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Cell Population Reduction of *Salmonella typhimurium* using Non-thermal Microwaves

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Abstract

Microwaves have been used to cause thermal destruction of various pathogens by heating the substrate they are found on. It has been found that microwaves also cause non-thermal destruction of bacterial cells thereby reducing the cellular population. The method to detect the non-thermal killing effects of microwaves is one of the highlight of the study undertaken. This has been an originally devised method (Barnabas et al, 2010) in our preceding work and has in this case been tested for the detection of non-thermal destructive effects on a specific pathogen. The study discusses the direct effect of microwaves on bacterial cells and the destruction quantification of the microwave exposure on cells, being laid out as a smear on slide than in a broth or solid media or food substrates. The impact of microwaves directly on cells at non-thermal levels proves that bacterial pathogenic cells can be destroyed at cellular levels without causing heating to substrates, this confronts the established science that microwaves could obliterate bacterial cells only by thermally heating the substrate. The quantification of cell population reduction in the smear slide were analyzed by growing the exposed smears slides in regular bacterial media and analyzing the surviving bacterial cell density in a spectrophotometer at 600nm.The study also exposed the other parameters such as the effect of varying incubation temperature after irradiation to analyze optimal incubation temperatures.

Keywords: Non-thermal microwaves, pathogen, bacteria, *Salmonella typhimurium*

Introduction

The destruction mechanism of microwaves required in a system to effect microbial cells has been proved to be due to heat generated in a medium; in a solution and/ or on solid substrate. But it not until recently, it was thought otherwise that microwaves themselves have a direct destructive effect on the microbial cells with contribution from the substrate heat generated. From the established protocols, it has been understood that specific parameters are required to heat up a medium in order to destroy microbial cells. Usually bacterial cells are destroyed by physical, chemical and antimicrobial agents from biological sources, further these microwaves have found immense application for a variety of uses, such as food processing and for inactivation of microorganisms (Kakita,*et al.,*1995, Rosenberg and Bogl;1987). It is widely believed that bacterial cells are destroyed by heat caused by microwave exposure (Fung and Cunningham;1980, Vela and Wu;1979).

Few bacterial species that were exterminated by heat, created on account of microwave irradiation, are *Escherichia coli*, *Streptococcus faecalis*, *Clostridium perfringens*, *Staphylococcus aureus*, *Salmonella*, and *Listeria* spp (Atmaca,*et al.,*1996; Blanco and Dawson,1974; Bookwalter *et.al.,*1982; Crespo,1977; Culkin and Fung, 1975; Farber,*et al.,*1998; Fujikawa *et al.,*1992*;* Heddleson. *et al* 1994; Ishitani *et al,* 1981;Khalil and Villota, 1989; Pothakamury,1995). Further fungal spores, bacteria and bacteriophage PL-1, specific to *Lactobacillus casei*, have been known to be susceptible to microwave radiation (Ishitani, *et al,* 1981;Kakatiya, 1985; Khalil and Villota, 1985). In a range (0.1–3 GHz, 10−3–10−4 eV) of energy absorption it has been observed to engross certain heating effect due to the credibly low energy of single photons (Shazman *et al.*, 2007). Microwaves tend to have an high impact on bacterial cells when they are concentrated (Wesierska and Trziska., 2007) whose finding were breakthrough, in that microbial cells were not destroyed with an increase in temperature.

The work on vacuum microwave kill rates on *Escherichia coli* by Yagmaee and Durance (2005) suggested parameters excluding heat contributed to the lethal effect of vacuum microwave kill rates on *Escherichia coli.* A low dose of microwave irradiation improved the microbial quality in ice creams (Jo *et al*. 2007) especially, *Escherichia coli*, *Salmonella typhimurium* and *Listeria ivanovii* which were found sensitive. Since ice creams are temperature sensitive, this raises the challenges for developing alternative methods for sterilization in these areas.

