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Effect of Low Power Microwave on Bacterial Growth, Protein Synthesis, and Intracellular Enzyme (Glucose-6-phosphatase and β-galactosidase) Activity

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Abstract – Effect of low power microwave (MW) radiation (2.45 GHz; 90 W) on growth, protein synthesis, and intracellular enzyme (glucose-6-phosphatase and β galactosidase) activity was investigated in *Bacillus subtilis*, *Lactobacillus acidophilus*, and *Escherichia coli.* Test parameters viz. growth, protein synthesis, and enzyme activity of MW treated cells were compared to that of untreated control. MW treatment for 3 min and 6 min duration had no effect on growth and total protein synthesis of any of the three test organisms. Glucose-6-phosphatase activity in all the test organisms experienced a significant increase following MW treatment for either one or both exposure times. β galactosidase activity in all the three test organisms experienced a significant decrease following MW treatment for either one or both exposure times. In the present study, estimations of protein content and enzyme activity were made on the cell population originated from MW treated inoculum, and not directly on the MW treated cells. Therefore, the alterations in protein content or enzyme activity might have been transferred from the originally MW treated cells to their daughter cells (who did not receive direct MW exposure). As thermal effect of MW was avoided by putting the inoculum in ice during MW treatment, whatever alterations have been observed are most likely a result of MW specific athermal effects.

Keywords – Microwave Specific Effects, Glucose-6-phosphatase, β-galactosidase, Protein Synthesis, Enzyme Activity, Non-thermal Effect

1. Introduction

Microwaves (MW) are ultra-high electromagnetic waves with wavelength range 1m-1mm, corresponding to frequency range 300 MHz-300 GHz. Since long they have been used for multiple applications [1], like sterilization [2-4], and extraction [5, 6] on account of their heating ability or thermal effect. MW are also suspected to exert non-thermal effects on biological systems, however existence of MW specific non-thermal effects is a matter of heated controversy among scientific community [7]. This controversy has been there for considerable time as reports suggesting existence of non-thermal effects of MW radiation [8-10], and those indicating otherwise [11, 12] both are available in plenty. On one hand reports suggesting no existence of non-thermal MW effects, and on the other hand reports suggesting such effects not only to be prevailing but also to be genetically stable, make the picture quite complicated. Microwave irradiation can alter the rate of enzyme catalyzed reactions, although the role of any non-thermal factors in such processes is controversial [7].

At present a large part of human population (and other life forms) is exposed chronically to non-thermal MWs from different types of mobile communication including GSM and UMTS/3G phones and base stations, WLAN (Wireless Local Area Networks), WPAN (Wireless Personal Area Networks e.g., Bluetooth), and DECT [Digital Enhanced (former European) Cordless Telecommunications] wireless phones. With such frequent exposure to MW radiation, the current safety standards set by various regulatory agencies for non-ionizing radiation protection, which are based largely on thermal effects of MW does not seem to be totally safe [12a]. In light of recent reports about MW effects (particularly athermal effects) on different life forms, the safety standard of exposure needs to be re-evaluated. Further research is needed on the exact mechanism(s) behind non-thermal effects of MW. Significance of acquiring reliable information regarding the effects of low power MW needs to be well acknowledged by the society. Due to the ease of handling them in laboratory, microorganisms can be conveniently used to study the effect of MW on living systems [10].

Present study was designed to investigate effect of low power MW radiation on bacterial growth, protein synthesis, and intracellular enzyme (glucose-6-phosphatase and β ga-

lactosidase) activity in selected gram-positive and gram-negative bacteria. Test parameters viz. growth, protein synthesis, and enzyme activity of MW treated cells were compared to that of untreated control.

2. Materials and Methods

2.1. Test organisms

Following microbial cultures were procured from Microbial Type Culture Collection (MTCC), Chandigarh: *Bacillus subtilis* (MTCC 619), *Lactobacillus acidophilus* (MTCC 447), and *Escherichia coli* (MTCC 1687).

