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Inactivation of Bacteriophage by Microwave Irradiation

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The effect of microwave irradiation at reduced temperature on the survival of bacteriophage T4 and T7, which is specific for *Escherial coli*, was investigated using a commercial 2,450 MHz microwave oven. Both phages were inactivated by microwave irradiation according to first-order reaction kinetics. Inactivation of phage by microwave irradiation is speculated to caused by heat or atomic vibration that is created by the electromagnetic wave. Analysis of the effect of microwave irradiation via agarose gel electrophoresis suggests that the vigorous shaking of molecules/atoms caused by microwave, instead of heat, inactivates the phage via disrupting the phage capsid and damaging the phage genome. Digestion of both unirradiated and irradiated phage samples with the restriction enzyme Kpn I and DNase I further confirm the theory.

The role of microwaves in sterilization of various types of materials has received increasing attention. To date, the inactivation of various types of micro-organisms by microwaves has been studied with *Saccharomyces cerevisiae* (1), *Candida albicans* (2), *Listeria monocytogenes* (3), *Proteus mirabilis* (3), *Psudomonas aeruginosa* (3), *Clostridium sporogene*s (4), *Escherichia coli* (5), *Bacillus sp*. (6), *Streptococcus sp*. (1) and *Staphylococcus aureus* (7). However, the mechanism of inactivation by microwave radiation has not been thoroughly clarified.

The mechanisms of sterilization by microwaves is could be due to either heat or atomic motion that is produced by microwaves. Microwaves are a form of electromagnetic wave that can easily be absorbed by water, fat, sugars, and various other types of small molecules (8). Absorption of microwave energy causes polar compounds to collide while rotating with great frequency to orient toward the electric field that was generated by the microwave (9). The effect of the motion that is generated by microwaves on microorganism is similar to the motion that is generated via sonication in the sense that they are both shaking the cells vigorously. The atomic motion also creates heat (9), which could also decontaminate or sterilize different types of materials. In general, microwave irradiation is considered to be a time and energy saving approach for sterilization of different types of materials.

Bacteriophage T4 and T7 were used to investigate the mechanistic detail of microwave irradiation. These viruses are both coliphage that belongs to T-phage families. T4 is a lytic phage whose virion particle is composed of a head constructed basically from a single major shell protein that is packed according to a complex icosahedral design (10). The virion also has a tail attached to the T4 capsid that is used for host cell recognition during infection. The capsid encloses the T4 genome, a single linear DNA duplex of about 170 kBP (10). T7 is a lytic phage that has an icosahedral head with short, stubby tail to which at attached six tail fibres. The genome is a linear DNA molecule containing 56 genes. It is approximately 30 kBP long.

Previous experiments have shown that T4 and T7 inactivation by microwave irradiation appears to be due to sonication instead of by heat. The objective of this research was to elucidate the sterilizing action of microwaves by microwave irradiatating the two bacteirophages at reduced temperature.

MATERIALS AND METHODS

Phage and host strain. *Escherichia coli* strains B23, bacteriophage T4 and bacteriophage T7 were used in this study as the host cell. All organisms were provided by Dr. W. Ramey of Department of Microbiology and Immunology of the University of British Columbia. The host cells were maintained on Luria plates (see appendix A) and all bacteriophage stocks were kept in either Luria broth (see appendix A) or TSG (see appendix A) with 0.2 volume of chloroform added.

Cell culture. Before each experiment, an overnight culture of *E. coli* was set up in Luria broth. The culture was achieved by re-suspending a single colony of *E. coli* B23 in 20 ml of broth and then incubated the suspension with aeration at 37^0C for 16-24 hours, or until saturation was reached.

Preparation of phage stock. Stock of bacteriophage T4 and T7 were first grown with a method adopted from Sambrook *et al* (12). An inoculum of 10^5 pfu of T4 or T7 mixed with 0.1 ml of saturated *E. coli* B23 culture was plated with standard plaque overlay technique and incubated for 24 hours at 37^0C to produce a confluent lysis of a bacterial lawn. After the lysis has occurred, overlay agar containing phage were scraped off into a sterile centrifuge tube via a sterile spatula. Five millilitre of TSG was then added to the plate to rinse off the remaining overlay agar and the rinse added to the centrifuge tube. One-tenth of a millilitre of chloroform was added to the agar suspension to kill any live bacteria that might be present in the culture. The suspension was incubated at 37^0C for 24 hours and then centrifuged at 4000g for 10 minutes at room temperature. The supernatant was recovered, titred, and used to grow new stocks of phage.

