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A demonstration of athermal effects of continuous microwave irradiation on the growth and antibiotic sensitivity of *Pseudomonas aeruginosa* PAO1

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Abstract:

Stress, caused by exposure to microwaves (2.45GHz) at constant temperature (37± 0.5°C), alters the growth profile of *Pseudomonas aeruginosa* PAO1. In the absence of microwave treatment a simple, highly reproducible growth curve was observed over 24 hours or more. Microwave treatment caused no reduction in growth during the first 6 hours, but at a later stage (>12hours) the growth was markedly different to the controls. Secondary growth, typical of the presence of persisters clearly became apparent, as judged by both the dissolved oxygen and the cell density profiles. These treated cells showed distinct morphological changes, but on re-growth these cells reverted to normal. The Microwave Induced Persisters were subject to antibiotic challenge (tobramycin) and showed increased sensitivity when compared to the un-stressed planktonic cells. This is in marked contrast to antibiotic induced persisters which show increased resistance. This provides evidence for both a non-thermal effect of microwaves and a previously undescribed route to a novel form of antibiotic susceptible persister cells.

Key words: *Pseudomonas aeruginosa* PAO1, microwave, 2.45GHz, constant temperature, persisters.

Abbreviations: MIPs: Microwave Induces persisters.

1. Introduction

1.1 Microwave effects on bacteria

Microwave heating has been extensively used for pasteurisation and sterilisation[1, 2]. It is well known that microwaves used in this way adversely affect the growth of bacteria, but this is usually ascribed solely to a thermal effect[1, 3, 4]. However, there is a continuing interest in exploring a possible non-thermal or "microwave component" particularly for any sub-lethal effects. Unfortunately, it has proved remarkably difficult to come to any firm conclusion, not least because of the practical difficulties of such experiments[5-8].

The argument for a "microwave effect" has been much better rehearsed in the attempts to understand the well-known microwave acceleration of chemical reactions[9-11]. Again, no entirely satisfactory conclusion has yet been reached. At the moment the balance of opinion favours understanding the effect of microwaves as a purely thermal phenomenon, with changes in reaction rate coming from differences in heat transfer. Indeed, even for chemical reactions it is very difficult to critically compare different experiments as they have been carried out under a wide range of conditions and are often reported in insufficient detail to allow reliable replication. However, there a few claims that microwaves affect the activation energy for a chemical reaction in way not easily attributable to purely thermal effects as predicted by the Arrhenius equation[12-15]. Interestingly, there are claims that microwaves do affect the rate of enzyme catalysed conducted athermally *in vitro* [16-19] which could have consequences for viability.

1.2 Persister cells

Persisters are a sub-population of cells which survive a variety of adverse conditions. They represent a small fraction (0.1-1 %) of the bacterial population and increase significantly

when the cell culture enter stationary phase [20]. The vast majority of studies on bacteria refer to persisters arising from bactericidal doses of an antibiotic and this is of particular importance in the aetiology of drug-resistant microbial infections. [21, 22]. Persisters are phenotypic, not genotypic, variants of the cell population and hence their drug resistance is non-heritable. They will give rise to a new population that is identical to the original one and equally sensitive to the antibiotic [23]. However it is now recognised that persisters themselves are not uniform and need to be considered stochastically to understand their phenotypic heterogeneity and the stability of cellular proliferation [24]. It is thought that persisters are selected for their resistance to stress through a period of dormancy in the early stage of population growth, coupled with a low growth rate, though the detailed mechanism is still subject of much discussion. A variety of effects at both environmental and cellular levels have been considered[20, 22, 23].