2.2. MW treatment

Bacterial suspensions were prepared from an actively growing culture, in sterile normal saline, whose turbidity was adjusted to that of 0.5 McFarland standard. Test cultures (5 mL) in sterile screw capped glass vials (15 mL, Merck) were exposed to MW radiation (90 W; 2450 MHz) in a domestic MW oven (Electrolux® EM30EC90SS) for 3, 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 5 min) inoculated into growth medium [lactose 10 g/L, peptone 1.5 g/L, yeast extract 1 g/L, KH_2PO_4 1 g/L, $(NH_4)_2 SO_4$ 7 g/L, $MgSO_4.7H_2O$ 1 g/L, $CaCl_2$ 0.3 g/L] following MW treatment. All the test organisms were incubated at 35 ºC for 24 h.

2.3. Cell lysis

Following incubation, growth was quantified by measurement of OD at 625 nm (Spectronic 20D+, Thermo scientific). The broth containing microbial growth was subjected to centrifugation (nüve®, NF 800R) at 7500 rpm for 10 min. The cell free supernatant (CFS) obtained after centrifugation was used for estimation of extracellular protein content, while the cell pellet was subjected to cell lysis, and the lysed content (after clarification by centrifugation at 7500 rpm for 10 min) was used for estimation of intracellular enzyme activity and intracellular protein content. Cell lysis was performed by using the method described in Harley and Prescott [13] with some modification. Briefly, the cell pellet obtained after centrifugation was suspended in 0.1 M phosphate buffer (pH 6.2), followed by addition of 0.05 mL of lysozyme (HiMedia, Mumbai) solution (2 mg/mL). This mixture was gently agitated and kept at room temperature for 1 h. Then the tubes containing this reaction mixture were put in chilled water for 1 h, followed by addition of chloroform (Merck) into it, and then incubated for 2 h at room temperature in case of *E. coli*. For *L. acidophilus* and *B. subtilis*, length of this incubation was kept 12 h (as 2 h incubation in their case was found to give incomplete lysis), following which the content was centrifuged (7500 rpm for 10 min), and the supernatant was used as cell lysate.

2.4. Protein estimation

Protein estimation was done by Biuret assay as described in Nigam and Ayyagari [14]. Briefly, 1 mL of CFS (for extracellular protein estimation) or cell lysate (for intracellular protein estimation) was mixed with 1.5 mL of Biuret reagent, and incubated at 37 º C for 10 min. Following incubation, absorbance was measured at 520 nm, and the protein concentration was found by using standard curve prepared with Bovine serum albumin (125-1250 µg/mL; HiMedia, Mumbai). Total protein was calculated as the sum of intracellular and extracellular protein content.

2.5. Assay for Glucose-6-phosphatase (G6P)

G6P activity in cell lysate was assayed as described in [14]. Briefly, 0.5 mL of cell lysate, 0.5 mL of 1mM glucose-6-phosphate, and 1 mL of 0.1 M citrate buffer (containing 1mM EDTA) was mixed in a test tube, followed by incubation at 50 ºC for 30 min. Then, 10 mL of 10% trichloroacetic acid (Central Drug House) was added to terminate the reaction. 1 mL content from this was mixed with 1.5 mL distilled water and 0.5 mL of 9 N H_2SO_4. This mixture was allowed to stand for 15 min, and then 0.5 mL of molybdate reagent (6% ammonium molybdate) was added, followed by incubation in dark for 15 min. Following incubation, 0.5 mL of ferrous sulfate reagent was added, and incubated in dark for 15 min. Following this, absorbance was measured at 660 nm. This absorbance value was used to find out amount of inorganic phosphate released, from the standard curve of inorganic phosphate.

2.6. Assay for β-galactosidase (β-gal)

β-gal activity in cell lysate was assayed as described by [12]. 1.1 mL of supernatant from each sample was added to a tube containing 0.2 mL of ONPG (o-nitrophenylβ-D-galactopyranoside; HiMedia, Mumbai) solution (5 mM in TM buffer) and 0.2 ml of TM buffer. The tubes were vortexed and then placed at room temp, and colour development was monitored. Upon noticeable colour formation, the reaction was stopped by adding 2.0 mL of 0.6 M Na_2CO_3, and the OD was measured at 410 nm. The molar extinction coefficient used to calculate amount of ONP (o-nitrophenol) released was 4500 $M^{-1}cm^{-1}$ [15].

