Effect of low power microwave radiation on pigment production in bacteria

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Abstract

Effect of low power (90 W) microwave (MW) radiation (2450 MHz) on bacterial growth and pigment production was studied in three different bacteria. Microwave exposure of 2-6 min duration was able to alter growth and pigment production (prodigiosin production by *Serratia marcescens*, violacein production by *Chromobacterium violaceum*, and staphyloxanthin production by *Staphylococcus aureus*) in the test organisms significantly. In this study, pigment production was estimated in the cell population originated from microwave treated inoculum, and not directly in the MW treated cells. Thus the alterations in pigment production and/or secretion might have been transferred from the originally MW treated cells to their daughter cells (who did not receive direct MW exposure), indicating the mutagenic influence of microwave radiation. Heavy prodigiosin overproduction observed in one of the test tubes inoculated with microwave treated *S. marcescens* could not be sustained by daughter populations corresponding to that tube, indicating the reversible nature of microwave induced mutation(s). The microwave effects observed in this study largely seem to be of athermal nature, as the thermal effect was minimized by use of ice during the microwave treatment.

Introduction

The part of electromagnetic radiation known as microwaves (MW), holds frequency ranging from 300 MHz-300 GHz, and can exert two types of effects on biological systems. One of these effects described as thermal effect is well established. Another effect described as non-thermal (also referred as microwave specific athermal effect) is controversial. Occurrence of the non-thermal effect has been a matter of debate over a long period of time.^{1,2} Reports suggesting the existence of non-thermal effect,³ as well as those indicating otherwise are available in literature.^{4,5} MW have also been reported to induce mutations in various biological systems.^{6,7} As the use of cell

phones and other devices using frequencies corresponding to the MW range is increasing, it becomes important to investigate biological effects of MW.

The present study aimed at investigating effect of low power MW on growth and pigment production in three different bacteria viz. *Chromobacterium violaceum*, *Serratia marcescens*, and *Staphylococcus aureus*. These bacteria respectively produce pigments namely violacein, prodigiosin, and staphyloxanthin.

Violacein is a bioactive pigment molecule produced by *C. violaceum*, whose production is regulated by cell density dependent quorum sensing (QS). *C. violaceum* has been used as model bacterium while screening natural products for anti QS property.⁸ Any alteration in the pigment producing ability of *C. violaceum* under the influence of any external treatment (e.g. antibiotics, radiofrequency, etc.) can easily be quantified photometrically. Violacein is an important bioactive molecule with attractive potential therapeutic applications. This pigment is known to possess antimicrobial and antitumor activities.⁹ The red-pigmented prodiginines are bioactive secondary metabolites produced by microorganisms like *Serratia marcescens* and *Vibrio* sp.¹⁰ These pigments are known to possess immunosuppressive and anticancer properties.¹¹ Prodigiosin have potential applications in textiles, cosmetics, etc. Prodigiosin production in *S. marcescens* is activated at high cell density by QS mechanisms.¹² *Staphylococcus aureus* produces a golden carotenoid virulence factor called staphyloxanthin. Bacterial carotenoids such as this can serve a protective function against hydrogen peroxide and singlet oxygen oxidation, as well as neutrophilic or phagocytic killing.¹³

Materials and Methods

Test organisms and culture condition

Chromobacterium violaceum (MTCC 2656), *Serratia marcescens* (MTCC 97), and *Staphylococcus aureus* (MTCC 737) were procured from Microbial Type Culture Collection (MTCC), Chandigarh. All the organisms were grown in nutrient broth (HiMedia, Mumbai). *S. marcescens* was incubated at 28°C, whereas incubation temperature for remaining two organisms was 35°C. Duration of incubation was kept 24 h for *C. violaceum*, 48 h for *S. marcescens*, and 72 h for *S. aureus*. Incubation was carried out under static condition (with intermittent manual shaking).