Phage titring. Phage suspensions were titred with soft-agar layer method. Small broth tube (13 x 100 mm) containing 3 ml of molten soft overlay agar (see Appendix A) was placed into a 50^0C water bath. Samples of phage to be titred were diluted to a concentration that, when plated, will give 30-300 plaques on a plate. 4 x 10^8 *E. coli* B23 (~0.1 ml of a saturated culture) was added to each tube as host cells, along with 0.1 ml or 0.2 ml of diluted phage samples, pending on the dilution. Tubes were mixed by phage style mixing and then rapidly transferred to a plate containing bottom agar (see Appendix A). Plates were incubated for 16-24 hours at 37^0C or until the plaques were visible.

Phage Purification. The method given below for purification was adopted from Sambrook *et al* (12). Pancreatic DNase I and RNAase were added to crude lysate cultures, each to a final concentration of 1μg/ml and the lysate was incubated for 30 minutes. After the digestion, phages were precipitated via adding NaCl and polyethylene glycol (PEG) 8000 to a final concentration of 1 M and 10%, respectively. The mixture was then cooled in ice water and let stand for 2 hours. Precipitated phage particles were recovered by centrifugation at 11,000g for 10 minutes at 4^0C. Phage pellets were then re-suspended with TSG and dialyzed against 50mM Tris and 10mM $MgCl_2$ for 24 hours. CsCl was added to the dialyzed phage to a density of 1.50 g/ml and the mixture was ultracentrifuged at 100,000g for 24 hours. A 21-gauge needle was used to recover the purified phage band and it was dialyzed against 1000-fold volume of a buffer composed of 10 mM NaCl, 50mM Tris-HCl (pH 8.0) and 10 mM $MgCl_2$ for 1 hour at room temperature. The number of surviving phages was assayed by soft-agar layer method, and expressed as plaque-forming units per ml (pfu/ml).

Bacteriphage DNA Extraction. The procedure presented below is adapted from Sambrook *et al* (12*)*. PEG precipitated phage or cesium purified phage were used for DNA extraction. With PEG precipitated phage, DNase I was added to the phage suspension in a centrifuge tube to a final concentration of 1μg/ml and the suspension was incubated at room temperature for 30 minutes. EDTA (pH 8.) was then added to the phage suspension to a final concentration of 20 mM. Proteinase K and SDS were added to a final concentration of 50 μg/ml and 0.5%, respectively. The mixture was mixed by inverting the tube several times and then was incubated for 1 hour at 56^0C. After 1 hour, the digested phage was cooled to room temperature and DNA was extracted by adding an equal volume of phenol equilibrated with 50 mM Tris (pH 8.0). The two phases were separated via centrifugation at 3000g for 5 minutes. The aqueous phase was transfer to a clean tube and extracted once again with 30:30 mixture of equilibrated phenol and chloroform. The final extraction was done with adding an equal volume of chloroform. The aqueous phase containing phage DNA was transferred to a dialysis sac and dialyze overnight at 4^0C against 1000-fold volume of TE (pH 8.0).

Microwave irradiation. A microwave oven (Samsung Model MW5592W) with a rotating table was used. 10 ml centrifuge tubes were filled with 5 ml of water first and then placed into 30 ml centrifuge tubes. The tubes were autoclaved and were left in the freezer until water in the smaller centrifuge tubes was completely frozen. Various volumes of PEG precipitated phage re-suspended in TSG were then placed in the annulus of the two centrifuge tubes. The tube containing phage suspension was placed in an empty 50 ml beaker and was microwave irradiated for various length of time at full power. Immediately after microwave irradiation, the temperature of the phage suspension was taken to ensure that the temperature remained below 70^0C. The suspension was then assayed by soft-agar layer method to obtain the percentage of surviving phage.

Electrophoresis. Samples of extracted DNA of bacteriophage, microwaved bacteriophage and non-microwaved bacteriophage were digested with either DNase I or restriction enzyme Kpn I (T7 only), or they were left untreated. Restriction digest was done by incubated 18 μl of T7 samples with 2 μl of digestion buffer and 2 μl of restriction enzyme at 37^0C for 80 minutes. Both restriction enzyme digestion and DNase I digestion were stopped by adding EDTA to the mixture to a final concentration of 10 mM. Agarose gel electrophoresis was done as follows: 15 μl of either digested or undigested samples were mixed with 3 μl of 6x sample buffer (see appendix A). The sample buffer contained detergent that would break open the phage capsid and release the encapsulated DNA. The samples were then loaded onto a 0.4% agarose gel in 45mM Tris-borate buffer (pH 8.5) containing 1 mM EDTA. Lambda phage DNA digested with Hind III was used as the DNA ladder. After electrophoretic migration at 100V for 90 minutes, the gel was stained with ethidium bromide at a concentration of 0.5 μg/ml. The stained bands were visualized using a UV-transilluminator and were photographed.

