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Effect of low power microwave on microbial growth, enzyme activity, and aflatoxin production

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This study aimed at investigating the effect of low power (90 W) microwave radiation (2450 MHz) on microbial growth, enzyme activity (protease and urease), and aflatoxin production. Thermal effect was avoided by keeping inoculum in ice while treating with microwave (for varying duration i.e. 2, 4, and 6 min). After 6 min MW treatment to *S. aureus*, its growth was stimulated over control by ~10%. Total protease activity in *Aeromonas hydrophila* witnessed a 33% decrease as compared to control after a microwave exposure of 2 min. Similar decrease of 24% in total protease activity of *Candida albicans* was observed after microwave exposure of 6 min. *Staphylococcus aureus* lost its urease activity completely after microwave treatment. Aflatoxin production was completely inhibited in *Aspergillus parasiticus* after microwave exposure of 2, 4, and 6 min. However, it required a 6 min microwave exposure for complete inhibition of aflatoxin production in *Aspergillus flavus*. Our results positively suggest existence of microwave specific non-thermal effect on microbial growth and metabolism.

Keywords: Microwave; Non-thermal effect; Aflatoxin; Enzyme activity

There has been considerable controversy over non-thermal effect of microwave (MW) radiation (Dreyfuss and Chipley, 1980; Welt *et al*., 1994; Wayland *et al*., 1997; Kothari *et al*., 2011; Trivedi *et al*., 2011). Non-thermal effect (MW specific athermal effect) was suggested to have an important role in the inactivation of microorganisms in suspension (Jeng *et al*., 1987). Bacterial species differ in their susceptibility to MW inactivation (Najdovski *et al*., 1991). Some reports have indicated that when temperature was controlled, no effects of microwave radiation could be seen, whereas others have indicated injury to cells regardless of temperature (Dreyfuss and Chipley, 1980).

Through present study we have attempted to investigate effect of low power MW radiation on microbial growth, enzyme activity (protease and urease), and aflatoxin production. Growth and enzyme activity of MW treated cells was compared to that of untreated control.

Materials and Methods

Test organisms: Following microbial culture were procured from Microbial Type Culture Collection (MTCC), Chandigarh: *Bacillus subtilis* (MTCC 169), *Staphylococcus aureus* (MTCC 737), *Escherichia coli* (MTCC 1687), *Aeromonas hydrophila* (MTCC 1739), *Saccharomyces cerevisiae* (MTCC 170), *Candida albicans* (MTCC 3017), *Malassezia furfur*

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(MTCC 1374), *Aspergillus parasiticus* (MTCC 411), *and Aspergillus flavus* (MTCC 2799).

Microwave treatment: Bacterial and yeast suspensions were prepared from a 24 h old culture in sterile normal saline, whose turbidity was adjusted to that of 0.5 McFarland standard. In case of mold, inoculum was prepared from a 48 h old culture in sterile normal saline, whose turbidity was adjusted between 0.09-0.11 at 530 nm. Test cultures in sterile screw capped glass vials (15 mL, Merck) were exposed to MW radiation (90 W; 2450 MHz) in an domestic MW oven (Electrolux® EM30EC90SS) for 2, 4, and 6 min. Vials inside the MW oven were placed in a ice containing beaker, so as to avoid any thermal heating. Temperature of the microbial suspension after MW treatment did not go beyond 15°C. The whole MW treatment was performed in an air-conditioned room. Untreated inoculum was used as control. Before MW treatment all the inoculum vials were put in ice for 5 min to nullify any variations in initial temperature. Test organisms were immediately (in less than 10 min) inoculated into respective growth media following MW treatment.