This study has been undertaken here whereby conditions are well defined. This is part of a programme aimed at examining the effects of microwaves on microbial growth. In this case the effect of continuous exposure to constant (non-pulsed) microwave field (2.45GHz) on a series of batch fermentations of *Pseudomonas aeruginosa* (*P. aeruginosa*) PAO1 have been examined. No attempt was made to use other frequencies. In an attempt to decouple any thermal effect from any microwave induced changes particular attention was paid to operating at constant bulk temperature ($37\pm0.5\ ^{o}C$) with other conditions being typical for the growth of this organism. The scale of the experiments (2 litres working volume) was such that, with stirring, bulk averaging ensured the homogeneity of the treatment of the contents, a feature lacking in many smaller scale experiments [25]. Here we report that non-thermal stress caused by continuous exposure of *P. aeruginosa* PAO1 to microwave (100 W)

at constant temperature causes the appearance of persisters. These cells demonstrate increased susceptibility to tobramycin, the aminoglycoside antibiotic typically used to eliminate *Pseudomonas* infections [26].

2. Materials and methods

2.1 Organism

A pure culture of *P. aeruginosa* PAO1 was stored in a bacterial preservation kit (Technical Service Consultants Ltd., Lancashire) at -80^0 C.

2.2 Culture media and fermentation

The *P. aeruginosa* PAO1 strain was maintained on nutrient agar (CM0309, Oxoid) at 37^0 C. Cultures were grown overnight in 50 ml of sterile nutrient broth (CM001, Oxoid) at 37^0 C in an orbital shaker (200 rpm). The main fermentation was carried out in an autoclavable 3 litre fermenter, (e-z controller, Applikon Biotechnology, UK) equipped with stirrer, heating jacket, cooling loop, condenser, pH, temperature and dissolved oxygen (DO) probes. Cooling was available by circulation of a coolant through the loop at 15^{o}C with the coolant maintained at that temperature by means of a Huber cooler (Polystat Control). Cell density was monitored on line using the non-invasive biomass monitor, "BugLab" (BugLab, LLC Ca). The monitor was calibrated in arbitrary units according to the manufacturer's instructions. Figure 1$_{a, b}$ shows the equipment.

In a typical experiment the bioreactor containing 2 litres of nutrient broth (Oxoid) was assembled and sterilised by autoclaving at 120^o C for 30 minutes. Following cooling the fermenter vessel was mounted on the microwave block, stirred at 200 rpm and sterile filtered air was supplied at a rate of 1 litre per minute. The microwaves were applied at the

required energy as described below. The system was then allowed to reach the pre-set temperature of 37°C, matching the heating effect of the microwaves with the cooling under the control of the Applikon Biotechnology software.

For control experiments, when no microwave energy was applied, heating was via an external electrical heating jacket. Temperature was monitored and controlled at all times. Only when the system had reached a steady state at the required temperature was the inoculum added to a final concentration of OD_{550} of 0.1 (BugLab unit: 0.1). The process was then monitored for at least 36 hours. Each fermentation was at least duplicated to examine run-to-run variability.

All parameters were logged on-line through the Applikon and Bug Lab proprietary software. Data was exported as Excel™ files in .csv format and further manipulated within Excel.

2.3 Microwaves

Microwaves were generated in a Sairem solid state microwave generator (model number: GMS200WSM56MPFCFST1IRWF AIT) operating at 2.45GHz capable of a continuous, non-pulsed output of up to 200W, with measurement of both forward and reverse power. This was coupled to the fermenter through an aperture in a dished aluminium block, shaped to match the profile of the bottom of the fermenter vessel as shown in Figure 1_b. The fermenter was surrounded by an aluminium mesh to prevent re-radiation of the microwaves. The absorption of the microwaves into the fermenter was assessed by measuring the reflected power. Microwave absorption was complete as the reflected power was very low, typically about 1% of forward power, so that no further matching or tuning was required.

2.4 Scanning Electron Microscopy (SEM)

A sample of *P. aeruginosa* PAO1 broth was treated with 1% gluteraldehyde overnight at 4^0 C, washed ten times with distilled water by centrifugation (10000 rpm) and dehydrated in air (24h). The sample was coated using an Emitech k550x gold sputter coater at 25mA for 2.5 mins, giving a thickness of 12nm and examined under the SEM (Fei quanta 200 esem).

