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Abstract
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Keywords
c, radiation, enzymatic, oxidase, influence, activity, escherichia, 18ghz, coli, lactate, microwave, dehydrogenase, cytochrome

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Influence of 18 GHz Microwave Radiation on the Enzymatic Activity of *Escherichia Coli* Lactate Dehydrogenase and Cytochrome c Oxidase

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Abstract: The catalytic activity of two common bacterial enzymes, lactate dehydrogenase (LDH) and cytochrome c oxidase (COX) from *Escherichia coli* was examined following bacterial exposure to microwave (MW) radiation under well-defined experimental conditions. The experiments were conducted in a specialized microwave processing apparatus, with an exposure frequency of 18 GHz, and a temperature profile that was restricted to below 40 °C to avoid thermal degradation of the bacteria. The absorbed power was calculated to be 1,500 kW/m^3 and the electric field was determined to be 300 V/m. Both values were theoretically confirmed using Computer Simulation Technology (CST) Microwave Studio 3D Electromagnetic Stimulation Software. Results showed that the activity of both enzymes was increased following MW radiation compared to negative controls and thermally treated samples subjected to similar temperature profiles. It is suggested that the increase in COX and LDH enzyme activity could not be explained by conventional heating alone, but was rather a result of micro-thermal effects that incorporated 'undetectable' thermal mechanisms.

Key words: Non-thermal, microwave radiation, specific microwave effects, enzyme kinetics, cytochrome c oxidase, lactate dehydrogenase, instantaneous temperature.

1. Introduction

The interaction of microwave (MW) radiation with biological material is an ongoing topic of interest. While the thermal effects of this interaction have generally been well defined ("Thermal" is here used to denote bulk temperature changes, as opposed to micro-thermal changes), the majority of discrepant scientific findings concern the existence of what have been termed "specific MW effects", or effects that are reportedly independent of thermal mechanisms.

The effects of MW radiation on cells have been studied by evaluating the interaction of the electromagnetic radiation with enzymes [1-5]. Perhaps the most comprehensive study exploring this interaction is by Dreyfuss and Chipley [2], which examined the effects of 2.45 GHz MW radiation at sub-lethal temperatures on the metabolic activity of a range of enzymes expressed in *Staphylococcus aureus*. That study reported a change in the activities of malate dehydrogenase, α-ketoglutarate dehydrogenase, cytochrome c oxidase (COX), cytoplasmic adenosine triphosphatase and glucose-6-phosphate dehydrogenase. Other enzymes such as lactate dehydrogenase (LDH) and alkaline phosphatase were found to be unaffected by MW radiation [2].

Another study focussing on enzyme kinetics reported the inactivation and denaturation of two

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thermophilic enzymes (S-adenosylhomocysteine hydrolase (AdoHcy) and 5P-methylthioadenosine phosphorylase (MTA)) under non-thermal MW settings at a frequency of 10.4 GHz [3]. The study also reported that the effect of the electromagnetic field on enzyme kinetics did not depend on enzymatic concentration. Given that both enzymes have previously been shown to be stable at the experimental temperatures, the irreversible denaturation and loss of activity for both enzymes as shown by fluorescence and circular dichroism were also described as non-thermal.

Continuous wave MW radiation at 2.45 GHz was used by Sun et al. [4] to determine its effect on the kinetics of the hydrolysis of adenosine triphosphate (ATP) to adenosine diphosphate (ADP). MW radiation was able to induce a 91% hydrolysis of ATP in a 4-min exposure, compared to only 6.9% with conventional heating. Similar results were found when examining the hydrolysis of uridine 5'-triphosphate (UTP), cytosine 5'-triphosphate (CTP), guanosine 5'-triphosphate (GTP) and formycin 5'-triphosphate (FoATP), which all exhibited a much higher rate of hydrolysis compared to controls.

