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Full Length Research Paper

Simple method to study the mechanism of thermal and non thermal bactericidal action of microwave radiations on different bacterial species

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The present study was based on the investigations to determine whether the bactericidal effect of microwave radiations on bacteria was either thermal or due to microwaves. It was also investigated which bacterial sp is more sensitive to microwave. Five bacterial species, *Proteus vulgaris PP25D, Staphylococcus aureus bgh010*, Bacillu *subtilis ase98, Escherchia coli TRE04* and *Corynebacterium spN33* were exposed to microwave radiation. These cultures were selected as test organisms due to their importance in food industry as human pathogens. Bacterial suspensions were exposed to microwave radiations (2450 MHz and 800W) for 60, 120 and 180 s to study the effect of heat generated by microwaves. The second experiment was designed to study the effect of microwaves only by maintaining temperature below 40°C. The degree of inactivation at uncontrolled and controlled temperature was compared quantitavely. The viable counts of all cell suspensions were found to reduce greatly with an increase in microwave heating time and temperature. *B. subtilis ase98* showed highest reduction at uncontrolled temperature. No significant reduction of cell density was observed in either cell suspension. The effect of microwave radiations was also studied in terms of morphological changes. No detectable change was observed in cell shape and morphology of colonies except *S. aureus bgh010*. Results indicated that the effect of microwave radiations on bacteria was purely thermal and no detectable change occurred in non-thermal treatment. *B. subtilis ase98* were the most sensitive species for microwave radiations. *B. subtilis ase98* can be used as an indicator bacterium to assess microwaves for sterilization.

Key words: Microwave radiation, thermal and non thermal effect, sensitive bacterial sp.

INTRODUCTION

Innovative approaches are required to inhibit and control food pathogens (Bevilacqua et al., 2008). Numerous studies address the effect of microwave heating on pathogenic microorganisms in foods. Microwave lamps have been reported for the disinfection of bacteria with promising results (Barkhudarov et al., 2007). Bacteria reported to be inactivated by microwave heating include *Bacillus cereus*, *Campylobacter jejuni*, *Clostridium perfringens*, pathogenic *Escherichia coli*, *Enterococcus*, *Listeria monocytogenes*, *Staphylococcus aureus*, and *Salmonella* (Heddleson et al., 1994; Rosenberg and Bogl, 1987; Knutson et al., 1987; Chipley, 1980).

A lot of work has been done on microwaves and their effect on several bacterial species (Vaid and Bishop, 1998). The actual mechanism of bacterial killing is still controversial. It is very difficult to precisely compare the effectiveness of microwave heating to conventional heating based on the literature, because of the different techniques employed or the lack of detail in the methods or materials used, especially in relation to temperature monitoring (Heddleson and Doores, 1994). Yeo et al. (1999) reported that the inactivation of *E. coli* was solely due to thermal effect, while other observed that heat generated during microwave exposure alone is inadequate to fully account for the nature of lethal effect (Barnes and Ho, 1977; Salvatorelli et al., 1996). Thus combined microwave

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heat may be involved in killing of bacteria. Some researchers used copper cooling coil to maintain temperature. But this treatment was not effective and copper ions were toxic to the bacterial cells and cause inactivation (Cha, 1993; Cooksey, 1990). Other workers also controlled the temperature of the irradiated specimen through various timing, pulsing or cooling techniques (Welt et al., 1994).

Kozempel et al., 2000 designed a new system that was capable of isolating thermal and non-thermal effects of microwave energy. The system was a double tube that allowed input of microwave energy but removed thermal energy with cooling water. Khalil and Villota, 1988 further studied the effect of microwaves (2450 MHz assumed) on injury of *S. aureus* FRI-100. They heated cells at a sub lethal temperature of 50°C and maintained microwave temperature using recirculated cooled kerosene. The aim of present study was to assess the thermal and non-thermal bactericidal action of microwave radiation. In order to investigate the effect of microwave radiations only, an ice bath was used for bacterial suspension to kept temperature below 40°C throughout the exposure to heating in the microwave. After exposure to different temperature cell viability and changes in morphology were studied. The study will help in differentiating the thermal and non thermal effect of microwave radiations.