2.5 OxoPlate®/statistical analysis and antibiotic susceptibility test

OxoPlate® (PreSens) is a 96 well microtitre plate, containing specific sensors that measure oxygen levels in each well using a fluorescence plate reader (BMG/OPTIMA.). Raw data was collected using an indicator filter (540/650 nm) and a reference filter (540/590 nm). The effect of tobramycin was evaluated by mixing 180 µl of sterile nutrient broth, 10 µl of tobramycin and 10 µl of the cell culture onto each of the OxoPlate® wells. The plate was incubated at 37^0 C in a horizontal shaking (2 g 5 $mins^{-1}$) fluorescent plate reader (BMG/OPTIMA). Six replicates were performed for each experiment and automatic fluorescent readings were obtained every 5 minutes. The data generated were collected over 24 hours and PO_2 (the percentage of O_2 saturation) was calculated using the following equation:

$$P_{O2} = 100\ (K_0/I_r - 1)/\ (K_0/K_{100} - 1) \qquad (1)$$

Where K_0 was the highest I_r (relative) number and K_{100} was the lowest *Ir* value.

$$I_r = I_{ind}/I_{ref} \qquad (2)$$

Where I_{ref} (reference) is the mean value of all the replicate samples measured at 540/590 nm at each time point and I_{ind} (indicator) is the mean number of all the replicate samples measured at 540/645nm at each time point.

2.5.1 Tobramycin

Tobramycin (T4014, Sigma), an aminoglycoside, was selected for the antibiotic challenge as it is very effective against *Pseudomonas*. Each dilution was freshly prepared and filter sterilised prior to use. Concentrations of 0.312 µg ml^{-1}, 0.625 µg ml^{-1}, 1.250 µg ml^{-1}, 2.500µg ml^{-1}, 5 µg ml^{-1} and 10 µg ml^{-1} were tested against all planktonic cells.

3. Results

3.1 Microwave absorption

With the fermenter filled, the microwave energy was completely absorbed, as judged by the reflected power which was less than 1W for an applied power of 100W. This was, of course, supported by the observation of a temperature rise of the contents when no cooling was applied.

3.2 The normal growth profile of *P. aeruginosa* PAO1

Typical results for the growth of the *P. aeruginosa* PAO1 without microwave exposure are shown in Fig. 2.

As expected the DO began to decrease rapidly due to microbial respiration in the first growth phase. This was accompanied by an increase in cell density as evident from the BugLab data (Fig. 3). After about 2 hours the DO was effectively zero but the biomass was rapidly increasing under these microaerophilic conditions. This continued for a further 17 hours after which the DO slowly rose above 0%, consistently reaching a plateau at about

80% saturation after 24 hours (final OD_{550} of 7). The final DO was less than 100%, but remarkably similar in every case, suggesting some endogenous metab9olism rather than probe drift, which might be expected to be more random. However, this was not investigated further. During this phase the biomass level also reached a plateau, indicating metabolic rest. Catabolising an amino-acid based broth, such as nutrient broth, without an added energy source will result in an increased deamination and subsequent pH increase. Figure 4 demonstrates that the PH rose only marginally over pH 7 and hence is unlikely to have had adverse metabolic consequences.

During the whole process the DO profile was well matched by the cell density data from the "Bug Lab", as far as could be observed. This latter data has the advantage of reporting the growth, whilst the system was still at 0% DO. The profile of each run was typical of normal growth and between-run variation for the controls was small. This is obvious from the data shown in Fig. 2 and 3.

3.3 Growth under continuous microwave exposure

When *P. aeruginosa* PAO1 was grown under the continuous exposure to microwaves (2.45GHz) at constant temperature ($37 \pm 0.5^{o}C$) the growth profile was markedly altered when compared to the controls, as is shown in Fig. 5 but the pH profile hasn't changed (Fig. 6). Treatment with microwaves at constant temperature did not stop the *P. aeruginosa* PAO1 from growing, but overall the results of the fermentations with and without microwave treatment were substantially different.

Between-run variation for the runs carried out with microwave application was again small during the first 2 hours. Moreover at this stage the DO and cell density profiles for the microwave treated growth closely matched the controls.