RESULTS

Time course of the inactivation of bacteriophage T4 and T7. One mL of T4 or T7 phage suspension was placed into annulus of the two centrifuge tubes and was then irradiated by microwaves at 2,600 MHz for different lengths of time. The results are shown in Fig.1, where the relative phage survivals are plotted as a function of time. From the figure, it is shown that T4 and T7 are inactivated at approximately the same rate with no lag periods, and the relative numbers of the surviving phage decreased to near 0% after 20 seconds of irradiation.

The microwave energy was absorbed by the phage suspension in the annulus and the ice in the inner centrifuge tube, theoretically, should remove the thermal energy from the phage suspension to prevent significant temperature rise. When the initial temperature of the suspension was kept at room temperature, the end-point temperature immediately after 20 seconds of irradiation rose to above 70^0C despite the cooling effect of the frozen inner centrifuge tube, as shown in Fig. 2. Due to the high end-point temperature, it cannot be concluded that phage inactivation under the condition was completely due to sonication.

Effect of volume of phage suspension on microwave irradiation. To eliminate the problem of reaching high-end point temperature after microwave irradiation, a larger volume of phage suspension was used for irradiation instead of the previous 1 ml. As shown in Fig.3, the end-point temperature of irradiated phage decreased as the volume of the sample increased for the same length of irradiation time. This implies that the temperature increase was indeed suppressed by larger volume of phage suspension, that of 2 ml giving about 68^0C and that of 4 ml 60^0C, respectively, after 20 seconds of irradiation. This is because microwaves penetrated more poorly into the inner part of the liquid with the increase of volume, thus the ice in the inner centrifuge tube was not melted and was able to cool the phage suspension. Rate of phage inactivation, presented in Fig. 4, was somewhat slower when the volume of liquid was larger, but 20 seconds of irradiation appeared to be able to inactive almost 100% of the phage for all volumes of phage suspension tested.

Fig. 1: Time course of inactivation of bacteriophage T4 and T7 by microwave irradiation.

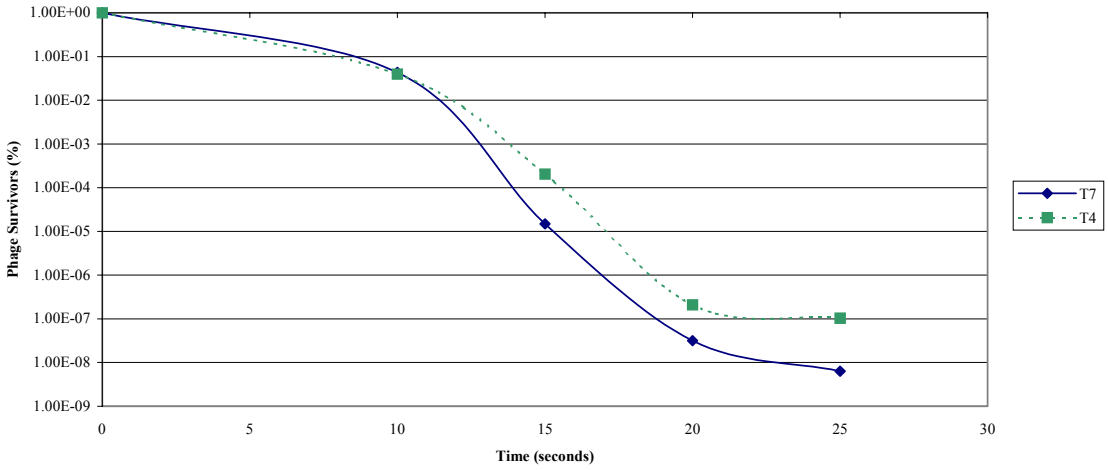


Fig. 2: Time course of temperature during microwave irradiation.