2.7. Statistical analysis

All the experiments were performed in triplicate, and measurements are reported as mean ± standard deviation (SD). Statistical significance of the data was evaluated by applying *t*-test using Microsoft Excel®. *P* values less than 0.05 were considered to be statistically significant.

3. Results and Discussion

Results of effect of MW on growth, protein synthesis, and

enzyme activity in *B. subtilis* (Fig. 1) are presented in Table 1-2. MW treatment had impact neither on growth of *B. subtilis*, nor on the amount of total protein synthesized by it. Though intracellular protein synthesis remained unaffected by MW, a decrease in the amount of extracellular protein was noted after MW treatment of 6 min duration. Change in the estimated amount of extracellular protein may be due to effect of MW on protein synthesis or on its secretion through modification of membrane permeability. Alteration of membrane permeability upon MW treatment in *E. coli* and *Staphylococcus aureus* has been reported by Chen et al [16]. However, Woo et al. [17] found no effect of MW radiation (till 40 ºC) on amount of protein released in *B. subtilis*. Although, no significant change was found following MW treatment in the total intracellular protein content of *B. subtilis* (Table 1), significant alterations in the activity of intracellular enzymes G6P and β-gal were noted after MW treatment of 6 min and 3 min duration respectively (Table2).

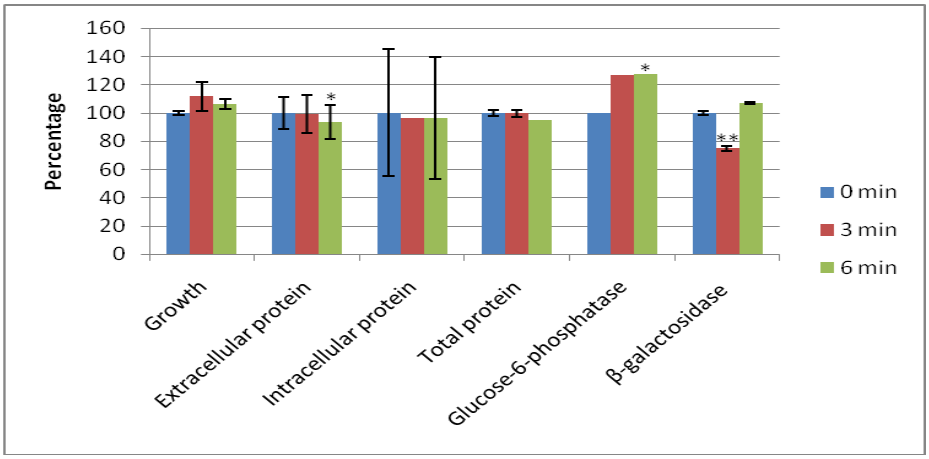

Fig. 1. Effect of MW radiation on *B. subtilis*

Table 1. Effect of MW radiation on growth and protein synthesis in *B. subtilis*

Duration of MW treatment (min)	OD_{625} (Mean ± SD)	% change compared to control	Extracellular protein (mg/ml) (Mean ± SD)	% change compared to control	Intracellular protein (mg/ml) (Mean ± SD)	% change compared to control	Total protein (mg/ml) (Mean ± SD)	% change compared to control
0 (control)	0.077 ± 0.001	0.00	1.127 ± 0.128	0.00	0.309 ± 0.139	0.00	1.425 ± 0.031	0.00
3	0.086 ± 0.009	11.68	1.116 ± 0.149	-0.97	0.298 ± 0.000	-3.55	1.419 ± 0.037	-0.42
6	0.082 ± 0.003	6.49	1.053 ± 0.128	-6.56*	0.298 ± 0.128	-3.55	1.350 ± 0.000	-5.26

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

Table 2. Effect of MW radiation on G6P and β-galactosidase activity in *B. subtilis*