The next stage of growth for both treated and untreated samples occurred at 0 % DO, indicating a very active respiration demand. However, whilst the reactions could not be monitored via DO during this period, the Bug-Lab data showed a continuing increase in cell density for both treated and untreated samples. Particularly though, it was clear that cell growth was notably smaller for the microwave treated sample (OD_{550} of 5) when compared with the untreated sample (OD_{550} of 7) (Fig. 7).

After the period at 0% DO the fermentation profiles for the treated samples diverged a little more between runs and very noticeably in comparison with the controls. In this stage the DO increased, indicating that the rate of oxygen consumption had fallen, as expected. Here there was a much bigger difference between treated and control samples. The increase in DO occurred at about 19 hours in the untreated samples and proceeded to a stationary level. However, for the microwave exposed samples the increase in DO commenced markedly earlier, at about 9 hours. This can be related to the cell-density measurements (Fig. 5). The microwave treated fermentation produced lower cell growth than the control, hence the lower cell density created a lower oxygen demand, allowing oxygenation to raise the DO above 0% at an earlier stage. However, importantly for the treated cells, after a period of about two to three hours during which the DO increased the DO then began to decrease again for about 3-4 hours, indicating renewed secondary growth. This was never observed with the untreated samples.

A sample was taken at that point in order to investigate the cells' morphology and antibiotic susceptibility. These cells will be called MIPs (Microwave Induced Persisters) from now on as they appeared stochastically during the fermentation and were tolerant to the microwave irradiation.

3.4 Cell morphology

After fixing, a sample of *P. aeruginosa* PAO1 the MIPs were examined under the SEM at a magnification of 13000 x as shown in Figs. 8-10. For the controls (untreated cells), a typical cell length was between 1-1.4µm whereas in comparison the persister cells where elongated to between 1.6-1.7 µm. After re-growth the cells returned to their normal size, which is a typical, non-inheritable trait of persister cells. The difference between persisters versus non microwaved cells was significant (Mann-Whitney $p=0.012$, $n_{1,2}=5$).

3.5 Antibiotic challenge

The effect of microwave treatment on subsequent growth under antibiotic challenge is shown in Fig. 9.

In order to assess the antibiotic sensitivity of the MIPs, the cells were exposed to an antibiotic challenge. Negative controls of both the planktonic cells and the isolated MIPs, with tobramycin omitted, rapidly consumed the oxygen reaching a PO_2 of zero in over 3 hours. These results are very similar for both types of cells and demonstrate a very active metabolic rate. Data shown in Fig. 11 represents a mean of six replicates.

The introduction of 0.3 µg ml^{-1} of tobramycin to MIPs produced a completely different metabolic profile for these treated cells. Oxygen levels diminished only partially then remains constant, indicating that the cells were being exposed to bacteriostatic levels of the antibiotic [27]. In contrast, planktonic cells were completely unaffected.

At higher levels, exposing the MIPs to 0.625 µg/ ml^{-1} of tobramycin and above caused cell death with PO_2 levels remaining at 100 %, whereas with the same treatment, planktonic cells carried on rapidly consuming and depleting oxygen for the first three hours. In contrast

planktonic cells were only affected at concentrations of tobramycin well above 0.625 µg ml^{-1} of tobramycin, with growth completely inhibited at 1.25 µg ml^{-1} of tobramycin.

Thus when MIPs were re-cultivated although they reverted back to their original shape, they demonstrated lowered drug tolerance. This is entirely different to antibiotic induced persisters which show an increased drug resistance, a major challenge for infection control [20, 22, 28, 29]. To our knowledge this is the first report of the synergetic impact of tobramycin on the microwave exposed *P.aeruginosa* PAO1 cells.

4 Discussion

The effect of microwaves on microbial growth, or more commonly on microbial death, has been quite widely studied, usually in connection with disinfection, particularly with foodstuffs [30]. In general the microwave energy is regarded as a source of heat, albeit with markedly different heat transfer characteristics to normal "thermal" process [2]. A few experiments have been described in which attempts have been made to disentangle the "thermal" component of microwave disinfection from a putative "microwave" component which might act in some as yet unspecified manner, but no definitive answer has been reached [31]. In part this is because the experiments are widely varied with parameters such as microwave frequency, energy density, field profile.