From the above information, it therefore makes an argument that it is the effect of microwaves at cellular level that need to be addressed rather than established concept of bulk heating by microwaves to destroy pathogens

Non-thermal effect of microwaves on microorganisms has been a subject of work and challenge of late (Carroll and Lopez;1969, Culkin and Fung; 1975, Kozempel *et al;* 1998, Shin and Pyun; 1997). Significant work has proved that microorganisms could be destroyed by microwaves at temperatures lower than thermal destruction point.(Cunningham 1978, Dreyfuss and Chipley;1980, Khalil and Villota, 1985, Khalil and Villota, 1989, Kozempel et al; 1998). Further, it has been noted that certain microwave-stressed cells of *S. aureus* had shown a greater degree of metabolic imbalance than conventionally heated cells (Kozempel et al; 1998). A significant

investigation expounded that non-thermal destruction of microbial cells by microwaves, arose at points beneath the precise thermal death point of the bacteria (Beckwith and Olsen, 1931; Yen and Lui, 1934; Fleming, 1944; Nyrop, 1946; Carpenter, 1958, and Susskind and Vogelhut, 1959).In connection to the above views on non-thermal microwave destruction of microorganisms, our studies were further conducted to prove the above by selecting a known common food pathogen *Salmonella typhimurium* MTCC 3214,

In the current study, the destruction of pathogen was studied by originally devised method from our previous work (Barnabas *et al*, 2010)

The smear slide exposure method was originally devised in-order to study the non thermal destruction of bacterial cells, this method was not known to have been previously tried other than our preceding work. The method excludes any substrate other than the bacterial cells as a freshly air dried smear on a glass slide that is impacted by microwaves at known parameters and studying the survival rates of specimen. Here we are using three different incubation temperatures 32°C, 35°C and 37°C to analyze any changes in the growth recovery of irradiated cells. In the present study the effect of bacterial destruction rate was investigated with regard to the effect of fixed microwave operation and varying time irradiation parameters in a controlled microwave field operation a common kitchen microwave. In the work that was undertaken, microwaves fixed at 55Hz for time exposure of 2 to 10 seconds were chosen to radiate single cells of bacteria on a coverslip used as a glass slide. These cells were exposed to the microwaves for a short periods of 2, 4, 6, 8 and 10 seconds and then examined for the effect of the microwave exposure, by spectrophotometric studies. This mechanism helped analyze the intensity of microwaves required to reduce cellular population of bacteria under such conditions.

Experimental Equipment, Consumables and Procedures

Microwave oven: LG. ECN: MS-1947/01, MOD: MS-1947C., 250v ~ 50Hz, Max. 1200W. 2,450MHz, 800W., S/N 208D MM V004

UV/Visible Spectrophotometer: ELICO SL159. Type-127. Ratings: 90-260V, 50/60Hz 170VA, 1ø FUSE: F4A

Petri dishes: Borosile®10cm. Coverslip-Blue star 22mm.

Nutrient broth and agar: NaCl(pure)-MERC, Beef extract –Chemport Pvt Ltd, Peptone-Fisher scientific. Agar-agar-Fisher scientific

Microbial cultures: *Salmonella typhimurium* MTCC 3214 used in the work were obtained from Microbial Type Culture Collection (MTCC), Institute for Microbial Technology (IMTECH) Chandigarh, Punjab. India.

Fresh over night colonies of *Salmonella typhimurium* MTCC 3214 were grown on nutrient agar slants to get a mat of colonies. Using an inoculation loop a single streak of bacterial culture was picked up from the mat, and streaked at the centre of a dry

sterile petriplate, to this streak 1ml of sterile distilled water was added and mixed well so that a uniform suspension was obtained, this became the stock culture of 1ml containing only *Salmonella typhimurium* MTCC 3214 cells in sterile water. A quantified inoculation loop of 5µl of the above culture was used to make a smear of the culture onto a glass coverslip. The smear was spread across the glass slide (cover-slip) so that the cells separated evenly on the slide. The slide was air dried in the laminar airflow bench by placing in an open sterile petriplate. The dried smear slides in the plate were placed at the centre floor of the microwave (Figure.1) oven where the microwaves converge to have maximum intensity of radiation for a specific time period of 2 seconds. Similarly for 4, 6,8 and 10 seconds.

Control smear slides were left in a petriplate in the Laminar Air flow bench for 2,4,6,8 and 10 seconds, so that they were not exposed to radiation of microwaves. The exposed and the unexposed slides were immediately dropped into 10ml of nutrient broth and incubated at 32°C , 35°C and 37°C for 5 hours thereby also to determine the influence of incubation temperatures on the growth recovery of irradiated cells. A total control consisting of 5µl of bacterial culture from stock was directly inoculated into the nutrient broth tubes and incubated at the same temperatures and incubation time so that a difference between the control dry smear and the direct wet inoculation could be analyzed. Blank tubes were also maintained for reference in spectrophotometry studies.