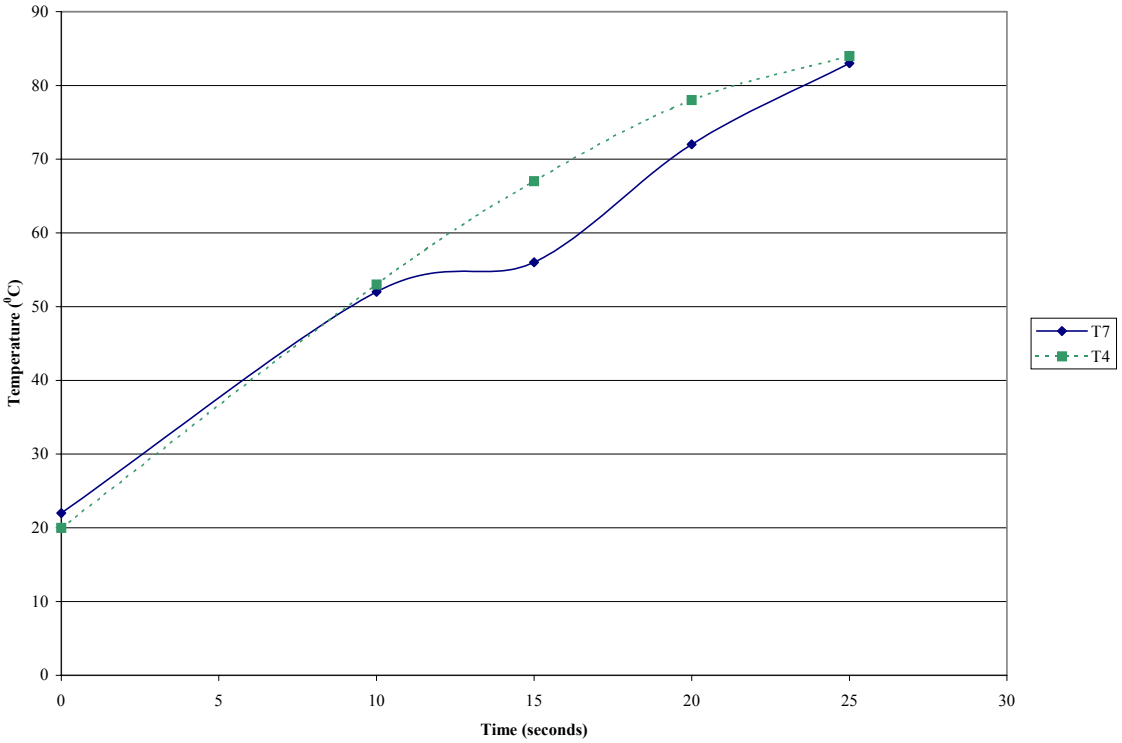


Fig. 3: Effect of volume of phage suspension on inactivation of bacteriophage T4 and T7 by microwave irradiation.

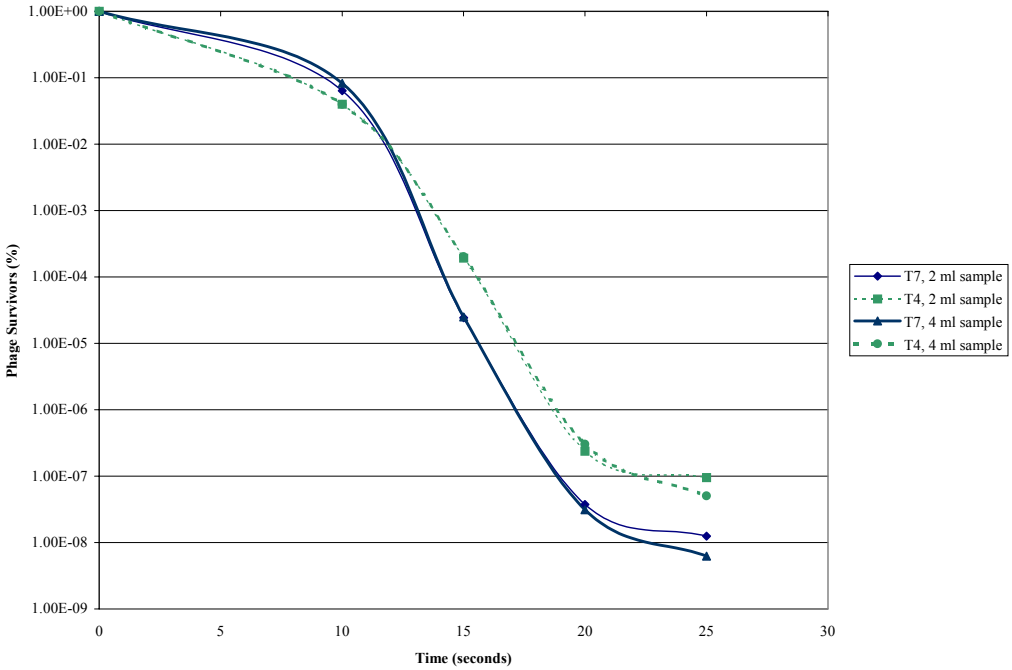
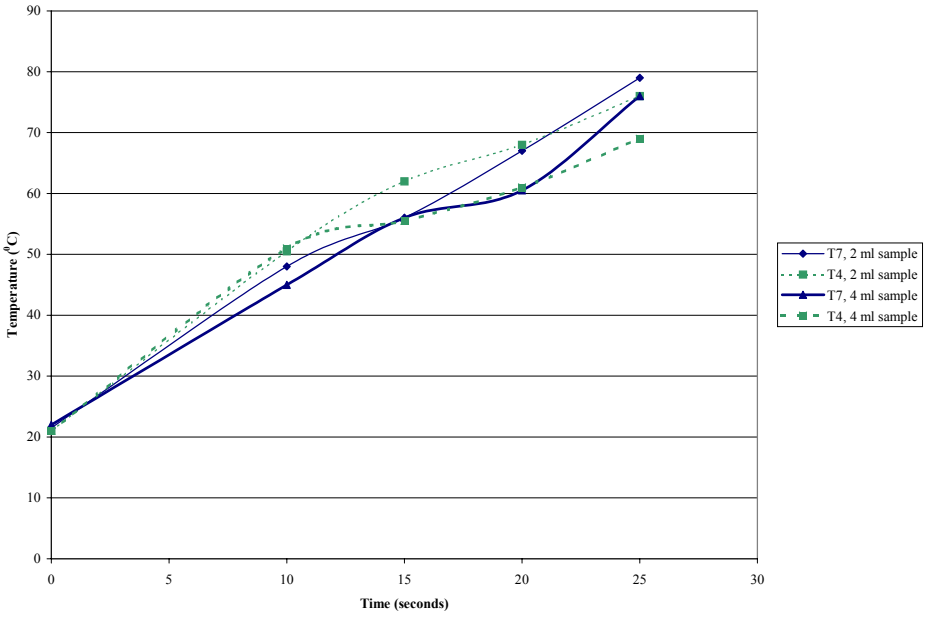