Protease activity: Bacteria were grown in nutrient broth for estimation of growth and protease activity, whereas yeast were grown in yeast peptone dextrose (YPD) broth. All the media were procured from HiMedia, Mumbai. Incubation (16-20 h for bacteria, and 36-48 h for yeast) was carried out under static condition at 35°C. Following incubation, growth was measured at 625 nm (Spectronic 20D+, Thermo scientific). Then the culture suspension was subjected to centrifugation (5,000 rpm for 10 min), and supernatant was used for acidic and alkaline protease activity determination by the method described in Nigam and Ayyagari (2007). Briefly, 1 mL of supernatant was mixed with 1 mL of casein (1%) - prepared in 0.1 M citrate buffer of pH 5, and 0.1 M glycine-NaOH buffer of pH 10 to estimate acidic and alkaline protease, respectively- followed by incubation at 50°C for 30 min. Then 5 mL of 5% trichloroacetic acid was added to precipitate the undigested protein. Contents were then centrifuged at 10,000 rpm for 10 min, and the supernatant was subjected to estimation of the amount of amino acids released by Lowry's method. International unit of protease were calculated as: IU= net amount of amino acid released (μg) × dilution factor/ 181× 30.

Urease activity: Test organisms were grown in urea broth (Atlas, 2010), which was supplemented with 0.5%v/v soya oil for *M. furfur* as it is a lipophilic organism. Incubation was carried out under static condition for 48 h at 35°C for *S. aureus*, and 30°C for *M. furfur*, followed by measurement of turbidity at 625 nm. Supernatant obtained after centrifugation was then used for estimation of urease activity as described by Nigam and Ayyagari (2007). Briefly, 1 mL of supernatant was mixed with 2 mL phosphate buffer and 2 mL urea solution followed by incubation at 50°C for 30 min. Thereafter the test tubes containing reaction mixture were quickly shifted to an ice jacket to arrest enzyme action. Finally the amount of ammonium ion released due to urease activity was estimated using nitroprusside test.

Aflatoxin estimation: *A. parasiticus* and *A. flavus* were grown in czepkdox medium contained in 20 ml test tube. Incubation was continued for 5 days at 30°C under static condition. This was followed by separation of mycelium from the broth. Mycelium was subjected to dry weight determination after drying at 55°C till constant weight was achieved. Aflatoxin was extracted in a mixture of toluene:acetonitrile (9:1) (Nesheim and Stack, 2001), which was mixed with the broth remaining after removal of mycelia, and kept on shaker for 12 h at room

temperature. This was followed by separation of solvent layer and measurement of aflatoxin at 350 nm. Amount of aflatoxin (µg/mL) was calculated as: A × MW× 1000/ε. Where, A: absorbance at 350 nm; MW: molecular weight of aflatoxin; ε: Molar absorptivity

Results and Discussion

Results of effect of MW on microbial growth and protease activity are presented through table 1-4. Percent change in the experimental tubes as compared to control has been reported. All experiments were performed in triplicate, and mean value was used to calculate % change.

MW radiation was able to cause change in the microbial growth of all organisms on at least one occasion except for *B. subtilis* and *A. flavus* (table 1). Growth of *M. furfur* was affected by MW exposure for all the three treatments i.e. 2, 4, and 6 min, and so was the case for *A. parasiticus*. However, in case of latter magnitude of MW effect remained the same irrespective of the duration of MW exposure. Maximum effect was observed after 6 min MW treatment to *S. aureus*, its growth was stimulated over control by ~10%.

Though *B. subtilis* growth was not affected by MW exposure, its total protease activity was significantly enhanced by MW exposure for all test durations (table 2). Thus, the MW radiation seem to act differently on growth and enzyme activity. *A. hydrophila* suffered the maximum loss (~33%) of total protease activity after MW exposure of 2 min. Alkaline protease activity in this case was reduced by more than half (table 4). There were no significant changes in the acidic protease activity of *S. aureus* and *A. hydrophila* due to MW treatment (table 3).

Table 1. Effect of MW on microbial growth

Duration of MW treatment (min)	% Change compared to control								
	B. subtilis	*S. aureus*	*E. coli*	*A. hydrophila*	*S. cereviseae*	*C. albicans*	*M. furfur*	*A. parasiticus*	*A. flavus*
2	-2.34	7.69*	7.52	-6.66*	-7.72*	-2.56	-3.03*	5.73*	15.75
4	3.08	2.66	5.85*	-2.73	-4.29	-1.99	2.77*	5.73*	10.96
6	-3.22	9.71*	2.30*	-4.44*	-0.86	4.84*	-4.65*	5.73*	-9.59

*p< 0.05; minus sign indicates a decrease over control.