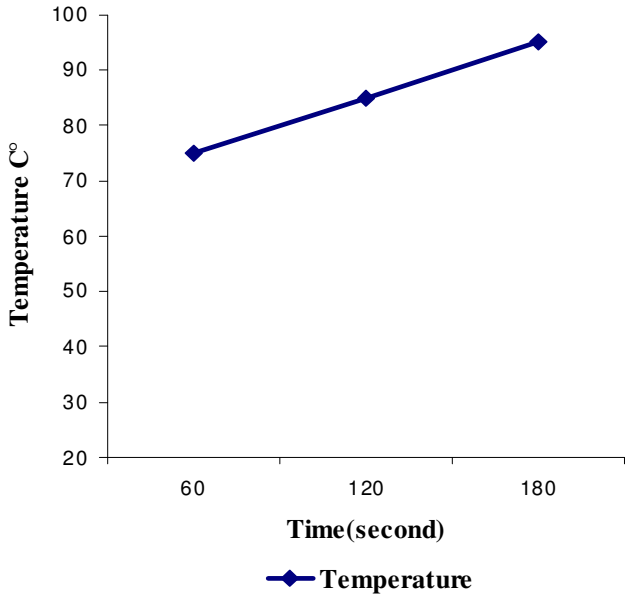

Figure 1. Change in temperature with increase in time of microwave irradiation.

MATERIALS AND METHODS

Collection of bacterial samples

The cultures of *P. vulgaris PP25D, S. aureus bgh010* , *B. subtilis ase98, E. coli TRE04* and *Corynebacterium spN33* were taken with the courtesy of DTL laboratory Lahore, Pakistan. These bacteria were used to study the effects of microwave radiations.

Preparation of inoculum

All the bacterial species were grown in Nutrient Broth in 250 ml cotton plugged conical flask. A loopful of pure bacterial culture was transferred by a sterilized wire loop and suspended into to 50 ml Nutrient Broth in 250 ml conical flask. The flask was then incubated at 35°C and rotated at 150 r.p.m for 20 h in a rotatory shaker (Model No. 3033, GFL, Germany). In order to obtain accurate results all indicator bacteria were cultured and reproduced for several generations, then prepared for the bacterial solution.

An amount of 10 ml of this suspension was used to check absorbance at 600 nm, 0.50 absorbance was used to get the approximately same number of microbes for further treatment.

Microwave source

Microwave treatment process was performed in a microwave oven (DW-131G; Dawlance). The full output power of the equipment was 800 W at 2450 MHz (frequency).

Exposure of bacteria to microwave radiations

Bacterial suspension of 0.50 absorbance was used to make dilutions (1×10^5) with sterilized phosphate buffer of pH 7.0. One of four test tubes (MW-C) represents control sample for viable counting without any treatment while the remaining (MW-60, MW-120, MW-180) were exposed to microwave radiation for 60, 120 and 180 s .

Bacterial suspension were exposed to radiation by placing test tubes in a 100 ml Pyrex beaker within a 500 ml Pyrex beaker containing 100 ml water and placed at the center of rotating plate of microwave oven. Water was used to prevent the evaporation of sample. Then samples were exposed to microwave for 60, 120 and 180 s separately. Temperature was measured after every treatment by measuring the temperature of suspension with the help of sterilized thermometer. The correlation between the microwave radiation time and temperature changes in bacterial suspension is shown in Figure 1.

In order to see the effect of microwave radiation only the bacterial suspension were taken in a test tube and placed in a 500 ml Pyrex beaker containing ice to keep the temperature below 40°C and placed at the center of rotating plate of microwave oven. Then samples were exposed to microwave for 60,120 and 180 s separately. In this series of experiment, there will be no effect of heat to these bacterial cultures and thus independent effect of radiation could be noted.