Fig. 4: Effect of volume of phage suspension on the temperature of phage inactivation by microwave irradiation.

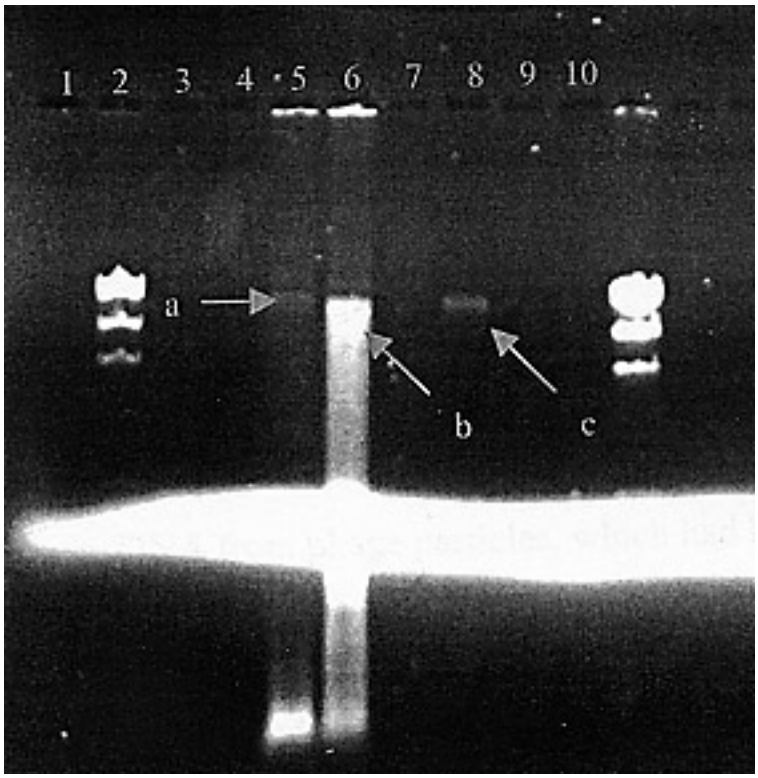


Effects of microwave irradiation on T4 analyzed by agarose gel electrophoresis. Bacteriophage T4 virions that were not subject to microwave irradiation were used as control against phage particles that were microwave irradiated. To investigate the mechanism of sterilization by microwave irradiation, DNase I was used to digest the samples as it was speculated that microwave irradiation breaks open the phage capsid and causes phage DNA release via shaking the phage particles vigorously.

As shown in Fig. 5, DNA of microwave irradiated T4 particles that were subject to microwave irradiation for 20 seconds (at this point, the temperature of 4 ml of phage suspension rose from room temperature to 60^0C), migrated as a discrete, fainter band compared to that of unirradiated phage. DNA of phage particles that were not microwave irradiated appeared as one discrete, bright, high molecular weight band and several fainter, lower molecular weight band, as shown in lane 5 of Figure 5. DNA of unirradiated T4 that was digested with DNase have, in general, similar migration pattern to one that was not treated with DNase I whereas DNA of irradiated phages that was digested with DNase I did not appeared after electrophoresis.

One difference between DNA from unirradiated phage that were treated with DNase I and one without was that the digested DNA did not show the long, bright smear that appeared in the lane containing undigested DNA. The bright smear may be caused by fragments of host chromosomal DNA that was present in the lysate. These residual chromosomal DNA fragments were probably digested when T4 particle was treated with DNase I, and hence the disappearance of the bands in lane 4 on Fig. 5.