The effect of MW radiation on enzyme catalysis (using Cutinase) in an organic media of ethyl butyrate and butanol has also been examined [6]. An organic media was used in order to measure changes to the enzyme bound water content as a function of thermodynamic water activity (a_w). Given that the solvent and enzyme water content were equilibrated to the same a_w, the effect of MW radiation at 2.45 GHz on enzyme activity as a function of hydration was determined. The study reported that for a_w of 0.58 and 0.69 (a_w of pure water = 1.0), and temperatures of 50 °C and 60 °C, reaction rates were 2-3 fold greater for MW radiated samples compared to conventionally heated controls. At equal a_w levels and at 70 °C, the observed reaction rates were reversed. Furthermore, increasing the a_w to 0.97 while maintaining the temperature at 60 °C, also resulted in a reversed reaction rate, with conventional heating delivering higher reaction rates (2-3 fold) than MW radiation.

Enzymes are sensitive to even slight alterations in environmental conditions. Therefore, measureable changes in enzyme activity following MW processing can be used to provide feedback regarding the MW-cell interaction. Furthermore, there are a number of established factors that influence enzyme activity. For example it is generally accepted that the specific rate constant of an enzyme increases with increasing temperature [7, 8]. At temperatures closer to the thermal degradation point of an organism, the enzyme starts to denature, thereby decreasing the amount of the active enzyme configuration, eventually leading to a complete loss of activity [7-10].

The second critical factor that affects enzyme activity is pH. The effect of acid (H^+) ions or basic (OH^-) ions on the activity of an enzyme is thought to be caused by a change in stereo configuration at or around the active sites [7]. Furthermore, ionogenic groups in an enzyme's active site are only able to provide for catalytic activity with a certain state of protonation. In this case, catalysis depends on the concentration of the enzyme active form and thus on pH of the medium [11]. Given that the physical properties of enzymes have been shown to vary depending on the hydration state of the protein, the thermodynamic water activity (a_w) has also been recognized as a key parameter which determines enzymatic activity [6, 12-14]. While most enzymes require relatively high water activity ($a_w > 0.7$) to achieve high catalytic rates [12], evidence suggests that enzymes such as lipases respond differently to increasing water activity. Some show activity optima at low a_w, some at high a_w and others have intermediate profiles with broader optima [12]. Therefore, the right combination of conditions, including temperature, pH and water activity are critical in order to derive high catalytic yields.

While there is no evidence that MW radiation can affect pH directly, it is well-established that changes in environmental conditions can induce bacterial cells to

alter the pH of their medium [15]. Therefore, examining the possibility that MW effects may be related to such a change in pH is warranted.

This is particularly important given that inconsistencies in enzyme activity levels following MW radiation, especially those reported to be "specific MW effects" are common and have generally been attributed to differences in experimental setups, such as MW frequency, absorbed power and the duration of exposure [16, 17]. In addition, the nature of the interaction of MW radiation at sub lethal temperatures with biomaterial has not been fully explored and the mechanisms of MW action are not wholly understood.

Furthermore, 18 GHz MW radiation has been previously demonstrated to have specific MW effects on bacteria cells and cellular components, with the mechanisms responsible for these not clearly understood. These include the formation of temporary pores within the *E. coli* cell membrane [17] and bacterial inactivation at sub-lethal temperatures [16, 18]. The aim of the present study was therefore to explore and interpret some of the specific MW effects reported to occur at a frequency of 18 GHz, using the activity of two enzymes (COX and LDH) from *Escherichia coli*. Changes in pH, water activity and temperature, as a result of MW radiation, were also monitored and interpreted. Precise modeling and theoretical determination of the specialized MW processing system as well as previously defined MW-sample interactions were used.

2. Experimental Setup

2.1 Procedure

Nine samples of *Escherichia coli* were randomly allocated to three treatment groups: a no-treatment control (Negative Control), a MW treated group (MW Treatment) and a conventionally heated positive control (Thermal Treatment) (Fig. 1). The pH and a_w measures were obtained before and after each of the three treatments. LDH and COX enzyme activity assays were used following each of the three treatments.