Duration of MW treatment (min)	G6P activity measured as amount of inorganic phosphate released (µg/mL) (Mean ± SD)	% change compared to control	β-galactosidase activity measured as amount of ONP released (M) (Mean ± SD) ($X\ 10^{-4}$)	% change compared to control
0 (control)	82.19 ± 0.00	0.00	1.44 ± 0.02	0.00
3	103.93 ± 0.00	26.45	1.08 ± 0.02	-25.00**
6	104.58 ± 0.00	27.24*	1.54 ± 0.01	6.94

*p< 0.05, **p< 0.01; minus sign indicates a decrease over control

Measurements regarding effect of MW on growth, protein synthesis, and enzyme activity in *L. acidophilus* (Fig. 2) are presented in Table 3-4. MW treatment did not cause any change in the capacity of *L. acidophilus* for growth, and

extracellular protein secretion. Total protein content remained unaffected following MW treatment; however a significant increase (> 40%) was observed in the intracellular protein content for both durations of MW treatment tested. G6P activity experienced a significant increase (27.01%) following a 6 min MW treatment, whereas almost identical decrease in the β-gal activity was observed following both durations of MW treatment (Table 4).

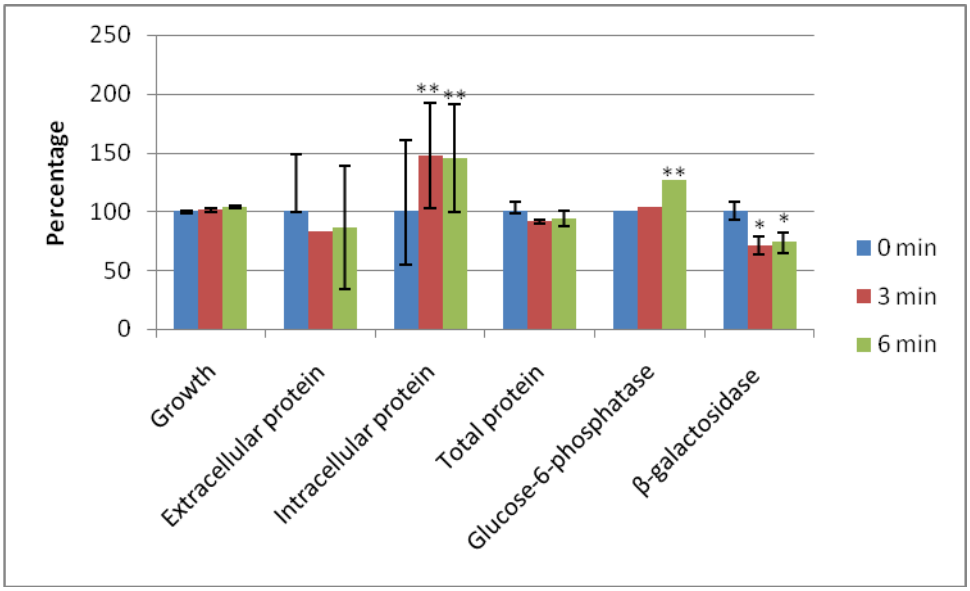

Fig. 2. Effect of MW radiation on L. acidophilus

Table 3. Effect of MW radiation on growth and protein synthesis in *L. acidophilus*

Duration of MW treatment (min)	OD_{625} (Mean ± SD)	% change compared to control	Extracellular protein (mg/ml) (Mean ± SD)	% change compared to control	Intracellular protein (mg/ml) (Mean ± SD)	% change compared to control	Total protein (mg/ml) (Mean ± SD)	% change compared to control
0 (control)	0.217 ± 0.002	0.00	0.927 ± 0.449	0.00	0.209 ± 0.127	0.00	1.136 ± 0.096	0.00
3	0.220 ± 0.003	1.38	0.768 ± 0.000	-17.15	0.309 ± 0.139	47.84**	1.043 ± 0.016	-8.18
6	0.208 ± 0.002	4.14	0.800 ± 0.417	-13.70	0.303 ± 0.139	44.97**	1.069 ± 0.067	-5.89

** $p < 0.01$; minus sign indicates a decrease over control

Table 4. Effect of MW radiation on G6P and β-galactosidase activity in *L. acidophilus*