The procedure was also repeated for a drop (wet slide method) of 5µl of bacterial culture placed at the centre of the cover-slip slides and by exposing the same parameters of microwaves and time is seconds. All the tubes were analyzed at 600nm in a UV/Visible spectrophotometer and the ocular density as absorption was noted for further analysis. Statistical analysis was executed on the spectrophometric data-obtained, Percentage killing of bacterial destruction due to irradiation was deduced. Destruction due to drying of the slides were calculated from the dry control slides from the wet control (total control) parameters.

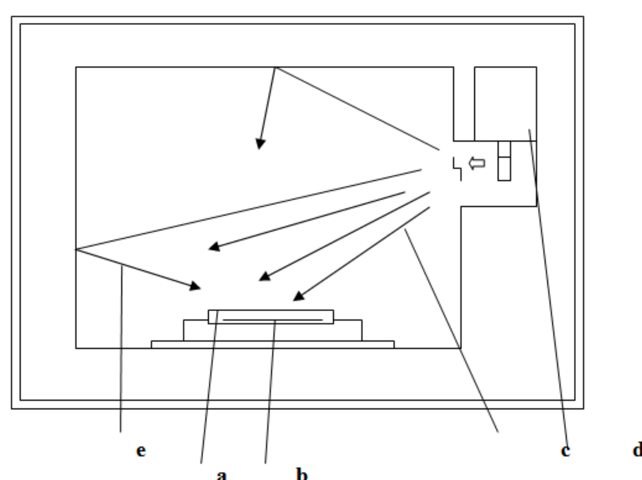

Figure 1: Principle and working of microwave apparatus. a–petriplate, b– smear slide, c–microwaves, d–microwave generator, e–reflected microwaves.

Results, Discussion and Conclusions

Table 1: Analytical analysis for results obtained for turbidometry:

	Mean	*Std.deviation*	*Variance*	*p-value*
A	*0.21372*	*0.09326*	*0.0087*	*0.01672*
B	*0.2943*	*0.06503*	*0.0042*	*0.01645*
C	*0.234*	*0.0594*	*0.0035*	*0.0161*
D	*0.2823*	*0.0575*	*0.0033*	*0.0165*
E	*0.2378*	*0.0528*	*0.0028*	*0.01601*
F	*0.2944*	*0.0564*	*0.0032*	*0.01658*

Incubation temperatures; *a*-37°C, *b*-37°C control, *c*-35°C, *d*-35°C control, *e*-32°C, *f*-32°C control. Analytical analysis to the obtained results; Mean, standard deviation, variance and P-value, describe the values obtained which were analytically significant. While the P-values were below 0.1 to 0.05, therefore considered highly relative

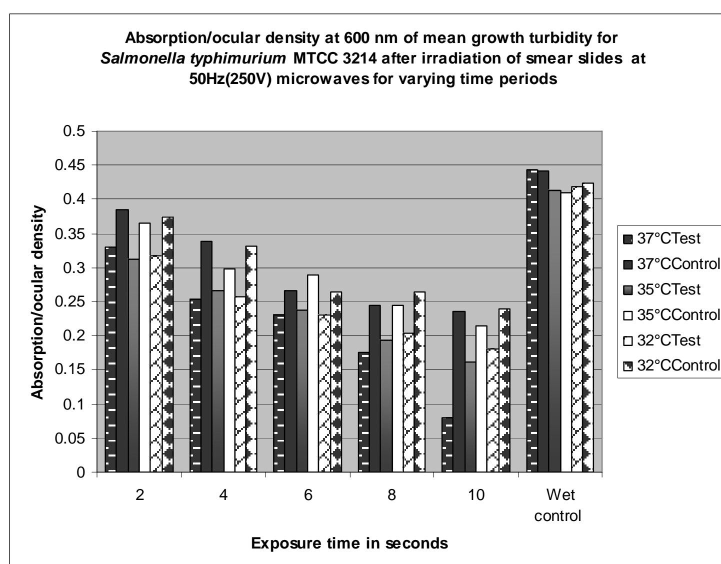

Figure 2: Spectrophotometric estimation of growth turbidity for *Salmonella typhimurium* MTCC 3214 after irradiation