Microwave treatment

Bacterial suspensions were prepared from an actively growing culture, in sterile normal-

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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 apparatus (Electrolux® EM30EC90SS) for 2, 4, and 6 min. Vials inside the MW apparatus were placed in a ice containing beaker (100 mL; Borosil®), so as to avoid any thermal heating. Temperature of the microbial suspension after MW treatment did not go beyond 10°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. Initial temperature of the vial content (after five min ice treatment) before being put in MW oven was measured to be 8°C. Following MW (90 W) exposure for 6 min the temperature reached 10°C. In case of control vial, put for 5 min in ice and then for 5 min at room temp (instead of MW treatment), the temp of inoculum was found to be 18°C. Test organisms were immediately (in less than 5 min) inoculated (at 10% v/v) into the growth medium following MW treatment.

Pigment extraction and estimation

Prodigiosin

After quantifying growth of *S. marcescens* at 625 nm, prodigiosin extraction was carried out as described in Pradeep *et al.*¹⁴ Briefly, 10 mL of the culture broth was centrifuged (Nüve NF 800 R) at 7500 rpm for 15 min. Centrifugation was carried out at 4°C, as prodigiosin is a temperature-sensitive compound.¹⁵ The resulting supernatant was discarded. Remaining cell pellet was re-suspended in 10 mL of acidified methanol (4 mL of HCl into 96 mL of methanol; Merck), followed by incubation in dark at room temperature for 30 min. This was followed by centrifugation at 7500 rpm for 15 min at 4°C. Prodigiosin in the resulting supernatant was estimated by measuring OD at 535 nm. Prodigiosin unit was calculated as the ratio (OD_{535}/OD_{625}) which gives an indication of prodigiosin production per unit of growth.

Violacein

Following estimation of *C. violaceum* growth by recording the OD at 625 nm the tubes were subjected to violacein extraction as described in Choo *et al.*⁸ Briefly, 2 mL of the

culture broth was centrifuged (Eppendorf 5417 R) at 10,000 rpm for 15 min, and the resulting supernatant was discarded. The remaining cell pellet was resuspended into 2 mL of DMSO (Merck, Mumbai), and vortexed, followed by centrifugation at 10,000 rpm for 15 min. The violacein extracted in the supernatant was estimated by measuring OD at 585 nm. Violacein unit was calculated as the ratio (OD_{585}/OD_{625}) which gives an indication of violacein production per unit of growth.

Staphyloxanthin

After measuring the growth of *S. aureus* at 625 nm, staphyloxanthin extraction was carried out as described in Rosado *et al.*¹⁶ with some modification. The culture broth was centrifuged at 4900 rpm for 5 min at 25°C. After discarding supernatant, the cell pellet was resuspended in 0.5 mL methanol (Merck), and vortexed. This was followed by centrifugation at 4900 rpm for 5 min at 25°C, and the quantification of staphyloxanthin in the resulting supernatant was done by measuring optical density (Agilent Cary 60) at 462 nm.

Concentration of the extracted pigments was calculated using their molar extinction

coefficient, whose value for prodigiosin, violacein, and staphyloxanthin was 1.12×10^5 , 5.6×10^2 , and 1920 respectively. The molar concentrations obtained thus, were then converted into $\mu\text{g/mL}$ using molecular weight of the individual pigment, whose value respectively for prodigiosin, violacein, and staphyloxanthin was taken as 323.23, 343.34 (<http://www.sigmaaldrich.com/catalog>), and 819.16 (<http://www.ncbi.nlm.nih.gov/pccompound/?term=staphyloxanthin>).

Statistical analysis

All the experiments were performed in triplicate, and measurements are reported as mean $\pm$ 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.

Results and Discussion

Results obtained while investigating the

Table 1. Effect of low power microwaves (MW) on growth and violacein production in *C. violaceum*.

Duration of MW treatment (min)	Growth (OD_{660}) (Mean $\pm$ SD)	Change compared to control (%)	Violacein ($\mu\text{g/mL}$) (Mean $\pm$ SD)	Change compared to control (%)	Violacein Unit (OD_{585}/OD_{625})	Change compared to control (%)
0 (Control)	0.45 $\pm$ 0.02	0.00	2.19 $\pm$ 0.01	0.00	0.26 $\pm$ 0.01	0.00
2	0.42 $\pm$ 0.00	-6.66	1.91 $\pm$ 0.07	-12.78	0.25 $\pm$ 0.00	-3.84
4	0.53 $\pm$ 0.03	17.77*	1.61 $\pm$ 0.07	-26.48*	0.14 $\pm$ 0.00	-46.15**
6	0.46 $\pm$ 0.00	2.22	0.84 $\pm$ 0.10	-61.64**	0.09 $\pm$ 0.00	-65.38**

*P<0.05; **P<0.01.

