



Effect of Microwave Radiation on Growth, Enzyme Activity (Amylase and Pectinase), and/or Exopolysaccharide Production in *Bacillus subtilis*, *Streptococcus mutans*, *Xanthomonas campestris* and *Pectobacterium carotovora*

Preemada Kushwah¹, Toshi Mishra¹ and Vijay Kothari^{1*}

¹*Institute of Science, Nirma University, Ahmedabad, India.*

Authors' contributions

This work was carried out in collaboration between all authors. Author VK designed the study and managed the analyses of the results. Authors PK and TM performed the statistical analysis, and executed the protocol in lab. All the three authors contributed towards literature searches, and manuscript writing. All authors read and approved the final manuscript.

Research Article

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ABSTRACT

Aim: To investigate effect of microwave (MW) radiation on bacterial growth, enzyme activity (amylase and pectinase), and exopolysaccharide production.

Study Design: The study was designed to investigate effect of MW radiation on bacterial growth, enzyme activity, and exopolysaccharide production. Particularly the non-thermal effects were focused. Thermal effects were avoided (minimized) by keeping the bacterial suspension in ice while exposing to MW radiation.

Place and Duration of Study: Institute of Science, Nirma University, Ahmedabad, India, between November 2012 and May 2013.

Methodology: The present study investigated the effect of MW (90 W) radiation on bacterial growth, enzyme activity (amylase and pectinase), and exopolysaccharide (EPS) production. Test parameters viz. growth, enzyme activity, and EPS production of populations originated from MW treated cells were compared to those originated from untreated control. Thermal effects of MW radiation were avoided (minimized) by placing

*Corresponding author: Email: vijay.kothari@nirmauni.ac.in, vijay23112004@yahoo.co.in;

inoculum vial(s) in a ice containing beaker during MW exposure.

Results: MW treatment was found to be capable of altering bacterial growth, enzyme activity, and EPS production significantly. Amylase activity in *B. subtilis* suffered a heavy loss of 67.43% ($P<0.01$) following 6 min MW exposure. Pectinase activity in MW treated (4 min duration) *B. subtilis* was 169.92 times higher ($P<0.01$) than that of control. MW treatment for 4 min and 6 min duration were able to induce EPS production in *Xanthomonas campestris* by 46.15% ($P<0.01$) and 53.84% ($P<0.05$) respectively.

Conclusion: MW treatment was found to alter growth, enzyme activity, and EPS production significantly in the test bacteria. This study positively suggests existence of non-thermal effects of MW radiation on biological entities. Further investigation on mode of action of these MW specific athermal effects, and on their genetic stability are warranted.

Keywords: Enzyme activity; exopolysaccharide; microwave; non-thermal effect.

DEFINITIONS

MW: Microwave; **EPS:** Exopolysaccharide; **Non-thermal MW effect(s):** The effect(s) of MW radiation which is due to its electromagnetic properties, and not due to its heating (thermal) capacity. These are also designated as MW specific athermal effects.

1. INTRODUCTION

Microwaves (MW) are component of electromagnetic radiation with frequency range of 300 MHz - 300 GHz corresponding to wavelength of 1 mm – 1 m. They can exert two types of effects on biological systems. First is the well established thermal effect, which is largely due to the electric field component of this part of electromagnetic radiation [1]. Second is the controversial non-thermal (also called microwave specific athermal effect) effect, whose occurrence has been a matter of debate since long [2,3]. Reports favouring the existence of non-thermal effect [4-8] as well as those suggesting against its existence [9-11] are available in literature. More research is warranted, particularly on the non-thermal MW effect, whether and how it affects biological entities, its possible mode of action, and whether these effects are heritable.

The present study investigated the effect of MW radiation on bacterial growth, enzyme activity (amylase and pectinase), and exopolysaccharide (EPS) production. Test parameters viz. growth, enzyme activity, and EPS production of populations originated from MW treated cells were compared to those originated from untreated control.