Table 2. Effect of MW radiation on total protease activity

Duration of MW treatment (min)	% Change compared to control					
	B. subtilis	*S. aureus*	*E. coli*	*A. hydrophila*	*S. cereviseae*	*C. albicans*
2	8.56*	-15.46*	-7.25*	-33.01*	-1.16	-1.59
4	11.28*	-11.05*	0.40	-8.21	-14.68*	-2.38
6	8.40*	-1.72	-2.40	-4.19	7.02*	-24.38*

*p< 0.05; minus sign indicates a decrease over control.

MW treatment resulted in complete loss of urease activity in *S. aureus* (table 5), irrespective of duration of treatment. However, 2 and 6 min MW treatment induced urease activity significantly in *M. furfur*. Urease has been considered among one of the important virulence factors of many pathogenic microorganisms such as

Proteus vulgaris, *Helicobacter pylori*, etc. (Madigan *et al*., 2009; Rao, 2010). Thus increase or decrease in urease activity due to MW treatment may have a significant effect on virulence of such organisms. In such cases, where MW radiation can reduce expression of particular virulence factor, it may be used for attenuation of pathogenic strains.The potential use of MW irradiation to improve vaccine preparation productivity and efficacy against *Fusobacterium necrophorum* has been indicated by Craciun *et al*. (2009).

Table 3 .Effect of MW radiation on acidic protease activity

Duration of MW treatment (min)	% Change compared to control					
	B. subtilis	*S. aureus*	*E. coli*	*A. hydrophila*	*S. cereviseae*	*C. albicans*
2	12.96*	-6.80	-4.07*	-10.4	1.45	-1.40
4	16.62*	0.15	8.42	-11.49	-28.55*	2.23
6	2.74*	0.45	7.14	-1.71	16.18*	-32.33*

*p< 0.05; minus sign indicates a decrease over control.

Table 4. Effect of MW radiation on alkaline protease activity

Duration of MW treatment (min)	% Change compared to control					
	B. subtilis	*S. aureus*	*E. coli*	*A. hydrophila*	*S. cereviseae*	*C. albicans*
2	3.70*	-23.93*	-10.47*	-57.36*	-3.21	-1.76
4	5.39	-22.01*	-7.70	-4.68	-3.77	-6.91
6	14.65*	-3.84	-12.05*	-6.86*	-0.18	-16.54*

*p< 0.05; minus sign indicates a decrease over control.

Table 5. Effect of MW radiation on urease activity

Duration of MW treatment (min)	*S. aureus*			*M. furfur*			
	OD_{625} (Mean ± SD)	% Change compared to control	Amount of ammonium ions released (µg/mL) (Mean ± SD)	OD_{625} (Mean ± SD)	% Change compared to control	Amount of ammonium ions released (µg/mL) (Mean ± SD)	% Change compared to control
0 (Control)	0.102 ± 0.001	0.00	2.460 ± 0.168	0.957 ± 0.001	-	19.405 ± 0.898	-
2	0.086 ± 0.006	-15.76	BDL	0.928 ± 0.003	-3.03*	23.254 ± 0.617	19.84*
4	0.081 ± 0.002	-20.69*	BDL	0.984 ± 0.001	2.77*	16.825 ± 0.281	-13.29
6	0.084 ± 0.006	-17.24	BDL	0.913 ± 0.004	-4.65*	23.041 ± 0.673	20.04*

BDL: Below detection limit; *p< 0.05; minus sign indicates a decrease over control.

After a 6 min MW treatment, both *A. parasiticus* and *A. flavus* completely lost their ability for aflatoxin production or secretion (Table 6). However, a 2 min MW treatment was found to significantly induce aflatoxin synthesis in *A. flavus*. Overall growth of *A. parasiticus* was enhanced due to MW treatment, with simultaneous loss of aflatoxin

production or synthesis, suggesting that growth and aflatoxin production are affected differently by MW exposure. Farag *et al*. (1996) had reported aflotoxin destruction by MW heating. In their study MW was applied onto pure aflatoxins directly. The rate of aflatoxin destruction was found to increase with the increase of MW power and exposure time. Non-thermal effect of MW on acid resistance and verocytotoxin productivity of entreohemorragic *E. coli* was investigated by Tsuji and Yokoigawa (2011). MW radiation clearly suppressed verocytotoxin productivity with slight reduction in acid resistance. They also found conventional heating less effective in reduction of virulence than the MW radiation. Further research is warranted on the mechanism of reduction in toxin production due to MW radiation.