Table 2. Effect of low power microwaves (MW) on growth and staphyloxanthin production in *S. aureus*.

Duration of MW treatment (min)	Growth OD_{625} (Mean $\pm$ SD)	Change compared to control (%)	Staphyloxanthin ($\mu\text{g/mL}$) (Mean $\pm$ SD)	Change compared to control (%)	Staphyloxanthin Unit (OD_{625}/OD_{462})	Change compared to control (%)
0 (Control)	0.59 $\pm$ 0.00	0.00	0.51 $\pm$ 0.01	0.00	1.01 $\pm$ 0.03	0.00
2	0.59 $\pm$ 0.06	0.00	0.60 $\pm$ 0.01	17.64*	1.27 $\pm$ 0.02	25.74**
4	0.64 $\pm$ 0.01	8.47*	0.58 $\pm$ 0.03	13.72*	1.07 $\pm$ 0.07	5.94
6	0.73 $\pm$ 0.01	23.72**	0.85 $\pm$ 0.18	66.66*	1.42 $\pm$ 0.08	40.59**

*P<0.05; **P<0.01.

Table 3. Effect of low power microwaves (MW) on growth and prodigiosin production in *S. marcescens*.

Duration of MW treatment (min)	Growth (OD_{625}) (Mean $\pm$ SD)	Change compared to control (%)	Prodigiosin ($\mu\text{g/mL}$) (Mean $\pm$ SD)	Change compared to control (%)	Prodigiosin Unit (OD_{535}/OD_{625})	Change compared to control (%)
0 (Control)	0.57 $\pm$ 0.00	0.00	1.03 $\pm$ 0.08	0.00	0.62 $\pm$ 0.04	0.00
2	0.56 $\pm$ 0.02	-1.75	0.41 $\pm$ 0.02	-60.19**	0.31 $\pm$ 0.05	-50.00**
4	0.54 $\pm$ 0.02	-5.26	0.53 $\pm$ 0.15	-48.54*	0.36 $\pm$ 0.12	-41.94
6	0.49 $\pm$ 0.00	-14.03**	0.52 $\pm$ 0.02	-49.51**	0.37 $\pm$ 0.01	-40.33*

*P<0.05; **P<0.01.

effect of low power MW on bacterial growth and pigment production are presented through Table 1-3. Growth of *C. violaceum* was significantly enhanced due to MW treatment for 4 min duration, with a simultaneous decrease in violacein production, which indicates considerable decrease in violacein production by individual cell (Table 1). As violacein production in *C. violaceum* is linked to cell density dependent QS,⁹ higher cell density in general should result in higher production of the pigment violacein. However, a reduced pigment production despite increased growth might have occurred due to effect of MW radiation on the violacein synthesis and/or secretion machinery of the cell. MW treatment for 2 min and 6 min duration was not able to alter growth of *C. violaceum* significantly, however a heavy decrease in violacein production was observed in case of the latter. The possible targets of MW radiation causing reduced production of violacein may be the members of *vioABCDE* gene cluster, or the pathways involved in QS signalling.¹⁷

MW treatment for all the three durations were able to promote staphyloxanthin production in *S. aureus* (Table 2). The six minute MW treatment had the highest impact on *S. aureus* growth as well as staphyloxanthin production. However, the magnitude of increase in these two quantities was not parallel to each other, suggesting that growth and staphyloxanthin production might be affected independently by MW treatment. Alteration in the staphyloxanthin producing ability of *S. aureus* can have considerable influence on its virulence.¹⁸ Higher carotenoid (of which staphyloxanthin is a type) biosynthesis may render the pathogenic strains

of *S. aureus* less susceptible to oxidant killing, and promote its virulence through antioxidant activity. Staphyloxanthin biosynthesis genes organized in the *crtOPQMN* operon¹⁹ may be the possible targets where MW radiation might have affected, resulting in altered staphyloxanthin production.