2.2 Bacterial Strain, Cultivation Procedure and Sample Preparation

E. coli ATCC 15034 was used as the test strain in all experiments. The bacterium was obtained from the American Type Culture Collection (Manassas, VA, USA). Pure cultures were stored at -80 °C in nutrient broth (NB) (Oxoid, Catalogue number: CM0067, Thermo Fisher Scientific, Melbourne, Australia) supplemented with 20% (v/v) of glycerol. The bacteria were routinely cultivated for 24 h on nutrient agar (NA), (Oxoid, Catalogue number: CM0003, Thermo Fisher Scientific). Working bacterial suspensions were freshly prepared for each independent experiment as described elsewhere [16, 18]. In brief, the cell density was adjusted to $OD_{600} = 0.8 \times 10^8$ colony forming units (cfu) per mL in phosphate buffered saline (PBS), 10 mM, pH = 7.4, using a spectrophotometer (GeneQuant Pro;

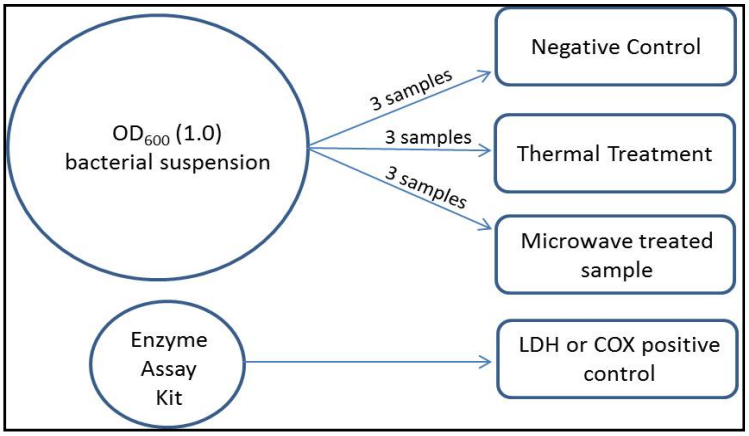

Fig. 1 Outline of the experimental setup.

model number: 80-2114-98, Amersham Biosciences, Buckinghamshire, England) from a bacterial culture grown overnight in 100 mL in NB. Bacterial cells were collected during the logarithmic phase of growth as confirmed by growth curves (data not shown). The bacterial cell suspensions were further subjected to direct counting using a haemocytometer to confirm the number of bacterial cells for each used strain in experiments [19]. Bacterial samples for MW analysis comprised of 2 mL of working suspensions that were transferred into a 35 mm × 10 mm plastic cell culture dish (Catalogue number: 627960, Greiner bio-one; Monroe, NC, USA).

2.3 Microwave Apparatus

The MW apparatus that was used in the present study had the option of a variable frequency ranging from 5 GHz to 18 GHz (Vari-Wave Model LT 1500, Lambda Technologies; Morrisville, NC, USA). The LT1500 is a computer controlled variable-frequency processing cavity for delivering precise levels of control and uniformity of energy distribution into a multi-mode microwave cavity. A schematic diagram of the MW apparatus setting was reported elsewhere [16]. Both the amplitude and frequency of the microwave power can be varied, allowing a significant expansion of the parameter space within which the system can be optimized. A data logging option allowed processed data capture (temperature, frequency, incident and reflected power) from the embedded computer system, over a standard RS-232-C serial interface (Lambda Technologies). A cavity characterization option was also available which allowed evaluation of the performance of a material in the cavity to assist in determining the optimum processing conditions.

2.4 Microwave Settings

All experiments were completed at a frequency of 18 GHz. Each bacterial sample was transferred into the MW chamber, which had its core temperature monitored and recorded in real-time through the attachment of a fibre optic probe. In order to minimize thermal MW effects (dielectric heating), the bulk temperature rise of the bacterial suspension during exposure was maintained below 40 °C as that is the temperature at which the bacteria were determined to be unaffected by heat [16]. Following MW processing, the sample was removed from the chamber and had its temperature immediately re-measured using an infrared temperature sensor to confirm that the temperature did not exceed 40 °C.

For uniformity of exposure, each sample was placed onto a ceramic pedestal (Pacific Ceramics Inc, Sunnyvale, CA, USA, PD160, $\varepsilon' = 160$, loss tangent < 10^{-3}) within the same region of the chamber that had been determined by electric field modeling to have the most even distribution of MW radiation using CST Microwave Studio 3D Electromagnetic Stimulation Software (CST Computer Simulation Technology, Oxley, QLD, Australia) (Fig. 2).