Table 6. Effect of MW on aflatoxin production

Duration of MW treatment (min)	*A. parasiticus*			*A. flavus*			
	Dry weight (mg) (Mean ± SD)	**% Change compared to control**	**Aflatoxin (µg/mL) (Mean ± SD)**	**Dry weight (mg) (Mean ± SD)**	**% Change compared to control**	**Aflatoxin (µg/mL) (Mean ± SD)**	**% Change compared to control**
0 (control)	78.5 ± 0.707	0.00	1.767 ± 0.132	73 ± 2.828	0.00	2.655 ± 0.148	0.00
2	83 ± 1.414	5.73*	BDL	84.5 ± 4.949	15.75	3.824 ± 0.029	44.01*
4	83 ± 0.000	5.73*	BDL	81 ± 2.828	10.96	1.103 ± 0.104	-73.05*
6	83 ± 1.414	5.73*	BDL	66 ± 5.657	-9.59	BDL	-

BDL: Below detection limit; *p< 0.05; minus sign indicates a decrease over control.

Our results positively suggest existence of non-thermal microwave effect on microbial growth and metabolism. It would be interesting to investigate whether these effects are heritable. There have been a number of reports dealing with the biological effects of low-level microwave radiation, which did not produce significant thermal induction. Such studies have suggested that molecular or membrane interaction leads to alteration of function (Buckle, 1985). Improvement in *E. coli* transformation by low power MW was reported by Fregel *et al*. (2008). Increase in the specific activities of several key enzyme systems due to MW radiation was reported by Dreyfuss and Chipley (1980) while comparing effects of sublethal MW radiation and conventional heating on the metabolic activity of *S. aureus* which could not be explained solely by thermal effects. MW treatment for short duration (1-7 s) was found to induce mutations and alter gene expression in *Vigna aconitifolia* by Jangid *et al*. (2010). Mutagenic potential of MW was also demonstrated by Li *et al*. (2009) with respect to cellulase production in *Trichoderma viride*. They employed a compound mutagenesis by MW and ultraviolet radiation, and the mutants were found to be stable up to 9 generations. Wen-jie and Hong-ping (2010) also used MW to induce mutation in a cellulase-producing *Bacillus* strain. Gene mutations with a high frequency after the MW treatment were detected in the culturable airborne bacteria by Wu and Yao (2011). Non-thermal MW reduction of pathogenic cellular population had been reported by Barnabas *et al*. (2010).

The effect of low–dose MW radiation on biochemical characteristics and mortality of *Aspergillus parasiticus* was investigated by Fang *et al*. (2011). MW was found to exert its effect through the increased Ca^{2+} permeability and DNA degradation. MW

irradiation was reported to considerably increase the enzymatic activity of bacterial (enterobacteriaceae) suspensions (Spencer *et al*., 1985). Ability of MW to interfere with the growth of *E. coli*, and absorbance of MWs by the cells of *E. coli* as well as isolated protein, RNA and DNA, and its effect on metabolic processes has been known (Webb and Booth, 1969). While analyzing the experiments on non-thermal effects, the possibility of non uniform distribution of MW radiation in different layers of irradiated microbial suspension cannot be ruled out (Rojavin and Ziskin, 1995), which may lead to lack of reproducibility in such experiments.

It seems difficult for the controversy over non-thermal effects of MW to end, as reports in favour of it, and those suggesting its absence, both keep accumulating in scientific literature. It will certainly be interesting and useful to continue investigating effect of microwaves of varying frequencies and power on living systems, with the objective of deciphering the mechanism of MW specific effects on cells and their biomolecules, and determining the type of effect i.e. reversible vs. heritable; morphological, physiological or genetic.

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