Measurement of viable cell count

After exposure the 0.1 ml sample from each of three test tubes containing treated bacterial suspensions were transferred into sterilized petriplates (100 mm) separately and about 15 ml of nutrient agar (maintained at about 40°C) was poured into each of petriplate. In order to mix the sample with medium, the petridishes were rotated uniformly clockwise and the medium was allowed to solidify and then incubated for about 20 h at 35°C.

Measurement of cell density

Cell density was measured at 600 nm using a spectrophotometer (UTECH PRODUCTS IN C.N.Y.12203.U.S.A).

Morphology changes

The slides were made from all the samples and stained (Gram Staining) to check morphological changes after treatment under simple microscope.

Data Analysis

The statistical analysis of the data was carried out using the Minitab v 13.0 software. Paired t- test was used to compare the data. The level of significance was taken at P = 0.05. The results are average of triplicates.

RESULTS

Effect of microwave radiations to *Escherchia coli TRE04*

E. coli is a rod shaped gram negative bacteria. They form pink colonies on MacConkey agar. The viable count of *E. coli TRE04* determined by pour plate method was 50 ± 4.36 × 10^5 CFU/ml before irradiation. The average reduction in viable count of *E. coli TRE04* after 60, 120 and 180 s of microwave exposure at uncontrolled temperature was 21.3 ± 2.08 × 10^5, 4 ± 1 × 10^5 and 2 ± 1× 10^5 CFU/ml respectively. Highly significant (P = 0.024) reduction was found in all experimental sample as compared to control. It was found that there was slight reduction in viable count of samples subjected to microwave irradiation at controlled temperature (<40°C). The observed values of samples after microwave exposure for 60, 120 and 180 s at controlled temperature was 47 ± 3.5 × 10^5, 45 ± 4.58 × 10^5 and 42 ± 2 × 10^5 while the control was 50 ± 4.36 × 10^5 CFU/ml. Non-significant (P = 0.067) reduction was found in all experimental samples as compared to control (Figure 2). No detectable change in colony morphology and cell shape was observed.

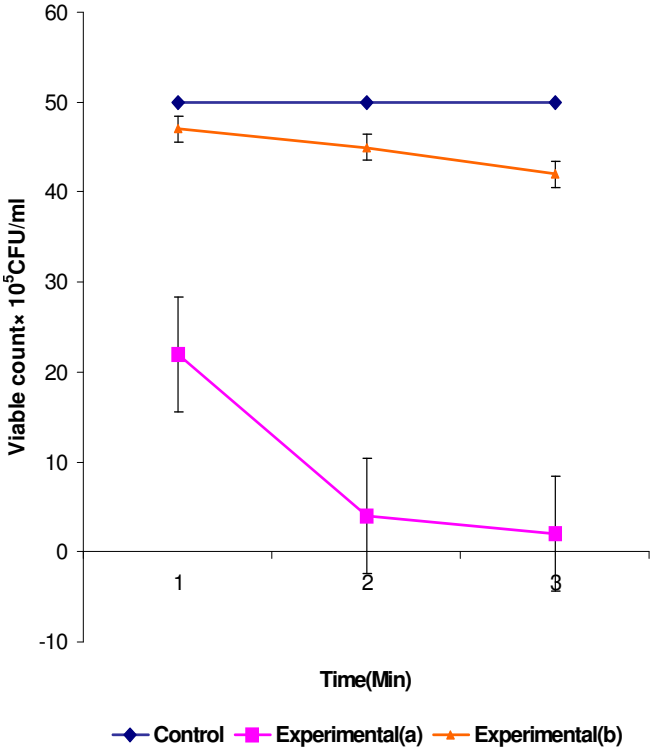

Figure 2. Change in viable count of *Escherchia coli* after microwave irradiation for 60, 120 and 180 s (a) at uncontrolled temperature (b) at controlled temperature.