Duration of MW treatment (min)	G6P activity measured as amount of inorganic phosphate released (μg/mL) (Mean ± SD)	% change compared to control	β-galactosidase activity measured as amount of ONP released (M) (Mean ± SD) ($X\ 10^{-4}$)	% change compared to control
0 (control)	86.10 ± 0.00	0.00	0.76 ± 0.06	0.00
3	89.58 ± 0.00	4.04	0.54 ± 0.04	-28.94*
6	109.36 ± 0.00	27.01**	0.56 ± 0.05	-26.31*

* $p<0.05$, ** $p<0.01$; minus sign indicates a decrease over control

Results of effect of MW on growth, protein synthesis, and enzyme activity in *E. coli* (Fig. 3) are presented in Table 5-6. As with other two test organisms, growth and total protein estimated in *E. coli* also remained unaffected by both durations of MW treatment. Activity of both the intracellular enzymes was affected by MW treatment for 3 min as well as 6

min. G6P activity experienced a significant-ant increase, whereas β-gal activity underwent significant decrease following MW treatment. This is in contrast to results reported by Asay et al. [12], they did not find non-thermal effects to alter β-gal activity in *E. coli* C29.

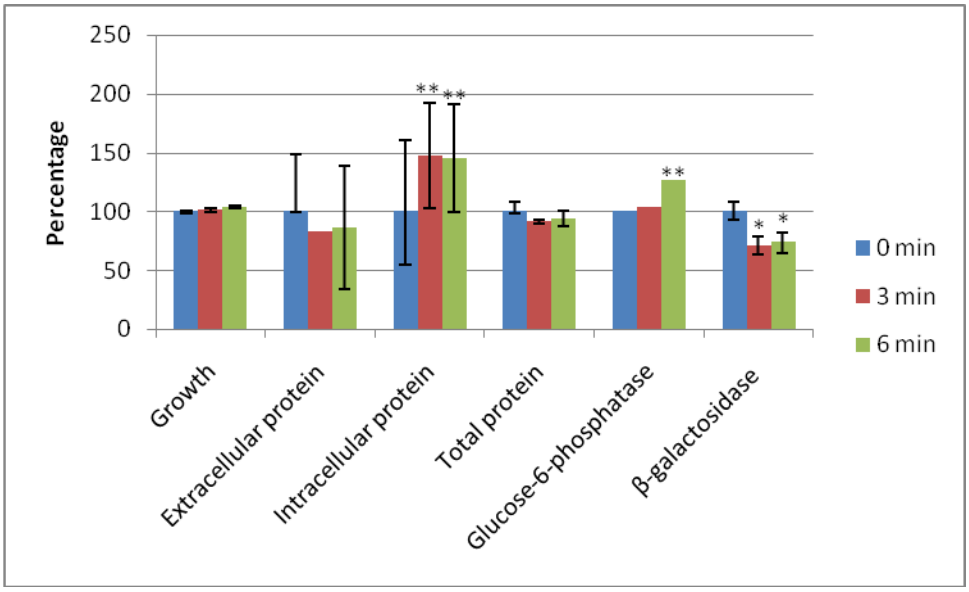

Fig. 3. Effect of MW radiation on E. coli

Table 5. Effect of MW radiation on growth and protein synthesis in *E. coli*

Duration of MW treatment (min)	OD_{625} (Mean ± SD)	% change compared to control	Extracellular protein (mg/mL) (Mean ± SD)	% change compared to control	Intracellular protein (mg/mL) (Mean ± SD)	% change compared to control (Mean ± SD)	Total protein (mg/mL) (Mean ± SD)	% change compared to control
0 (control)	0.206 ± 0.000	0.00	0.819 ± 0.128	0.00	0.245 ± 0.000	0.00	1.074 ± 0.030	0.00
3	0.213 ± 0.004	3.39	0.798 ± 0.128	-2.56	0.234 ± 0.128	-4.48	1.014 ± 0.031	-5.58
6	0.221 ± 0.009	7.28	0.787 ± 0.149	-3.90	0.171 ± 0.000	-30.20**	0.958 ± 0.045	-10.80