By examining the distribution of MW power and electric field throughout the sample, the distribution of heat was also evaluated. The experimental setup for MW treatment involved placing the sample in the predetermined location within the MW chamber and subjecting it to the specified radiation treatment at a heating rate of 20 °C/min for 1 min, thereby maintaining the temperature rise from 20 °C to 40 °C.

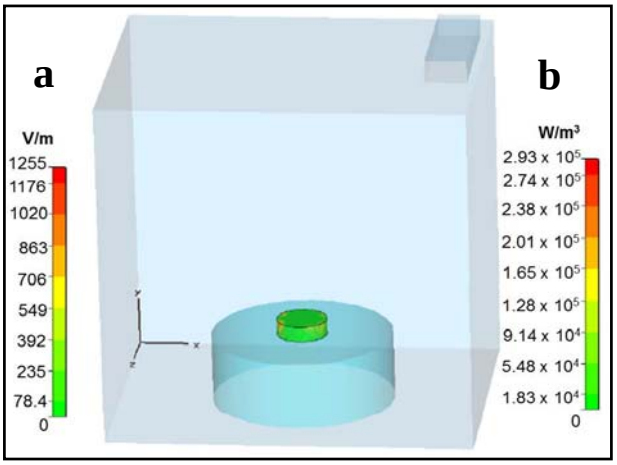

Fig. 2 Modelling of (a) electric field (V/m) and (b) absorbed power (W/m^3) is shown using CST Microwave Studio 3D Electromagnetic Simulation Software.

In order to maximize the MW effect, a previously optimized repeated exposure technique was employed [16, 18]. Each sample was exposed to MW radiation for three consecutive exposures allowing the sample to cool to 20 °C on ice (at a rate of 10 °C/min), in between exposures. The temperature profile (by time) is illustrated in Fig. 3. Modeling of the theoretical distribution of electric field and absorbed MW power at similar experimental parameters has been included in our previous study [17]. That work also determined that the dielectric constant and specific heat of the bacterial suspension would be the same as that of water given that the dielectric loss factor and the rate of change in temperature of the bacterial sample would be lower than for the surrounding medium. Calculations of the depth of penetration of MW radiation also led to the conclusions that the temperature inside the cell would be equivalent to that of the surrounding medium [17].

2.5 Conventionally Heated Positive Control (Thermal Treatment)

In order to ensure that any caused effects by MW radiation were not purely a result of conventional heating, a positive control was developed. A Peltier plate heating/cooling system (Model AR-G2, TA Instruments; Melbourne, VIC, Australia) was used to replicate the obtained temperature profiles during MW processing. A total volume of 2 mL of working bacterial suspension was spread across the Peltier plate and subjected to heating from 20 °C to 40 °C at a rate of 20 °C/min for three consecutive exposures, with identical cooling times as for MW treatment (at a rate of 10 °C/min) in between trials (Fig. 3).

Ensuring that bacterial suspension was spread across the entire surface of the Peltier plate increased surface area and allowed for an even distribution of heat. All experiments using MW treated samples were run in parallel with Peltier plate heated samples.

2.6 Determination of pH and Water Activity

The pH was measured using a pH meter (Model number: 901-pH, TPS; Brisbane, QLD, Australia) and the water activity was determined using a water activity meter (Decagon Pa$_w$kit Water Activity Meter, Graintec Scientific; Toowoomba, QLD, Australia). The pH and a_w were both measured at 20 °C. Measurements were taken prior to and following each experiment, as well as for the PBS solution.