Figure 5: **Comparison of Bacteriophage T4 DNA from Unirradiated Phage to DNA from Irradiated Phage.** Lane 2 – Lambda DNA digested with Hind III, Lane 5 – Unirradiated T4 digested with DNase I, Lane 6 – Unirradiated T4, Lane 7 – Irradiated T4 digested with DNase I, Lane 8 – Irradiated T4. Unlabeled lanes had no samples

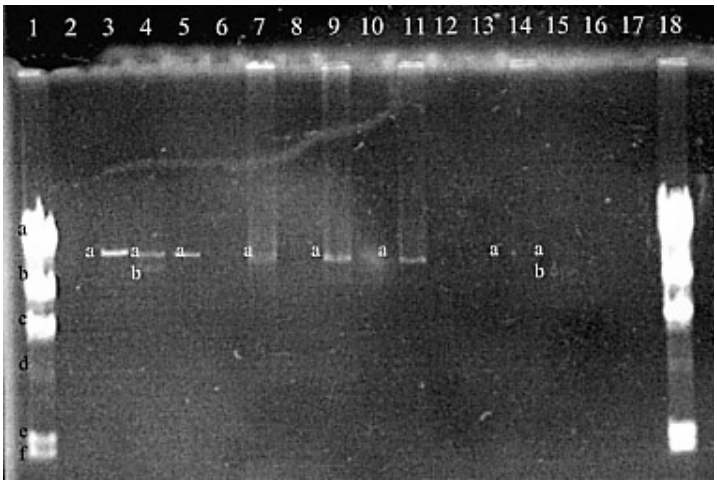


Effects of microwave irradiation on T7 analyzed by agarose gel electrophoresis Bacteriophage T7 that was not subject to microwave irradiation was used as control against phage particles that were microwave irradiated. DNase I and restriction enzyme Kpn I were used to digest the samples to verify the exact mechanism of phage inactivation by microwave irradiation, which was speculated to be that microwave irradiation caused vibration of phage particles. As T7 is rather sensitive to strong mechanical forces, the vigorous shaking may break open the

phage capsid and cause release of phage DNA molecule. DNA extracted from phage particles that were not subject to microwave irradiation was also used as a control to ensure that the bands on the gel were indeed T7 genome. Table 1 provides the relative amount of DNA that was in each sample, analyzed by KODAK 1D Gel Analysis program.

As shown in Fig. 6, in contrast of the discrete band formed by DNA of unirradiated phage, DNA from phage particles, which had been microwave irradiated for 20 seconds (at this point, the temperature of 4 ml of phage suspension rose from room temperature to 60^0C), migrated as fainter and more diffused bands. Also, DNA of irradiated phages that was digested with DNase I did not appeared after electrophoresis (lane 16, Figure 6) whereas the DNA of unirradiated T7 that undergone the same treatment did appear. Both control and irradiated phage were digested with Kpn I and the pattern obtained was compared to the pattern shown from extracted T7 DNA that was digested with Kpn I. Figure 6 and 6a showed that only the DNA of irradiated T7 had the restriction pattern, contrasting DNA of unirradiated, Kpn I digested phage. DNA from the latter migrated as one, discrete band that was similar to DNA from unirradiated T7 that was not subject to any type of digestion.

Fig. 6A: Comparison between Extracted T7 DNA, DNA from Unirradiated Phage and DNA from Irradiated Phage. Lane 1 is a lambda DNA cut with Hind III restriction endonuclease to provide a molecular size standard. Bands a, b,c,d,e and f in lane 1 correspond to 23.1, 9.4, 6.5, 4.3, 2.3, and 2.0 respectively. Bands a and b in the other lanes correspond to a molecular size of 21.0 and 7.3 kBP. Lane 3 – Extracted T7 DNA, Lane 4 – Extracted T7 DNA digested with Kpn I, Lane 5 – Extracted T7 DNA, Lane 7, 8 – Unirradiated T7, Lane 9, 10 – Unirradiated T7 digested with Kpn I, Lane 11, 12 – Unirradiated T7 digested with DNase I, Lane 14 – Irradiated T7, Lane 15 – Irradiated T7 digested with Kpn I, Lane 16 – Irradiated T7 digested with DNase I, unidentified lanes were empty.