**p<0.01; minus sign indicates a decrease over control

Table 6. Effect of MW radiation on G6P and β-galactosidase activity in *E. coli*

Duration of MW treatment (min)	G6P activity measured as amount of inorganic phosphate released (µg/mL) (Mean ± SD)	% change compared to control	β-galactosidase activity measured as amount of ONP released (M) (Mean ± SD) ($X\ 10^{-4}$)	% change compared to control
0 (control)	235.69 ± 0.00	0.00	2.67 ± 0.07	0.00
3	283.52 ± 0.00	20.29*	2.02 ± 0.06	-24.34**
6	280.91 ± 0.00	19.18*	2.41 ± 0.01	-9.73*

*p<0.05, **p<0.01; minus sign indicates a decrease over control

Our results (Table 1-6) indicate that MW treatment (2.45 GHz; 90 W) for 3 min and 6 min duration had no effect on growth and total protein synthesis of any of the three test organisms. A 6 min MW exposure was able to cause a significant decrease in extracellular and intracellular protein content of *B. subtilis* (Table 1) and *E. coli* (Table 5) respectively. Reduction in protein synthesis in microorganisms subjected to MW irradiation has also been reported earlier [18, 19]. G6P activity in all the test organisms experienced a significant increase following MW treatment for either one or both exposure times.

β-gal activity in all the three test organisms experienced a significant decrease following MW treatment for either one or both exposure times. MW exposure of 3 min was able to significantly reduce β-gal activity in all three test organisms. MW exposure of 6 min was able to significantly induce G6P activity in all the three test organisms. Increase in the catalytic activity of intracellular enzymes (lactate dehydrogenase and cytochrome c oxidase) in *E. coli*, following MW (18 GHz) treatment was reported by Shamis et al. [20]. Involvement of non thermal effects of MW in altering activity of many intracellular enzymes in *Staphylococcus aureus* was suggested by Dreyfuss and Chipley [21]. Effect of MW treatment on extracellular and intracellular protein content varied from organism to organism. The observed alterations in protein content or enzyme activity may be due to mutagenic effects of MW, or it might have resulted from changes in the membrane permeability of the test organisms induced by MW radiation. Alterations in the permeability of cell walls and cell membranes of both gram-positive and gram-negative microbes, induced by MW irradiation has been reported by Bollet et al. [22]. Excitation of cell macromolecules by MW has been suggested to change enzyme activity and nucleic acid synthesis [23, 24]. Otludil et al. [19], and Marha et al. [25] did report ability of MW radiation to induce alterations in enzyme activity and protein synthesis.

In the present study, estimations of protein content and enzyme activity were made on the cell population originated from MW treated inoculum, and not directly on the MW treated cells. Therefore, the alterations in protein content or enzyme activity might have been transferred from the originally MW treated cells to their daughter cells (who did not receive direct MW exposure). The cells receiving direct MW exposure may get their biomolecules such as DNA altered in some manner. Maximum absorption of MW by DNA has been indicated at 2.45 GHz, and the dielectric properties of DNA are reported to reduce at this frequency [26]. However, the effect exerted by MW on DNA can be repairable [27]. As in this study, thermal effect of MW was avoided by putting the inoculum in ice during MW treatment, whatever alterations have been observed are most likely a result of *MW specific effects* (athermal effects).

This study detected changes in the protein content, and intracellular enzyme activity in three test bacteria following MW treatment under temperature controlled condition, where thermal effects were avoided by putting bacterial suspension in ice during MW treatment. As these changes occurred in absence of any noticeable heat generation, they can be said to be induced by MW specific athermal effects. Exact mechanism by which MW exert their athermal effects on biological systems is still not clear. Many of these effects may be reversible, but their existence indicated by sufficient number of reports [19, 28, 29] has attracted considerable attention. As low intensity MW are believed not to possess sufficient energy for breaking chemical bonds directly, alternative mechanisms of interaction between MW and biological entities are likely to prevail. Owing to their rapid growth rate and ease of handling, microorganisms can be most suitable test organisms for study of MW effects on biological systems. Research on effect of MW radiation on biological entities at varying frequencies, power, and for different durations can yield interesting data. Further probe into whether these MW effects are reversible seems to be an exciting and useful scientific exercise.

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Note

Toshi Mishra and Preemada Kushwah contributed equally to this work.

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