In case of *S. marcescens*, the 2 min and 4 min MW treatment had no significant effect on growth, but it could reduce pigment production heavily. The six min MW treatment could reduce the growth as well as pigment production, however the latter was affected more than the former. Out of the tubes put in triplicate for 6 min MW treatment, 2 tubes were showing reduced growth and prodigiosin production, whose data is presented in Table 3. Whereas the third tube showed no change in growth (OD_{625} : 0.57), but notable increase in prodigiosin (OD_{535} : 0.48) production. As the prodigiosin concentration in this tube (1.40 $\mu\text{g/mL}$) was 35.92% higher than the control, we picked up this tube for further investigation that if we can isolate any prodigiosin overproducing mutant(s) from this tube. From the test tube showing higher prodigiosin production corresponding to six min MW treatment, one nutrient agar plate was streaked and incubated at 28°C for 48 h. This was done under aseptic conditions based on visibly high intensity of red color, before opening this tube for estimation of growth and prodigiosin.

From the resulting growth on the nutrient agar plate, three colonies were picked randomly, and designated as VHVS_PR1, VHVS_PR2, and VHVS_PR3. Each of these three colonies were streaked onto a new nutrient agar plate (1 plate per colony) and incubated. Inoculum

prepared from the resulting growth was inoculated into nutrient broth tubes, followed by estimation of growth and prodigiosin production upon incubation. The strain designated as VHVS_PR2 showed prodigiosin production more than control (Table 4). This strain was further studied to check whether it can maintain the trait of prodigiosin overproduction over next few generations. However eventually this strain (*i.e.* its daughter population) lost the prodigiosin overproducing ability (Table 5). The small increase in prodigiosin production shown over control was not found to be statistically significant. Thus it can be said that MW-induced mutation(s) leading to prodigiosin overproduction was not retained by the mutant, who soon reverted back to the parent phenotype. This is in contrast to the report by Liu *et al.*⁶, wherein they have claimed the prodigiosin overproducing mutant obtained by MW mutagenesis to be stable for 10 generations. The possible cellular targets of MW radiation, whose interaction with MW resulted in altered prodigiosin production include genes like *pswP* or those coding for enzymes (*e.g.* O-methyltransferase) involved in prodigiosin biosynthesis pathway,²⁰ or any of the loci of the *pig* operon.²¹

This study has shown that MW radiation (2.45 GHz) when applied at low power (90 W in this study) and for short durations (2-6 min) can considerably alter bacterial pigment production. Two of the pigments (violacein and prodigiosin) included in this study are produced in a quorum sensing-regulated fashion by the producer organism.^{9,21} It may be possible that MW had exerted its effect on production of these two pigments by influencing the

Table 4. Prodigiosin production in populations generated from the colonies obtained from prodigiosin overproducing broth.

Colony designation	Growth (OD_{625}) (Mean $\pm$ SD)	Change compared to control (%)	Prodigiosin ($\mu\text{g/mL}$) (Mean $\pm$ SD)	Change compared to control (%)	Prodigiosin Unit (OD_{535}/OD_{625})	Change compared to control (%)
Control	0.50 $\pm$ 0.010	0.00	0.60 $\pm$ 0.010	0.00	0.55 $\pm$ 0.010	0.00
VHVS_PR1	0.43 $\pm$ 0.003	-14**	0.11 $\pm$ 0.001	-81.67**	0.12 $\pm$ 0.000	-78.18**
VHVS_PR2	0.42 $\pm$ 0.008	-16**	1.17 $\pm$ 0.005	95**	1.27 $\pm$ 0.020	130.90**
VHVS_PR3	0.44 $\pm$ 0.001	-12**	0.17 $\pm$ 0.006	-71.67**	0.16 $\pm$ 0.007	-70.90**

*P<0.05; **P<0.01.

Table 5. Loss of prodigiosin overproduction by daughter generations of VHVS_PR2.