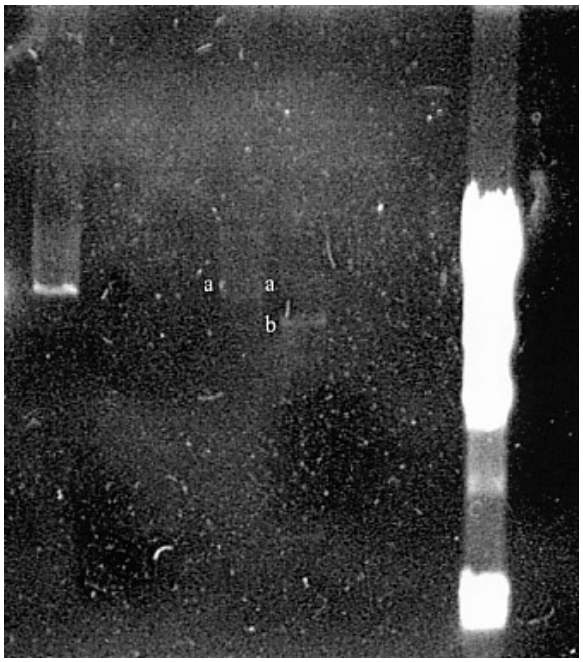
DISCUSSION

Studies have reported the disruption of microorganisms with electromagnetic energy such as microwave and radio frequency (13, 14), but little has been reported concerning the mechanism of sterilization of individual species of microorganism. It has been shown here that coliphage T4 and T7 appear to be inactivated by microwave irradiation through almost first-order reaction kinetics, implying that the phage particles are inactivated or lose the ability to replicate by only one "shot" of absorbed microwaves, as proposed previously by Stahl (15).

There are four predominant theories that explain the possible mechanism for phage inactivation: heating, capsid rupturing, breakage of phage tail/tail fibres and magnetic field coupling. Heating theory postulates that phage particles absorb the electromagnetic energy, which causes vibration at the atomic level and transforms electromagnetic energy into thermal energy. When the temperature reaches a certain level, the amount of thermal energy that is present is sufficient to break the bonds between amino acids and denature the proteins that forms the phage particle, hence the phage inactivation. The atomic motion created by microwaves is also responsible for the rupturing of phage capsid and separation of phage tail/tail fibres. Bacteriophage T4 and T7 are both relatively fragile and are sensitive to strong mechanical forces. When the absorbed electromagnetic energy causes atoms/molecules to vibrate vigorously, phage capsid or phage tail become unstable and eventually break apart. The

rupturing of capsid causes DNA release and the tail breakage causes inability for host cell adsorption, both of which can inactivate phage particles. In the fourth theory, a coupling of the electromagnetic energy with critical molecules within the phage, such as protein or DNA, disrupts the internal component of phage particle and thus inactivates them (16).

Fig. 6B: Enlargement of Lanes 11 through 18 from Figure 6A. Comparison between Extracted T7 DNA, DNA from Unirradiated Phage and DNA from Irradiated Phage. In lane 14 the letter a marks the band for irradiated T7, in lane 15 the letter b marks the band when irradiated T7 was digested with the restriction endonuclease Kpn I, in lane 16 there was no visible band after irradiated T7 was digested with Dnase I.



Even though several studies have shown that microorganisms are inactivated by heat that is created by microwave irradiation (10, 17), bacteriophage T4 and T7 appear to be inactivated by microwaves through a mechanism more similar to sonication. This effect is shown by previous experiments (19) that heating from outside at equivalent high temperatures did not inactivate the two coliphages, and thus suggesting that the inactivation is independent of temperature. In this particular experiment, the temperature variable was eliminated by keeping the temperature of phage suspension below a certain point (70^0C) since the two phages are known to be able to survive for an extended period of time at that particular temperature. This is achieved by a tube-in-tube system, where the inner tube is filled with ice. The microwave energy was absorbed by the phage suspension that was placed in the space between the walls of the two tubes but was less absorbed by the inner tube. The ice in the inner tube removed the thermal energy from the phage suspension to prevent significant temperature rise.

In this study, the results obtained appear to support the theory that phage are inactivated by microwave irradiation through an effect that resembles sonication and disrupts the phage capsid to a point that it cannot offer protection to the phage genome and thus, the genome becomes disposed to the action of restriction enzymes or DNase I. As shown in Figure 5 and 6, DNA from irradiated phage particles that were digested with DNase I did not appear on the gel, indicating that the phage genome has become susceptible to the enzyme and that it has been digested. On the other hand, capsids of virions that were not microwave irradiated were undisrupted and thus able to protect phage DNA from the enzyme. This resulted in a discrete band that resembled the DNA band from phage particles that were not digested

T4 and T7 showed similarities in their electrophoretic migration. DNA of both irradiated T4 and T7 migrated as a more diffused band than the unirradiated phage. This showed that the DNA molecule within the phage particles was fragmented by microwave irradiation, resulting in the loss of the ability of phages to form plaques. In the case of T4, after electrophoresis, the irradiated phage that was not digested by DNase I showed on faint band at around 9

kBP position whereas the unirradiated phage had several fainter bands and two bright bands in both undigested and DNase digested samples: one also at 9 kBP position and the other one relatively close to the well of the gel (high molecular weight). The disappearance of the high molecular weight band in the irradiated phage samples also suggests that the phage genome was fragmented by microwave irradiation and thus, only the bands at lower molecular weight appeared.