In the experiments described here a further attempt has been made to examine the athermal effect of microwaves on a culture actively growing at constant temperature. To our knowledge this is the first report of a fully automated batch system operated at constant temperature with continuous microwave irradiation. The measurements of bulk temperature were such that it is certain that the bacteria were growing at constant

temperature of 37± 0.5°C, within the accuracy of the measuring system. Complete transfer of energy from microwave radiation to the fermenter contents occurred. The energy was absorbed directly by the fermenter contents and not indirectly via microwave heating of the fermenter vessel, followed by heat transfer to the contents via conduction and diffusion. Glass is largely transparent to microwaves whilst water is extremely lossy and readily absorbs microwave energy. This was supported by measurements of the surface temperature of the vessel using a fibre optic contact probe which showed the exterior surface was at 37°C. Moreover, even if some indirect heating of this sort had occurred, it would then only resemble the heating via the electric wrapper used when microwaves were not applied and could not be responsible for the significant change in growth seen here. It is important to emphasise that in these experiments microwave energy was supplied continuously at the constant, i.e. non-pulsed, power level quoted here. This is unlike the continuous application of microwaves from pulsed fields where the power is a time-weighted average of high and low power states e.g. as in the domestic oven[32, 33]. Under those conditions average power figures may disguise very high instantaneous powers. Moreover, in our experiments the microwaves were applied during the whole of each fermentation so that during the run any transient effects would be eliminated.

The changes in both growth profile and morphology between treated and untreated samples observed in these experiments lead to the conclusion that at the energy used there is a distinct, sub-lethal, adverse effect of microwaves on growth. Growth under microwave irradiation certainly occurs. In the first 6 hours the rate of growth for treated and untreated samples is broadly the same but at a reduced cell density, which cannot be ascribed to any change in bulk temperature. The next stage for both treated and untreated samples

occurred at 0 % DO, indicating a high metabolic rate. During this period the cell density of the cultures increased although it was obvious that growth was much smaller for the microwave treated cells compared to the untreated one. As the DO started to increase the treated cells demonstrated a decrease in DO, which was indicative of secondary growth phase. This was never observed with the untreated cells. It was this point that the culture broth was sampled and we called these cells MIPS. The MIPS were observed under the SEM and appeared elongated. Rosenberg has previously reported that *Escherichia coli* cells become elongated following treatment with electrical currents but to our knowledge there are no reports of a similar microwave effect [34]. The persisters were ephemeral and on re-growth in the absence of microwave stress they reverted to normal size and metabolic active stage as seen in the electron micrographs and the Oxoplate data. This observation supports the theory that persister cells are a small population that express a temporary phenotypic characteristic, a variant of the planktonic cells, and revert back to their original growing state once the environmental stress is removed [20, 23].

When challenged with antibiotics during re-growth the treated cells appeared to be more susceptible to antibiotics. More specifically introducing 0.3 µg ml^{-1} of tobramycin had bacteriostatic effect to the MIPS compared to the untreated cells that continued growing with no obvious effect on their metabolism. Therefore we conclude that microwave treatment leads to the appearance of *P. aeruginosa* PAO1 persisters with decreased tolerance to tobramycin. Phenotypic resistance presents a major challenge to the development of anti-persister drugs.

Previously it has been reported by Niepa *et al* that drug tolerance of viable planktonic *P. aeruginosa* PAO1 persister cells can be eradicated by a combination of weak

electrochemical currents and tobramycin (1.5µg ml^{-1}) [35]. This mechanism, known as bioelectric effect, is poorly understood and has also been reported to be efficient against *P. aeruginosa* biofilms [36-39]. However this is the first report of electromagnetic waves applied to eliminate *P. aeruginosa* PAO1 persister cells in synergy with tobramycin. The effect of microwaves on the membrane of the MIPS and the permeability to tobramycin is part of on-going research.

5 Conclusions

Distinct changes in growth compared to untreated controls are induced by microwave treatment of *P. aeruginosa* PAO1 under the conditions used. However these changes only become apparent at later stage in the growth cycle. Cells treated with microwaves show a distinct change in morphology which disappears on re-growth. They also demonstrated increased sensitivity to tobramycin compared to the planktonic cells.