Effect of microwave irradiation on *Bacillus subtilis ase98*

B. subtilis is a rod shaped Gram positive spore former bacteria. They form white convex shaped colonies on Nutrient agar. *B. subtilis ase98* was found highly sensitive to thermal treatment. The viable count of *B. subtilis ase98* was determined by pour plate method. The average reduction in viable count of *B. subtilis (*69 ± 4.36 × 10^5 CFU/ml before irradiation) after 60, 120 and 180 s microwave exposure at uncontrolled temperature was found to be 30 ± 3 × 10^5, 15 ± 2.65 × 10^5 and 5 ± 1.73 × 10^5 CFU/ml respectively. The reduction found in all experimental samples was highly significant (P = 0.01).

It was observed that there was slight reduction in viable count of samples subjected to microwave irradiation at controlled temperature (< 40°C). The observed values of viable count after microwave exposure for 60, 120 and 180 s at controlled temperature was 65 ± 1.73 × 10^5, 64 ± 4 × 10^5 and 58 ± 2 × 10^5 CFU/ml while in control, viable count was 69 ± 2 × 10^5 CFU/ml (Figure 3). Non-significant (P = 0.09) reduction was found in all experimental samples. No detectable change in colony morphology and cell shape was observed

Effect of microwave irradiation on *Staphylococcus aureus bgh010*

S. aureus was round shaped Gram positive cocci. They form yellowish slimy and convex shaped colonies on Nutrient agar. A gradual reduction was observed in viable count of *S. aureus bgh010* (67 ± 3 × 10^5 CFU/ml before irradiation) with increase in exposure time to microwave radiation (Figure 4). The observed values of reduction in viable count of *S. aureus bgh010* after 60,120 and 180 s microwave exposure at uncontrolled temperature was found to be 38 ± 2 × 10^5, 12 ± 3 × 10^5 and 2 ± 1 × 10^5 CFU/ml respectively. Significant (P = 0.044) reduction was found in all suspensions.

According to observed values there was small reduction in viable count of specimens subjected to microwave irradiation at controlled temperature (< 40°C). The observed values of viable count after microwave exposure for 60, 120 and 180 s at controlled temperature was 63 ± 2 × 10^5, 62 ± 2 × $10^5$and 58 ± 2 × 10^5 while in control, viable count was 67 ± 3 × 10^5 CFU/ml. Non-

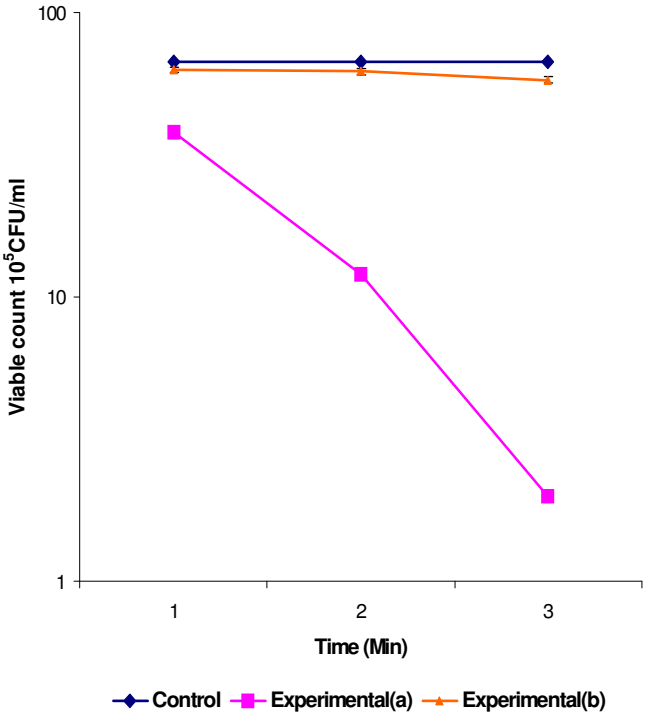

Figure 3. Change in viable count of *Bacillus subtilis* after microwave irradiation for 60, 120 and 180 s (a) at uncontrolled temperature (b) at controlled temperature.