2. METHODOLOGIES

2.1 Test Organisms

Following microbial cultures were procured from Microbial Type Culture Collection (MTCC), Chandigarh: *Bacillus subtilis* (MTCC 619), *Streptococcus mutans* (MTCC 497), *Xanthomonas campestris* (MTCC 2286), and *Pectobacterium carotovora* (MTCC 1428).

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 2, 4, and 6 min. Vials inside the MW oven were placed in a ice containing beaker (100 mL; Borosil®), so as to avoid (minimize) 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 into respective growth media following MW treatment.

2.3 Amylase Activity

Effect of MW on amylase activity was investigated in *B. subtilis* and *S. mutans*. The growth medium containing 1% w/v starch [K_2HPO_4 7 g/L, KH_2PO_4 2 g/L, $(NH_4)_2SO_4$ 1 g/L, sodium citrate 0.5 g/L, $MgSO_4 \cdot 7H_2O$ 0.1 g/L, yeast extract 0.1 g/L, starch powder 10 g/L, pH 6.8] was inoculated with actively growing culture of the test organism. Incubation for 24 h under static condition was carried out at 30°C for *B. subtilis*, and at 35°C for *S. mutans*. Following estimation of growth by measuring optical density (OD) at 625 nm (Spectronic 20D+, Thermo scientific), culture broth was subjected to centrifugation (nüve®, NF 800R) at 7500 rpm for 10 min, and the cell free supernatant (CFS) was used for amylase estimation using the method described by Jayaraman [12]. Briefly, 0.5 mL of phosphate buffer (pH 6) was added into test tubes followed by 0.5 mL of CFS, and 1 mL of starch solution (1% w/v). This mixture was incubated at 50°C for 30 min, followed by addition of 2 mL of DNSA (3,5-dinitrosalicylic acid) reagent, and then kept for 5 min in boiling water bath. Amount of maltose released from starch degradation was estimated by measuring OD at 540 nm. Enzyme activity was calculated as [13]:

Enzyme activity (IU) = Net amount of sugar produced (μ g) / (MW X T)

where, MW= molecular weight of maltose (342.30 g/mol); T = reaction time (30 min).

2.4 Pectinase Activity

Effect of MW on pectinase activity was investigated in *B. subtilis* and *P. carotovora*. The growth medium containing 1% w/v pectin [K_2HPO_4 7 g/L, KH_2PO_4 2 g/L, $(NH_4)_2SO_4$ 1 g/L, sodium citrate 0.5 g/L, $MgSO_4 \cdot 7H_2O$ 0.1 g/L, yeast extract 0.1 g/L, pectin powder 10 g/L, pH 6.8] was inoculated with actively growing culture of the test organism. Incubation for 96 h under static condition was carried out at 30°C. Following estimation of growth by measuring OD at 625 nm, culture broth was subjected to centrifugation at 7500 rpm for 10 min, and the CFS was used for pectinase assay [14]. Briefly, 0.5 mL of CFS was mixed with 0.5 mL of 0.5% pectin solution (prepared in 0.025 M acetate buffer of pH 6), and incubated at 50°C for 10 min. Following incubation, 1 mL DNSA reagent was added in assay tubes, and then kept in boiling water bath for 5 min. Following this, amount of monogalacturonic acid released

from pectin degradation was estimated by measuring OD at 575 nm. Enzyme activity was calculated as:

$$\text{Enzyme activity (IU)} = \text{Net amount of sugar produced } (\mu\text{g}) / (\text{MW} \times \text{T})$$

where, MW= molecular weight of monogalacturonic acid (212.12 g/mol); T = reaction time (10 min).