Conflict of interest:

There is no conflict of interest related to this work.

Permission statements:

The manuscript does not contain human or animal studies.

6. References

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Figure legends:

Figues 1 a, b Fermenter assembly showing microwave cavity and tuning section beneath (left) and the dished coupling section (right).

Figure 2 The growth profile of *P. aeruginosa* PAO1 during control fermentations.

Figure 3 BugLab data for *P. aeruginosa* PAO1 growth profile. The culture reached an average final OD_{550} of 7.

Figure 4 The relationship between dissolved oxygen and pH during control fermentations.

Figure 5 The profile of *P. aeruginosa* PAO1 during constant exposure to microwaves (2.45GHz). Temperature was controlled at ($37 \pm 0.5^{o}C$).

Figure 6 The relationship between dissolved oxygen and pH during microwave exposure.

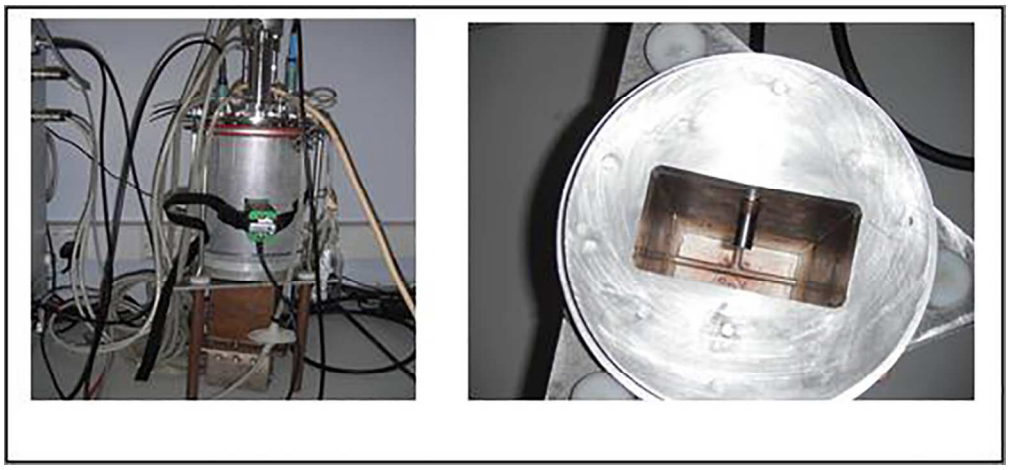
Figure 7 Growth of *P. aeruginosa* PAO1 during microwave treatment.

Figure 8 Scanning electron micrograph of *P. aeruginosa* PAO1 grown without microwave treatment.

Figure 9 A scanning electron micrograph of the *P. aeruginosa* PAO1 persisters.

Figure 10 Persisters re-grown.

Figure 11 Metabolic profile of planktonic cells and persisters in the presence of tobramycin. When the persisters were subjected to an antibiotic challenge (tobramycin), they demonstrated increased sensitivity (evident from the slow metabolic profile) compared to the un-stressed planktonic cells.
W: planctonic cells with no tobramycin present. P: Isolated persisters with no tobramycin present.Negative: Un-inoculated sterile media.



Figues 1 a, b Fermenter assembly showing microwave cavity and tuning section beneath (left) and the dished coupling section (right).

468x215mm (72 x 72 DPI)