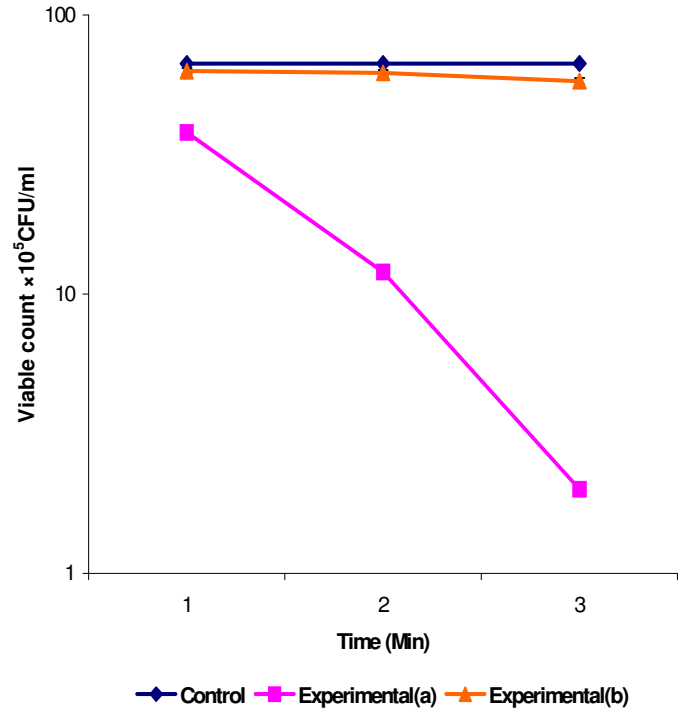

Figure 4. Change in viable count of *Staphylococcus aureus* after microwave irradiation for 60,120 and 180 s (a) at uncontrolled temperature (b) at controlled temperature.

significant (P = 0.059) reduction was found in all experimental samples (Figure 4). The cells grown on petriplate after exposure form white while the untreated sample formed yellowish colonies. The change was observed in both irradiated samples at uncontrolled and controlled temperature. No visible change in cell shape was observed.

Effect of microwave irradiation on *Proteus vulgaris PP25D*

P. vulgaris PP25D was a rod shaped Gram negative bacteria. They form white irregular shaped colonies on Nutrient agar**.** The viable counts of all cell suspensions (57 ± 3 × 10^5 CFU/ml before irradiation) were found to reduce relatively with an increase in microwave heating time and temperature (Figure 5). The average reduction in viable count of *P. vulgaris PP25D* after 60, 120 and 180 s after microwave exposure at uncontrolled temperature was 27 ± 4.58 × 10^5, 20 ± 3.79 × 10^5 and 12 ± 2 × 10^5 CFU/ml respectively. Highly significant (P = 0.01) reduction was found in all experimental sample. The observed values of viable count after microwave exposure for 60, 120 and 180 s at controlled temperature was 54 ± 4 × 10^5, 50 ± 4 × 10^5 and 46 ± 4 × 10^5 while in control, viable count was 57 ± 3 × 10^5 CFU/ml . Non-significant (P = 0.09) reduction was found in all experimental samples. No detectable change in colony morphology and cell shape was observed.

Effect of microwave irradiation on *Corynebacterium sp N33*

Corynebacterium sp N33 was a rod shaped Gram positive bacteria. They form white irregular shaped and rough colonies on Nutrient agar. The average reduction in viable count of *Corynebacterium sp N33* (65 ± 3 × 10^5 CFU/ml before irradiation) after 60, 120 and 180 s after microwave exposure at uncontrolled temperature was 46 ± 4 × 10^5, 35 ± 2 × 10^5 and 25 ± 2.65 × 10^5 CFU/ml respectively. Highly significant (P = 0.03) reduction was found in all experimental sample. The observed values of viable count after microwave exposure for 60, 120 and 180 s at controlled temperature was 62 ± 3.61 × 10^5, 60 ± 2 × 10^5 and 57 ± 2.65 × 10^5 while in control, viable count was 65 ± 3 × 10^5 CFU/ml. Non-significant (P = 0.067) reduction was found in all experimental samples as compare with control (Figure 6). No detectable change in colony morphology and cell shape was observed.