2.5 EPS Quantification

Influence of MW on EPS production was investigated in *X. campestris*, and *S. mutans*. *X. campestris* was grown in TY broth (tryptone 5 g/L, yeast extract 3 g/L, CaCl₂ 0.7 g/L), whereas *S. mutans* was grown in brain heart infusion (BHI) broth (HiMedia, Mumbai) supplemented with 2% sucrose. Incubation was made for 72 h in shaking (100 rpm) condition, at 28°C and 35°C for *X. campestris* and *S. mutans* respectively. Following estimation of growth by measuring OD at 625 nm, culture broth was subjected to centrifugation at 7500 rpm for 10 min, and the CFS was used for EPS quantification using the method described in Li et al. [15] with some modification. Briefly, 40 mL of chilled acetone (Merck) was added to 20 mL of CFS, and allowed to stand for 30 min. In case of *X. campestris*, the EPS precipitated thus was separated by filtration through pre-weighed Whatman # 1 filter paper (Whatman International Ltd., England). Filter paper was dried at 60°C for 24 h, and weight of EPS on paper was calculated.

In case of *S. mutans*, after mixing chilled acetone with CFS, the precipitated EPS settled down in form of a thin film at the bottom of the pre-weighed flask. The liquid above the EPS film was decanted, and the flask containing EPS film was subjected to drying at 60°C for 24 h. Post-drying weight of the flask was used to calculate the amount of EPS produced.

2.6 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

Growth of *B. subtilis* remained unaffected by MW exposure for all durations (Table 1). However amylase activity suffered a significant decrease after MW exposure for all three test durations. *S. mutans* responded differently to MW treatment than *B. subtilis*. Growth of the former experienced a little enhancement after 2 min treatment, whereas the amylase activity was influenced by 4 min and 6 min duration of MW exposure. Albeit small, but significant increase in amylase activity of *S. mutans* was observed after 4 min MW treatment. Amylase has been among the most important microbial enzymes with wide-ranging applications in baking, brewing, and textiles [16]. In case of both the organisms growth and amylase activity were affected by MW exposure independently, despite starch being the major carbon source in the medium.

Table 1. Effect of MW on growth and amylase activity in *B. subtilis* and *S. mutans*

Duration of MW treatment (min)	Growth (OD ₆₂₅) (Mean ± SD)	% change compared to control	Amylase activity (IU/mL) (Mean ± SD)(X 10 ⁻³)	% change compared to control
<i>B. subtilis</i>				
0 (control)	0.093 ± 0.005	0.00	44.28 ± 1.60	0.00
2	0.086 ± 0.005	-7.52 (P= .23)	23.34 ± 0.00	-47.28 (P= .05)
4	0.086 ± 0.005	-7.52 (P= .23)	34.50 ± 0.44	-22.08 (P= .001)
6	0.100 ± 0.000	7.52 (P= .21)	14.42 ± 0.00	-67.43 (P < .001)
<i>S. mutans</i>				
0 (control)	0.077 ± 0.000	0.00	26.06 ± 0.00	0.00
2	0.079 ± 0.000	2.59 (P= .03)	27.98 ± 0.00	7.36 (P= .06)
4	0.077 ± 0.001	0.00 (P= 1)	28.38 ± 0.00	8.90 (P= .04)
6	0.074 ± 0.000	-3.89 (P= .03)	22.56 ± 0.00	-13.43 (P= .04)

#minus sign indicates a decrease over control.

Table 2. Effect of MW on growth and pectinase activity in *P. carotovora* and *B. subtilis*

Duration of MW treatment (min)	Growth (OD ₆₂₅) (Mean ± SD)	% change compared to control	Pectinase activity (IU/mL) (Mean ± SD) (X 10 ⁻²)	% change compared to control
<i>P. carotovora</i>				
0 (control)	0.483 ± 0.007	0.00	55.08 ± 8.44	0.00
2	0.523 ± 0.021	8.28 (P= .12)	173.56 ± 5.82	215.10 (P= .003)
4	0.367 ± 0.036	-24.01 (P= .04)	30.64 ± 4.30	-44.37 (P= .016)
6	0.488 ± 0.007	1.03 (P= .55)	26.88 ± 0.00	-51.19 (P= .03)
<i>B. subtilis</i>				
0 (control)	0.416 ± 0.017	0.00	1.30 ± 0.00	0.00
2	0.417 ± 0.001	0.24 (P= .94)	0.00 ± 0.00	-100 (P= .03)
4	0.421 ± 0.001	1.20 (P= .72)	220.90 ± 2.06	16892.30 (P < .001)
6	0.416 ± 0.008	0.00 (P= 1)	84.56 ± 2.06	6404.61 (P= .03)

#minus sign indicates a decrease over control.