2.7 Lactate Dehydrogenase Assay

Changes in LDH activity were detected using an enzyme assay kit (In *Vitro* Toxicology Assay Kit, lactate dehydrogenase based, Cat number: TOX7, Sigma-Aldrich; St. Louis, MO, USA). This assay is based on the reduction of nicotinamide adenine dinucleotide (NAD) by LDH. The reduced NAD (NADH) is utilized in the stoichiometric conversion of

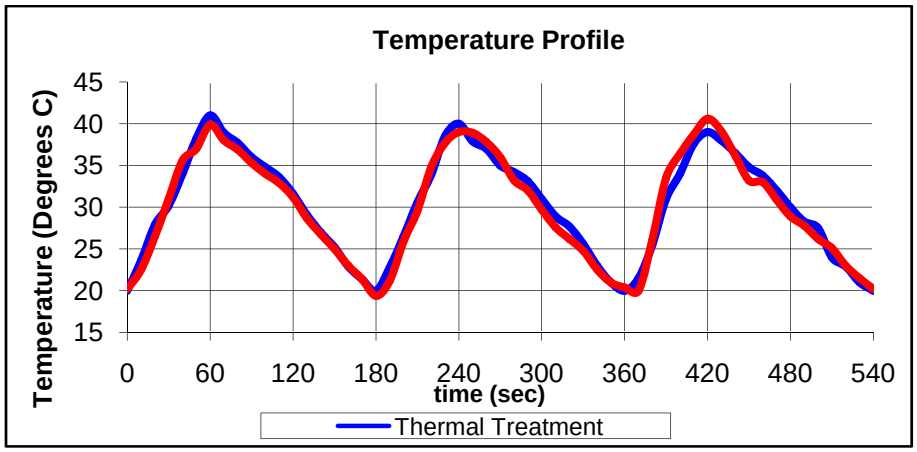

Fig. 3 Temperature profile of the 18 GHz microwave heating system and Peltier plate heating system (with subsequent ice cooling).

a tetrazolium dye. The ensuing coloured compound is then measured spectrophometrically. All treatment group samples as well as the LDH positive controls were processed simultaneously. The provided LDH positive control in the assay kit was used only to test whether the enzyme reaction was taking place.

Assay reagents were prepared and stored as per manufacturer's specifications. Following each treatment (and for the LDH positive control), volumes of 1.8 mL of the treated bacterial suspension were mixed with 200 µL of LDH lysis buffer and incubated at 37 °C for 45 min. The samples were then centrifuged at 250 g for 4 min to pellet the cells, mixed with the LDH assay at a ratio of 1:2 and incubated for 30 min. The reaction was then stopped by adding 150 µL of 1 M HCL and the absorbance was read at 490 nm every 30 sec for 10 min. Enzyme absorbance was then converted into Units/mL. Given that all samples were prepared from the same bacterial suspension and that pre-treatment conditions were matching, the initial quantity of active enzymes (and their activity) was assumed to be identical.

2.8 Cytochrome c Oxidase Assay

Enzyme activity was determined using an enzyme assay kit (cytochrome c oxidase Assay Kit, Cat number: CYTOCOX1, Sigma-Aldrich). The colorimetric assay is based on the observation of the decrease in absorbance at 550 nm of ferrocytochrome c caused by its oxidation to ferricytochrome c by cytochrome c oxidase. All treatment group samples as well as the COX positive controls were processed simultaneously (Fig. 1). The COX positive control provided in the assay kit was used only to determine whether the enzyme reaction was taking place. Assay reagents were prepared and stored as per manufacturer's specifications.

Following each experimental treatment, bacterial samples (in 2 mL aliquots) were transferred into centrifuge tubes and lysed as per the LDH assay protocol. The pelleted bacterial suspensions were then freeze-dried overnight in order to concentrate the target enzyme. Each sample was then resuspended in 50 µL of ultrapure water (18.2 Ω·cm).

Preparation of samples for spectrophotometric analysis was completed as per manufacturer's specifications. All samples were stored on ice prior to analysis and processed sequentially. Cytochrome c oxidase activity for each sample was then calculated from the obtained data using spectrophotometric analysis. Each experiment was performed in triplicate. Enzyme absorbance was then converted into Units/mL.

2.9 Statistical Analyses

The measurement of pH and a_w was included to test whether the treatments had a biologically meaningful effect on these (that in turn may have affected LDH and COX activity levels). As changes of at least 0.1 [7] and 0.05 [12-13] are required to produce biologically meaningful effects for pH and a_w respectively, the authors tested whether the range of each of pH and a_w were less than these values. The LDH and COX positive controls were not enzymes derived from *E. coli*, hence their pH and a_w levels were not analyzed. The two enzyme assays had four treatment conditions, each of which was repeated three times. All initial obtained values were averaged, and the average for each sample was used as the data inputted into the statistical analysis.