Generation	Growth (OD_{625}) (Mean $\pm$ SD)		Change compared to control (%)	Prodigiosin ($\mu\text{g/mL}$) (Mean $\pm$ SD)		Change compared to control (%)	Prodigiosin Unit		Change compared to control (%)
	C	E		C	E		C	E	
1 (Parent)	0.50 $\pm$ 0.01	0.42 $\pm$ 0.00	-16.00**	0.60 $\pm$ 0.01	1.17 $\pm$ 0.00	95.00**	0.55	1.27	130.90**
2	0.39 $\pm$ 0.03	0.41 $\pm$ 0.03	5.12*	3.03 $\pm$ 0.10	3.31 $\pm$ 0.09	9.24	3.56	3.67	3.08
3	0.56 $\pm$ 0.04	0.57 $\pm$ 0.01	1.78	2.05 $\pm$ 0.24	2.16 $\pm$ 0.17	5.36	1.74	1.98	13.79

C, control; E, experimental; *P<0.05; **P<0.01.

QS signaling in the producing bacteria. In this study, pigment production was estimated in the cell population originated from MW treated inoculum, and not directly in the MW treated cells. Thus the alterations in pigment production might have been transferred from the originally MW treated cells to their daughter cells (who did not receive direct MW exposure). The effect of MW on prodigiosin production in *S. marcescens* was not found to be stable over daughter generations. MW effects on DNA can be repairable.²² Reports indicating reversible nature of MW effects, as well as those suggesting MW induced mutations to be stable, both have appeared in literature. Kothari *et al.*²³ reported mutagenic effect of MW radiation on exopolysaccharide production in *Xanthomonas campestris*, however the xanthan overproducing mutants were shown to revert back to the parent phenotype. Pasiuga *et al.*²⁴ reported disappearance of low-level MW induced effects after few generations in *Drosophila melanogaster*. MW treatment might have a profound effect on mutation repair system of a cell for initial few generations, but thereafter the repair system may restore its efficiency. Exploitation of MW mutagenesis resulting in genetically stable mutants has also been reported by few workers. Lactic acid overproducing mutants of *Lactobacillus rhamnosus* using MW radiations (2450 MHz; 400W for 3 min) were obtained by Lin *et al.*⁷, and these mutants were found to be stable for up to 9 generations. Li *et al.*²⁵ claimed *Klebsella pneumoniae* mutants with superior nitrogen fixing and P-solubilising ability, obtained through MW (250 W for 36 s) mutagenesis, to be genetically stable.

In our experiments thermal effect of MW was avoided by putting the inoculum in ice during MW treatment, thus whatever alterations in bacterial growth and/or pigmentation have been observed are most likely owing to MW specific effects (athermal effects). MW radiation at 2.45 GHz does have the ability to disrupt hydrogen bonds in a non-thermal fashion.²⁶ MW exposure can alter the biomolecules such as DNA of the cells receiving direct MW exposure. Maximum absorption of MW by DNA has been suggested at 2.45 GHz, and dielectric properties of DNA have been shown to be reduced at this frequency.²⁷

Biological systems can exhibit resonance behavior involving the mechanical vibration of system elements. The natural frequencies of such resonances will, generally, be in the microwave frequency range, indicating the possibility that microwave exposures may generate physiological effects in living cells.²⁸ The vibrations generated due to MW exposure (or any other factor) can affect microbial growth and properties like antibiotic resistance.²⁹

Conclusions

Within last few years, there has been a tremendous increase in the use of radiofrequency devices, such as cellular phones which transmit radiofrequency waves of very low intensity. There have been heated debates over possible adverse effects of these radiations on human health and biodiversity.³⁰ It has become important to assess the effect of non-ionising electromagnetic radiation (300MHz to 3GHz) on biota and ecology, using appropriate living organisms as model, of which microorganisms are the easiest to be handled in laboratory. Interest of the research community regarding biological effects of MW radiation is evident from the good number of relevant papers being published in this field in recent times. Identifying the MW frequencies which are most likely to interact with biological molecules will not only help in a meaningful assessment of the possible biological effects, but also may pave way for development of effective MW mutagenesis programs for industrial strain improvement.

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