Pectinase activity was influenced significantly by all durations of MW exposure in both the test organisms, *P. carotovora* and *B. subtilis* (Table 2). MW exposure of 2 min resulted in a significant increase in the pectinase activity in *P. carotovora*, whereas 4 min and 6 min MW exposure almost halved the pectinase activity. Pectinase has been implicated as an important virulence factor for plant pathogens like *P. carotovora* [17]. If a reliable protocol can be developed for attenuation of pectinase activity in such phytopathogens, it can be interesting with respect to control of crop damage by these organisms. In such cases, where MW radiation reduces 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* was indicated by Craciun et al. [18].

Though the pectinase activity in *B. subtilis* was heavily influenced by MW treatment, its growth remained unaffected. Inability of MW radiation to cause any change in growth of *B. subtilis* was observed while growing this organism in both pectin containing medium (Table 2), as well as in starch containing medium (Table 1). Previously also, while investigating

influence of MW on multiple prokaryotic and eukaryotic microorganisms, we found MW radiation unable to influence growth of *B. subtilis* [7]. Tahir et al. [19] also reported that viability of *B. subtilis* remained unaffected by short MW exposures in controlled temperature experiments. Neither colony morphology nor cell shape was altered due to MW exposure for 60-180 s.

It is interesting to note that MW treatment for the same duration had quite opposite impact on the pectinase activity of *B. subtilis* and *P. carotovora*. The former completely lost its pectinase activity after 2 min MW exposure (it may be due to effect of MW radiation on gene(s) involved in synthesis and/or export of pectinase), the latter experienced a heavy increase (215.10%) in the same (Table 2). MW exposure of 4 and 6 min caused a significant decrease in the pectinase activity of *P. carotovora*, whereas same duration of MW exposures caused a significant increase in the pectinase activity of *B. subtilis*. MW exposure of 4 min and 6 min duration caused a 169.92 times and 65.04 times increase (as compared to control) respectively in the pectinase activity of *B. subtilis*. Pectinases are enzymes of commercial importance, particularly for depectinization in fruit juice industry [20].

MW treatment for all the durations was able to cause a significant increase in growth of both the EPS producers, *X. campestris* and *S. mutans* (Table 3). A 2 min MW exposure to *S. mutans* resulted in a significant decrease (23.48%) in EPS production by *S. mutans*, whereas the same duration of MW treatment had no significant effect on EPS production by *X. campestris*. Similarly, MW treatments of 4 and 6 min duration significantly enhanced EPS production by *X. campestris*, but had no effect on EPS production by *S. mutans*. This variation in response of different microbial species to MW radiation is likely to be due to the fact that different species have varying susceptibilities to MW radiation [21]. There seemed to be no correlation between effect of MW on EPS production, and that on growth i. e. growth and EPS production were affected by MW treatment independent of each other. Enhancement of microbial polysaccharide production by MW treatment or any other method can be of considerable industrial interest, as they have found multiple applications in fields including food, pharmaceutical, medical (e.g. development of vaccines), cosmetics, etc. [22]. EPS (xanthan gum) produced by *X. campestris* has been a product of commercial importance since long. It is useful as a packaging material, in tooth-pastes, in oil well drilling, and has a market value of approximately US\$ 14 per kg [23].