Effect of microwave radiation on cell density

Change in cell density of bacterial suspensions was observed by taking its absorbance value at 600 nm. The average reduction in cell density of all cell suspensions after microwave irradiation was not detectable (data not

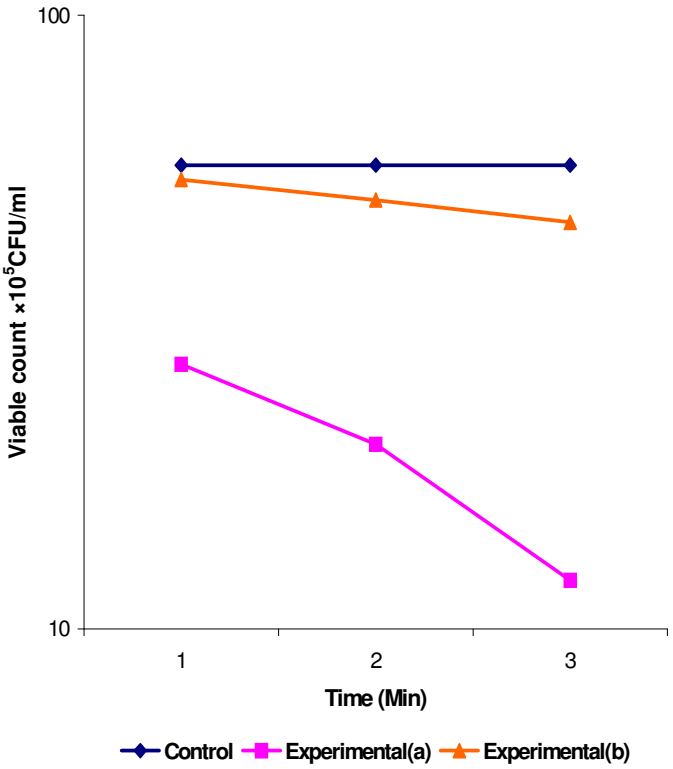

Figure 5. Change in viable count of *Proteus vulgaris* after microwave irradiation for 60, 120 and 180 s (a) at uncontrolled temperature (b) at controlled temperature.

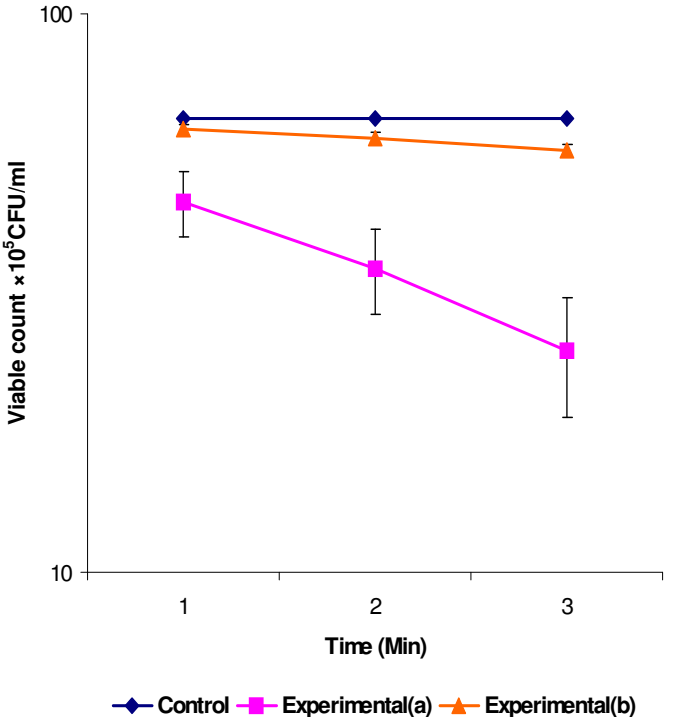

Figure 6. Change in viable count of *Corynebacterium* after microwave irradiation for 60, 120 and 180 s (a) at uncontrolled temperature (b) at controlled temperature.

shown). The rate of reduction was lower in both irradiations at uncontrolled and controlled temperature.