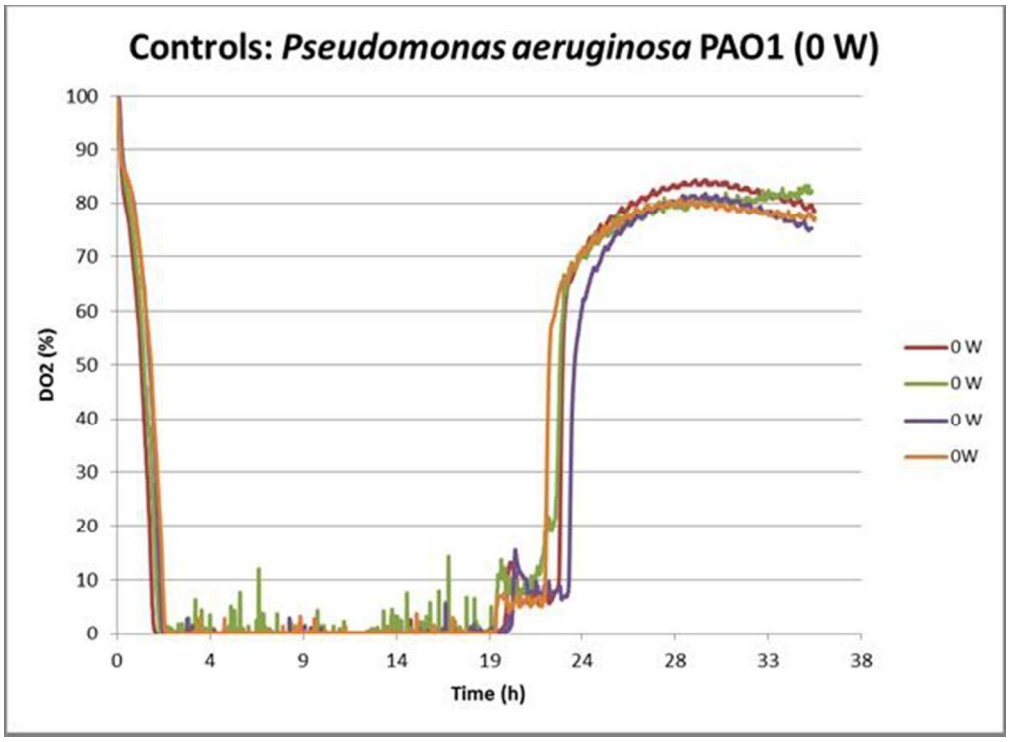


Fig. 2 The growth profile of P. aeruginosa PAO1 during control fermentations.

159x116mm (96 x 96 DPI)

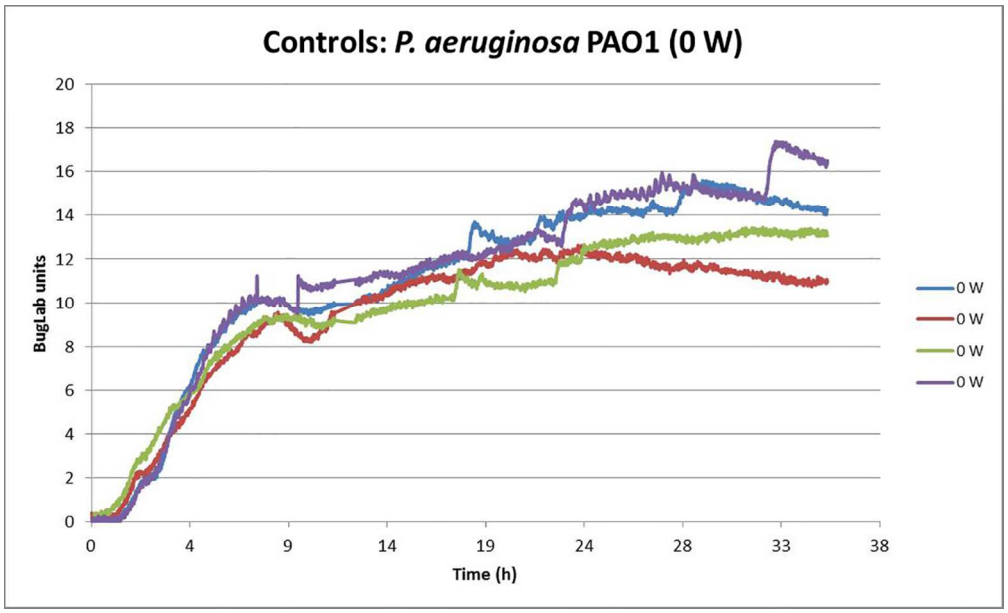


Figure 3 BugLab data for P. aeruginosa PAO1 growth profile.