Response of *S. mutans* to MW treatment was not the same, when grown in two different media. MW exposure of 4 min was not able to induce any change in growth of *S. mutans* in starch containing medium (Table 1), whereas this exposure of 4 min heavily induced its growth in sucrose containing BHI (Table 3). Medium composition may not necessarily have a role in this, because effect of MW radiation on microbial cells can be random, as can happen with ultraviolet radiation or any other part of electromagnetic spectrum. The possibility of non-uniform distribution of MW radiation in different layers of irradiated microbial suspension can be the major reason for random effect of MW (which may be difficult to reproduce) on given test organism even when experiments on non-thermal effects are performed under identical conditions [24].

Table 3. Effect of MW on growth and EPS production by *X. campestris* and *S. mutans*

Duration of MW treatment (min)	Growth (OD ₆₂₅) (Mean ± SD)	% change compared to control	Amount of EPS produced (g/L) (Mean ± SD)	% change compared to control
<i>X. campestris</i>				
0 (control)	0.735 ± 0.007	0.00	0.52 ± 0.04	0.00
2	0.775 ± 0.007	5.44 (P= .02)	0.56 ± 0.00	7.69 (P= .49)
4	0.805 ± 0.007	9.52 (P= .01)	0.76 ± 0.00	46.15 (P= .004)
6	0.815 ± 0.007	10.88 (P= .007)	0.80 ± 0.04	53.84 (P= .03)
<i>S. mutans</i>				
0 (control)	0.730 ± 0.024	0.00	6.60 ± 0.20	0.00
2	0.870 ± 0.005	19.17 (P= .004)	5.05 ± 0.10	-23.48 (P= .014)
4	1.790 ± 0.014	145.20 (P < .001)	7.85 ± 0.70	18.93 (P= .12)
6	0.830 ± 0.007	13.69 (P= .012)	6.35 ± 1.25	-3.78 (P= .80)

[#]minus sign indicates a decrease over control

Increase in enzyme activity following MW treatment may be due to direct effect of MW radiation on genetic or cellular machinery of the test organism, and this effect may or may not be heritable. However, increased enzyme activity, particularly in case of extracellular enzymes (like those investigated in this study) may not necessarily be due to genetic effect of MW radiation. It may be due to increased secretion of extracellular enzymes, as MW are known to alter membrane permeability in both gram-positive and gram-negative bacteria [25]. Reports dealing with the biological effects of low-level MW radiation, which did not produce significant thermal induction, suggest that molecular or membrane interaction of MW with living systems can lead to alteration of function [26]. While comparing effects of sub-lethal MW radiation and conventional heating on the metabolic activity of *Staphylococcus aureus*, Dreyfuss and Chipley [4] reported increase in specific activities of many key enzyme systems due to MW radiation, which could not be explained solely by thermal effects. Ability of MW irradiation to increase the enzymatic activity of bacterial suspensions has also been demonstrated in members of the family Enterobacteriaceae [27].

4. CONCLUSION

Results of present study indicate the possibility of the existence of MW specific athermal effects on microbial systems. However, such studies (either in favour of non-thermal MW effects or otherwise) at present can only add fuel to the controversy over non-thermal effects of MW radiation rather than settling it. For this controversy to end, it is required to have enough data about impact of microwaves of varying frequencies and power for different time durations on both prokaryotic and eukaryotic biological systems. Such data can be of help in deciphering the mechanism of MW specific effects on cells and their biomolecules. Due to the ease of handling them in laboratory, microorganisms can be conveniently used to study the effect of MW on living systems, and further probing into the underlying mode of their action. Whether the MW effects are heritable, also remains an interesting problem to investigate. The MW-induced effects observed in the present study were measured not directly on the cells which received the MW treatment, but on their daughter cells obtained after inoculation of MW-treated cells in appropriate growth media, allowing for transfer of MW-induced changes into next generation(s). Identifying mutagenic frequencies of MW radiation, and employing them for microbial strain improvement can be of considerable industrial significance.

CONSENT

Not applicable.

ETHICAL APPROVAL

Not applicable.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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