DISCUSSION

It was observed that the viable cell count reduced relatively with an increase in time of microwave irradiation at different temperatures. It might be due to microwave energy absorption by biological materials with increase in temperature. It was observed that the exposure up to 90°C resulted in a highest reduction of the viable count in all bacterial strains. The highest reduction ratio in the viable counts was observed when the temperature was increased from 70 to 90°C. Our findings are in agreement with other researchers (Woo et al., 2000; Jeng et al., 1987; Yeo et al., 1999), who reported that the reduction effect on exposing species was due to higher increase in temperature. The temperature was increased because substances with high dielectric constant such as water absorb microwaves and convert the energy to heat. As the temperature of the material increases, its ability to absorb microwaves also goes up, increasing the temperature. This can lead to uncontrolled heating (Zhang et al., 2000). Microbial lethality of microwave radiation may be due to the penetration of electromagnetic waves into a biological wet material, heating up the intra and extra cellular fluids by the transfer of energy from polar water molecules and dissolved ions. This results in the generation of heat within the material itself due to molecular activity (Mudgett and Schwartzberg, 1982).

It was observed that exposure of bacterial suspensions to microwaves for different time periods did little or no damage in terms of the viability. The non-thermal effects of the microwave energy are of no significance. Our findings are in agreement with other workers (Ramaswamy et al., 2000). It was found that bacteria respond differently to inactivation by microwaves. Microwave treatment has been reported to cause protein denaturation and aggregation in cytoplasm as well as to induce heat shock proteins, which may cause microbial inactivation.

It was very interesting to observe that Gram positive bacteria were found more sensitive to microwave radiations than Gram negative. It might be due to the difference in chemical composition of cell walls. It has been reported that the lipid content of gram negative bacterial cell wall make them more resistant to microwave. *B. subtilis ase98* was found highly sensitive to microwave radiation because it showed highest reduction in viable count. *B. subtilis ase98* should be considered as an indicator bacterium for inactivation by microwave energy (Deng et al., 1990).

The sensitivity of cells to lyses by microwave irradiation was also investigated at 600 nm. It was observed that cell density in all microwave heated cell suspensions slightly decreased for increasing and controlled temperature. The reduction in cell density in all cell suspensions was not significant. Our findings are in agreement with other

workers (Woo et al., 2000). This insignificant change in cell density may be due to the fact that the microwave-treated cells were not completely lysed even when they were inactivated by microwave radiation, and thus the cell density did not decrease.

During the current research work a change in bacterial colony was observed, white colonies of *S. aureus bgh010* were observed after microwave irradiation while yellowish colonies were observed before irradiation. Similar types of changes in character were reported by other workers (Dreyfuss and Chipley, 1980; Atmaca et al., 1996). These changes might be due to microwave radiations effect on the genetic make up of the bacterial cells as destruction of DNA has been reported by Kakita et al., 1995. Other bacterial species did not show any detectable change in colonial morphology and cell shape.

Conclusion

It was evident from the present study that microwave radiations might affect the microorganisms up to significant level. Thus, the microwave can be used after maintaining the temperature to control the required microbes. Microwave radiations caused high reduction in viable count of bacteria with increase in exposure time. But no significant change was found by irradiation at controlled temperature, which shows that the effect of microwaves was highly thermal. *B. subtilis ase98* can be considered as an optimum indicator bacterium for microwave sterilization.

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