The effects of MW treatment and heat treatment were defined as ratios relative to the Negative Control (i.e., MW Treatment/Negative Control, and Heat Treatment/Negative Control for the MW and conventional heating conditions respectively). As the small number of samples did not provide enough data to assume normality, non-parametric independent groups tests were performed (Wilcoxon's Rank Sum), comparing the above two ratios.

3. Experimental Results

3.1 pH and Water Activity Analysis

The pH and water activity (a_w) of the Negative

Control was 7.41 and 0.95, respectively. Since the range of the values of pH and a_w were 0.03 and 0.02, respectively, it was concluded that these changes were too small to impact the LDH and COX results.

3.2 Enzyme Activity Analysis

Catalytic activity of COX and LDH in *E. coli* are shown in Fig. 4. As shown in Table 1, both enzymes exhibited significantly higher activity levels for MW treated samples compared to conventionally heated controls, with a 3.6 fold increase for COX ($Z(6) = 1.96$, $p < 0.05$) and 2.4 fold increase for LDH ($Z(6) = 1.96$, $p < 0.05$).

The findings in the present study are partially consistent with previously reported data [2] showing that the enzyme activity of LDH was higher following MW radiation (2.81 U/mg) compared to conventionally heated controls (1.50 U/mg). It is however noted that in that study the negative control had higher LDH activity level than either MW or conventionally heated controls (2.89 U/mg). Dreyfuss and Chipley [2] also reported changes in the activity of several other enzymes including COX, but no change in activity was found for glucose-6-phosphate dehydrogenase. While no interpretation was given for these findings, it is likely that such a difference in results is due to a poor experimental setup: no modeling was performed to investigate the distribution of the electrical field throughout the MW chamber, and thus could not eliminate possible hotspot formations throughout the sample. Hotspots are thermal irregularities that arise as a result of uneven field distribution throughout a sample. The omission of such an analysis would lead to uneven temperature distribution with time and thus affect the catalytic activity of enzymes.

Furthermore, conventionally heated controls were submerged in a 100 °C water bath in order to reach similar temperature profiles obtained with MW processing [2]. No stirring mechanisms were adopted and thus uneven heating would have occurred. Also, cells in close proximity to the water bath would be inactivated or damaged due to contact with very high temperatures. Hence the heating mechanism adopted in that study did not achieve similar processing to MW radiation and would have led to unpredictable effects on enzyme kinetics.

In the present study, each sample was prepared from the same bacterial suspension which contained approximately 0.8×10^8 cfu/mL. Therefore, the enzyme concentration of samples and controls was assumed to

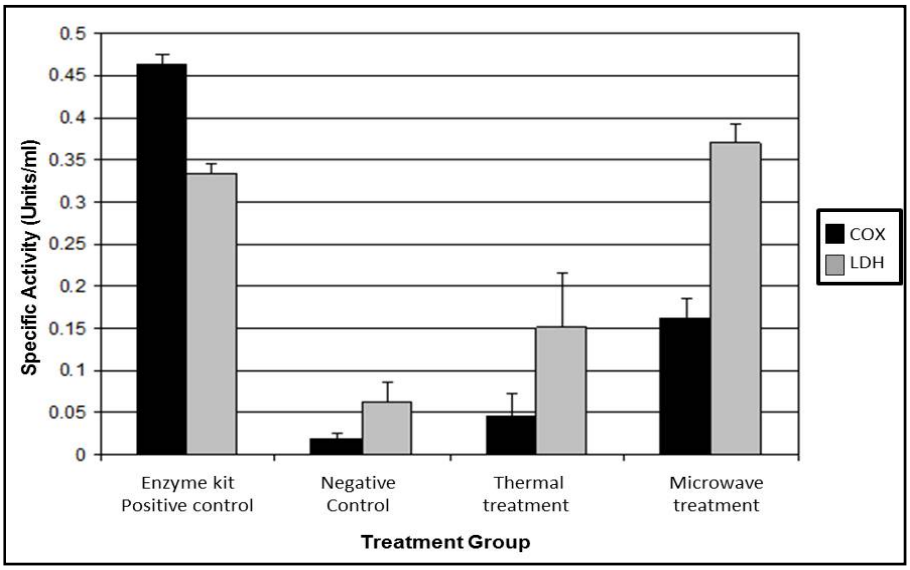

Fig. 4 Enzyme activity of cytochrome c oxidase and lactate dehydrogenase after repeated treatment with either conventional or microwave heating and subsequent cooling. Also shown are the no treatment (Negative Control) and Positive Control conditions.