In contrast of T4, though fainter, irradiated T7 retained the high molecular weight bands that were also present in the unirradiated phage samples. The faintness of the bands were probably due to the fact that a phage suspension with lower titre was used for microwave irradiation compared to the tire of the T7 that was not microwave irradiated. Thus, naturally, the irradiated phage would contain a lower concentration of DNA material and as a result, fainter bands appear.

To confirm that the bands seen on the gel were indeed T7 genome, restriction digest was used. The pattern obtained after digestion by Kpn I verified that it was T7 genome that was seen. Kpn I cleaves T7 genome at four sites: at 0.2 kBP, 5.0 kBP, 10.0 kBP, 13.0 kBP positions (18) and results in five DNA fragments that have the size of 0.2 kBP, 3.0 kBP, 4.8 kBP, 5.0 kBP and 27.0 kBP. The digestion pattern that appeared on the gel resembled the pattern sited on literatures with slight variations in the size of the fragments (see Table 1). These variations were probably caused by difference in the strain of T7 used in the experiment as genome of each T7 strain varies slight from each other. The strain of T7 used in this experiment may not necessary be the same as the strain used in literatures, and thus variation in digestion pattern arises. There is also the probability that, since the T7 that was used in this experiment has been cultured many times, mutation may have arose and changed the restriction site which would then resulted in a slight different digestion pattern from the sited ones.

A double comb system was used for agarose electrophoresis of T7. Since the unirradiated DNA was still encapsulated before it was mixed with sample buffer, it was possible that the sample buffer was unable to completely break open the phage capsid and results in fragments of DNA that were still entangled with proteins. Some DNA-protein complex could have an overall positive charge and thus migrated off the gel under electrophoresis. The double comb system creates wells at both ends of the gel and by loading samples of unirradiated T7 into them, positively charged DNA-protein complex can also be visualized, as it would migrate from the bottom of the gel toward the negative electrode. Since no bands appeared in the lanes that samples were loaded in the wells at the bottom of the gel instead of the top, it is safe to assume that such positively charged protein-DNA complex probably did not exist.

Microwave irradiation has been used for sterilization of various materials. The results obtained in this experiment showed that the mechanism of sterilization of viruses is via the sonication effect that is created by the electromagnetic wave instead of by heat and that the phage capsid was disrupted by microwaves to the degree that the phage was inactivated. However, it remains undetermined whether sonication by microwave irradiation would actually break apart the phage capsid and further study is required to clarify the problem. It is also possible that microwave irradiation damages the phage tail/tail fibre to release the genome by triggering DNA release mechanisms that phages possess without attachment to host cells, or that the damaged phage tail/tail fibres are simply unable to adsorption to host cells anymore. These possibilities can be checked via running irradiated bacteriophage samples through density gradient to separate the broken parts of irradiated phage and then running the parts though protein gel.

One problem for this experiment was that the high molecular weight DNA molecule cannot be well visualized by agarose gel electrophoresis, and this can be solved by running a pulse field electrophoresis on the phage samples instead. Labelling the phage genome with radioactive isotope and detecting bands with autoradiography may also be used to investigate the effect of microwave irradiation on microorganisms since it eliminates the problem that ethidium bromide may not be able to properly stain encapsulated phage.

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Appendix A

a. Hershey's Broth

Nutrient broth	8g
Peptone	5g
Sodium chloride	5g
Glucose	1g
Water to	1000 ml
	pH adjusted to 7.4 with 1 M NaOH

b. Phage Bottom Agar

Tryptone	13g
Sodium chloride	8g
Sodium citrate	2g
Glucose	1.3g
Agar	15g
Water to	1000 ml
	pH adjusted to 7.2

c. Phage Top Agar

Tryptone	13g
Sodium chloride	8g
Sodium citrate	2g
Glucose	3g
Agar	7.5g
Water to	1000 ml
	pH adjusted to 7.2

d. Luria Broth

Tryptone	0.5g
Yease Extract	7.0g
NaCl	3.0g
Glucose	1.3g
Water to	1000 ml
	pH adjusted to 7.2

e. TSG

Tryptone	0.5g
Yease Extract	7.0g
NaCl	3.0g
Glucose	1.3g
Water to	1000 ml
	pH adjusted to 7.2

f. Sample Buffer

Bromophenol blue	0.025g
Xylene cyanol	0.025g
50% glycerol	6 mls
50 xTBE buffer	1.2 mls
Distilled water	2.8 ml