Fig.2 describes the absorption/ocular density at 600nm after mean growth turbidity for *Salmonella typhimurium* MTCC 3214, it describes the absorption/ocular density at 600nm, a the mean value of growth turbidity for *S.typhimurium* MTCC 3214 after irradiation at 50Hz(250V) and as well as by varying the incubation temperatures in order to determine optimum temperature for cellular irradiation

recovery, at 2 seconds of microwave exposure all the three controls 37°C, 35°C, 32°C, had shown maximum turbidity. The growth turbidity at 37°C test had shown higher than the growth turbidity incubated at 35°C and at 32°C, being higher than 35°C. At 4 seconds of exposure, 35°C of incubation appeared higher than 37°C and 32°C. At 6 seconds of microwave exposure growth turbidity at incubation of 35°C appeared with higher turbidity than 32°C and 37°C. At 8 seconds, 32°C had shown higher turbidity than 35°C and 37°C. Smear slide exposure to microwaves for 10 seconds had shown higher growth recovery at 32°C than 35°C and 37°C. From the observation of the moist slide control to the dry slide controls it has been observed that there has been a little difference at each of the incubation temperature parameters. But it has been observed that as the radiation time increased the dryness of slide increased and there was a decrease in turbidity from 2 seconds to 10 seconds. This shows the effect of decrease in turbidity as the increase in the time of exposure to microwaves. Further from Fig.1 there has been significant decrease in the turbidity from 2 to 10 seconds of microwave exposure for all the 3 incubation temperatures. The most significant observation from Fig.1 includes, at lower exposure time, there was a higher turbidity at 37°C incubation and as the increase in exposure time continued it was observed that there was maximum turbidity at 32°C to 35°C to 37°C. Therefore it infers that as the cells being exposed to microwaves for longer periods, they were found to prefer lower incubation temperatures. Table.1 describes the analytical analysis of the data obtained, hence proposes the significance of the work.

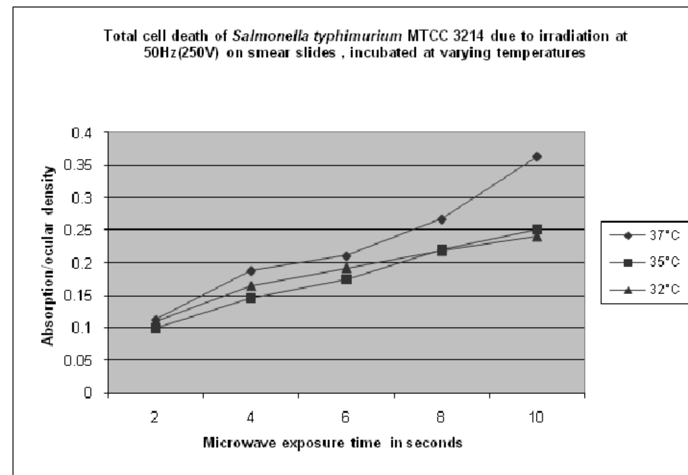

Figure 3: Analysis of total cell death of *Salmonella typhimurium* MTCC3214

From fig.3 it is observed that the actual destruction in cell population due to microwaves at 50Hz (250V) on smear slides comparatively at different incubation temperatures 37°C, 35°C and 32°C. It is noted that at 2 seconds of microwave radiation, turbidity was observed to be the same for all 3 incubation temperatures. At, 4 seconds of exposure to microwave, at 37°C incubation temperature, the turbidity was higher followed by incubation at 32°C and 35°C. At 6 seconds of exposure time

cell population reduction at 37°C was higher followed by cell reduction at 32°C and 35°C. At 8 seconds of exposure, maximum destruction was observed to occur at 35°C and 32°C. A far higher destruction was observed to occur at 37°C for exposure to 10 seconds followed by 35°C and 32°C closely. The above description of cell population reduction shows that the actual cell population reduction at all three incubation temperatures was significantly clear observed at incubation temperature of 37°C, hence an account of cell death had been clearly distinguished at optimum temperatures for bacterial growth. Cell death is calculated from deleting test values from the control values of dry smear slide turbidity.

