently. Secondly, the transducer attached with two O rings for no leakage of water was held in a center of an attachable funnel shaped cylinder as shown in Fig. 3. Thirdly, the socket and the nut were attached to the transducer, and a hot water of ca. 60 °C for setting Tf was poured not into the chamber but into the cylinder. Fourthly, after ca. 5 minutes, a solution including a kind of bacteria was poured into the chamber with a micro-pipet, and a ball was put onto a cylindrical top edge of the chamber. Finally, the ball was compressed with the screw until the ball was deformed plastically by ca. 20 Nm with a hand torque wrench. T_f was adjusted at ca. 50 °C.

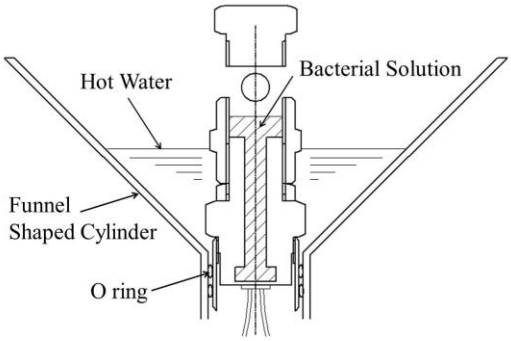


Figure 3: Sealing operation

The bacteria in the solution were Bacillus subtilis subsp. Spizizenii (JCM 2499, RIKEN). According to the supplier's manual, a solution including 2.5 % nutrient broth No.2 (Kanto Kagaku Co.) was prepared and was tested as the sample liquid.

After sealing operation, the funnel shaped cylinder was detached, the tube was immersed in a hot bath, and temperature cycles were repeated automatically as shown reported before [9]. Temperature cycles were carried out in a temperature range from ca. 10 °C to ca. 55 °C. A period for a temperature cycle took ca. 5 min.

3 Result and discussion

Fig. 4 shows a trend in negative pressures for 15 temperature cycles. Negative pressures from ca. 1.5 MPa to ca. 6.5 MPa in magnitude were measured. The average and the standard deviation were 4.23 MPa and 1.54 MPa, respectively. Table 1 showed averages and standard deviations for different temperature cycles. They were almost the same values each other.

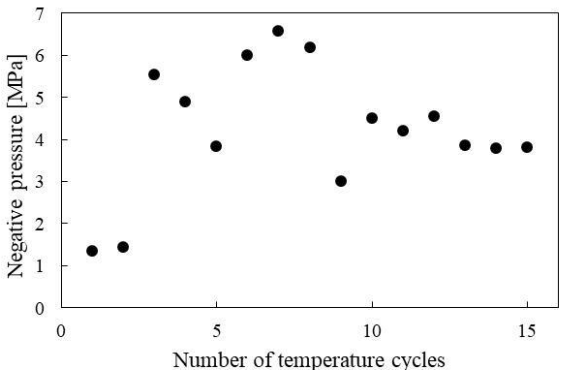
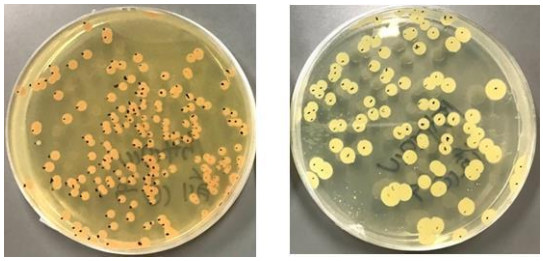


Figure 4: Trend in negative pressures for 15 cycles
Table 1:Average and standard deviation of negative pressures

		Ave. [MPa]	St. Dev. [MPa]
No. of temperature cycles	13	4.5	1.2
	15	4.2	1.5
	20	4.5	1.5
	30	5.3	1.5

Fig. 5 show typical photographs by an agar dilution plate method. In order to count a number of conies, they were marked with a black pen. Dilution rates were 10^{-9}. The number of colonies for the 15 cycles was less than those for no cycle.



(a) (b)

Figure 5: Typical photographs by an agar dilution method, (a) no cycle (b) 15 cycle

Table 2 shows reduction ratios for different temperature cycles. In the table, reduction rate (*R*) was calculated by following equation;

$$R = \frac{(N_0 - N_C)}{N_0} \times 100$$

,where N_0 and N_C represent numbers of colonies with no temperature cycles and with a definite ones. Reduction ratios increased with cycles.

Table 2: Numbers of colonies for no cycle and constant cycles, and reduction rates

		Reduction Rate[%]
No. of temperature cycles	13	33
	15	52
	20	88
	30	96

Table 2 indicates a possibility of the metal Berthelot method as a new means for an inactivation of bacteria. The method generated static negative pressures of ca. 4.5 MPa with temperature cycles which mean repeated cavitation. In general, it has been known that cavitation events have inactivation effects of bacteria [10]. The effects have been attributed to shock waves, micro jets, and chemical radicals accompanied with bubbles' collapses during extremely short times. In the present study, cavitation bubbles occurred and grew steeply so that the sample liquid co-existed with vapor as shown in Fig. 1. In this experiment, a typical period from cavitation inception at T_b in Fig. 1(a) to bubbles' disappearance at T_f in Fig. 1(a) took ca. 5 sec; the bubbles disappeared slowly through dissolution to the sample liquid with the heating processes of temperature cycles. Therefore, the waves, jets, and radicals caused by the collapses would not occur in the present study.

Any effects of bubbles' growth with cavitation inception on an inactivation of bacteria were not evaluated in this study. Future work should reveal the effects. Even if the growth with cavitation inception inactivates bacteria, the application of the Berthelot method using a metal tube to inactivation of bacteria will lead to a new method without any chemical compounds.

4 Conclusion

In order to check inactivation effects of negative pressures on bacteria, solutions of Bacillus subtilis underwent temperature cycles repeatedly using a metal Berthelot tube. As a result of an agar dilute plate method, numbers of colonies which underwent temperature cycles were less than those with no cycle. Furthermore, reduction ratios of bacteria increased with cycles. The inactivation effects were confirmed. The application of the Berthelot method will lead to a new means without any chemical compounds in future.

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