Table 1 Enzymatic activity of cytochrome c oxidase and lactate dehydrogenase following different treatment condition.

Enzyme	Enzyme activity (Units/mL)				Ratios	
	Positive* control	Negative control	Thermal	MW	MW/negative control	CH/negative control
COX	0.463 ±0.008	0.019 ±0.006	0.045 ±0.025	0.162 ±0.017	8.53 ±3.65	2.37 ±0.58
LDH	0.333 ±0.012	0.063 ±0.018	0.152 ±0.028	0.371 ±0.024	5.89 ±1.62	2.41 ±0.89
PBS solution						
pH	7.41 ±0.01	7.40 ±0.02	7.43 ±0.00	7.41 ±0.01	-	-
a_w	0.96 ±0.01	0.96 ±0.02	0.97 ±0.01	0.96 ±0.01	-	-

*The positive control is only included as a reference.

be identical. Furthermore, the temperature profiles were kept constant for the MW radiated samples and conventionally heated controls (Fig. 3), and the pH and water activity levels which measured prior and post treatment and were determined to be unchanged (Table 1). Therefore, it can be assumed that increased the enzyme activity found for MW treated samples could only be attributed to direct electromagnetic interaction (or the interaction of MW radiation with dielectric heating).

4. General Discussion

Other than Bohr and Bohr [1], who suggested that the application of non-thermal external electromagnetic radiation close to the frequency of an intrinsic mode of a protein can lead to an enhancement of kinetics, very little supportive evidence is available suggesting that MW radiation alters enzyme kinetics using heat independent mechanisms. However, based on experimental data in the field of organic chemistry [20], it has been found that microwave-enhanced chemical and kinetic reaction rates are significantly faster compared to those using conventional heating methods. The explanation for this phenomenon can be found by revisiting the definition of temperature and energy transfer to a sample. In conventionally heated reactions, energy is driven from the heat source to the sample until the energy difference is eliminated, thereby bringing the heat source-sample system to an equilibrium. In conventionally heated reactions, this temperature is a bulk temperature (T_B). Energy transfer using MW radiation transpires as a direct interaction of the sample with the electromagnetic field occurring at an extremely fast rate of less than 10^{-9} sec (at 2.45 GHz) [21]. The reactant molecules of the sample receive MW energy at a higher rate than can be dissipated, thus creating a state of non-equilibrium. This state leads to a high instantaneous temperature (T_i) of the reactant molecules. The T_i is a function of MW power input and is not measurable directly due to its short existence and molecular nature, but is much greater than the bulk temperature (T_B) of the system, T_B ($T_i > T_B$), to satisfy the Arrhenius equation ($k = Ae^{-Ea/RT}$). Thus, the greater the intensity of MW power being administered to a reaction, the higher and more consistent T_i will be, which leads to a greater rate of enzyme catalysis at the same T_B.

This hypothesis challenges available literature that suggests that specific MW effects are non-thermal in nature. Rather, it appears that a currently undetectable thermal force may be at play that is not directly comparable to conventional heating. Future work which focused on delineating the bio-effects of MW radiation would require the development of novel control systems to accommodate instantaneous temperature principles.

5. Conclusions

The present study examined the effects of MW radiation at 18 GHz, on the kinetic activity of LDH and

COX in *E. coli*. Results indicated that MW radiation increased the activity of both enzymes to a greater rate than conventional heating alone. It can be proposed that the MW effects may be the result of micro-thermal heating that is importantly different to conventional heating. The development of novel control and detection systems would be required to test this hypothesis.

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