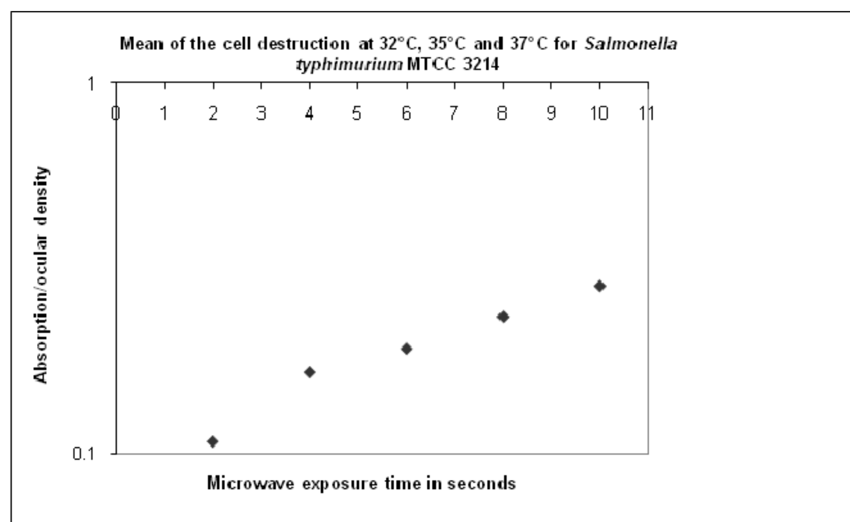

Figure 4: Cell destruction of *Salmonella typhimurium* MTCC 3214.

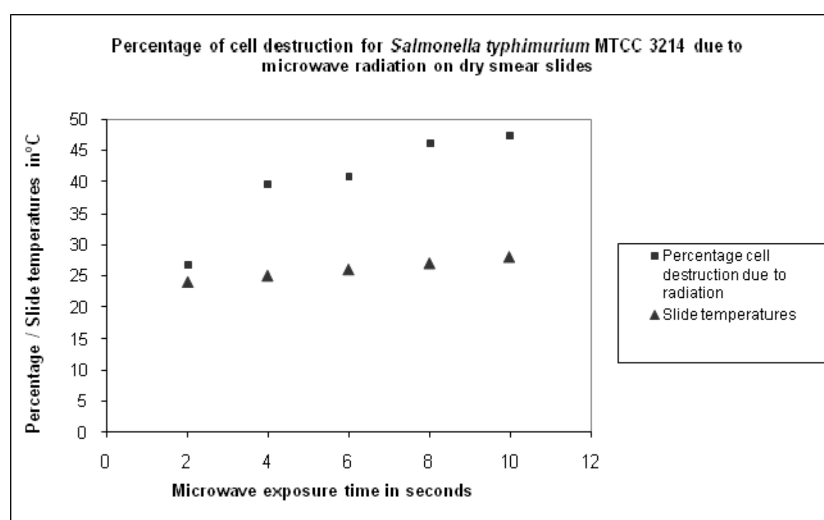

Figure 5: Percentage cell destruction of *Salmonella typhimurium* MTCC 3214.

Fig.4 and Fig.5 describe the mean of cell destruction and percentage of cell destruction. In Fig.4 the mean of cell destruction is observed to raise as the increase in time of exposure in seconds. There was a gradual raise in cell destruction for *Salmonella typhimurium* MTCC 3214, in Fig5; when compared to the percentage of cell destruction for *Salmonella typhimurium* MTCC 3214 along the slide temperature which observed to be negligible indicating only a slight changes there-by not contributing heat to a result to cell destruction. Percentage cell destruction was observed to raise well upto 4 seconds from initial 2 seconds of exposure time and at 6 seconds there was a very negligible increase in percentage cell death compared to 4 seconds. In observation there is a definite raise of cell death between 6 to 8 seconds of exposure and a slight increase of cell death at10 seconds. It has therefore been observed that the cell death due to non thermal microwaves was significant as in fig.4 and that the contribution of heat of the slide was negligible here ruling out the significance of heat due to microwave impaction on cells. From the above work it is evident non thermal microwaves have definite impact on microbial cell rather than the conventional concept of being destroyed by the heat caused by microwaves. It still remains to known, the kind of effect that the non-thermal microwaves could have inside the cellular systems, the effect on the genetics, the proteomics, the lipids, the intercellular inclusions, the chemistry and the biochemisty of the cellular organisation.

The conventional method of bulk heating by microwaves has been thought otherwise and chalked out to prove otherwise that microwaves could destroy cellular bacteria without causing the heating of the substrate by using certain di-electric substrates such as a glass slide in this case, thus leaving out any organic substrate being heated during operation. The method has tremendous advantage to sterilize the surface without causing the substrate to be disturbed by heat, this technique could be further devised to address non-thermal killing of pathogens. The technique has an upper application to surface sterilize even equipment in medical field. In the above context *Salmonella typhimurium* MTCC 3214 being a food pathogen belongs to the family of *Salmonella* pathogens, the outcome of the work has been proved that these pathogens can be to be destroyed by this technique which is opened to be consolidated. Different incubation temperatures were monitored to analyze the recovery of the surviving cells, these parameters had shown significant outcome. Methods to devise such new techniques are wanting, especially for the food, medicine and pharmaceutical industry to bring forth advancement of cutting edge technologies.

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