468x282mm (72 x 72 DPI)

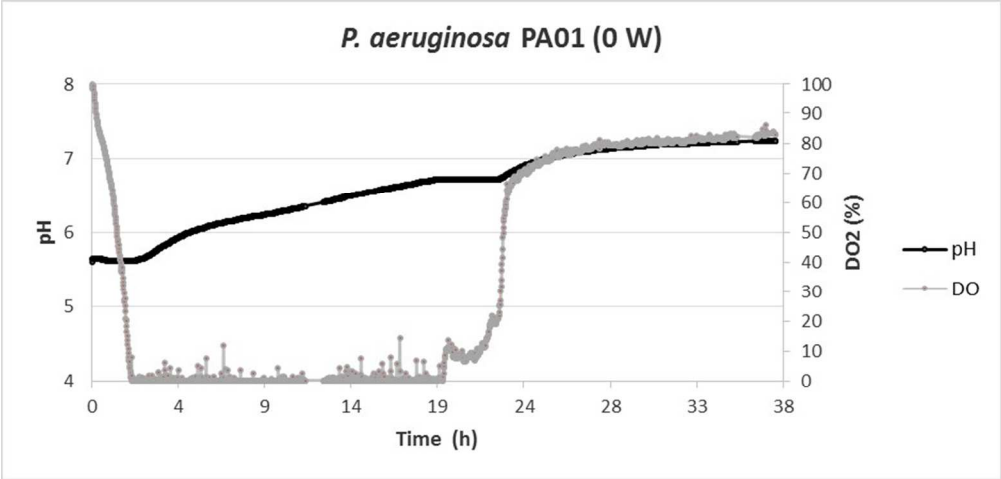


Figure 4 The relationship between dissolved oxygen and pH during control fermentations.

159x76mm (150 x 150 DPI)

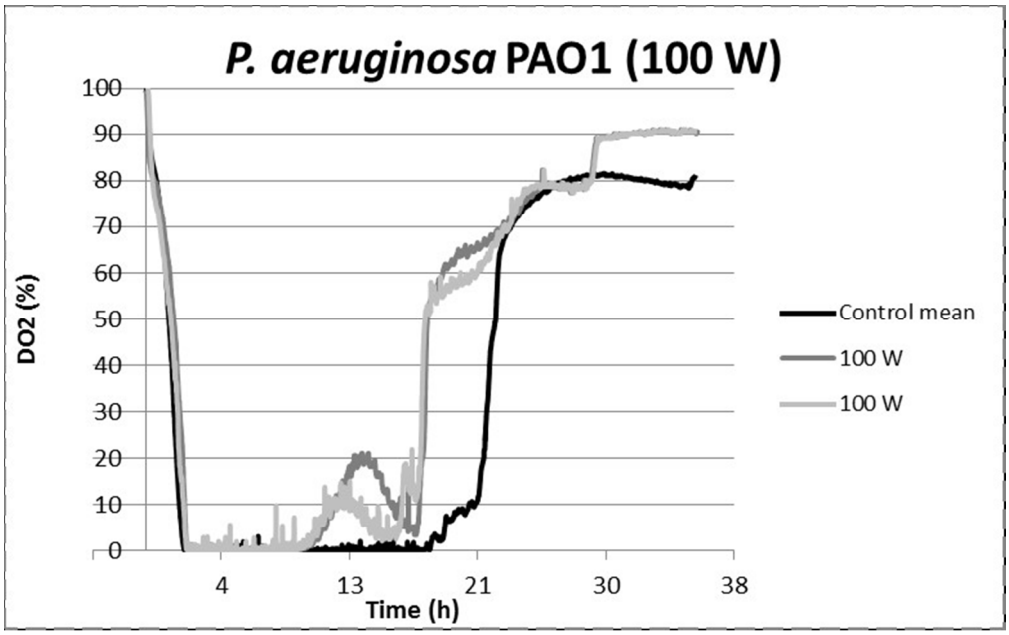


Figure 5 The profile of P. aeruginosa PAO1 during constant exposure to microwaves (2.45GHz). Temperature was controlled at (37+ 0.5oC).

131x82mm (150 x 150 DPI)

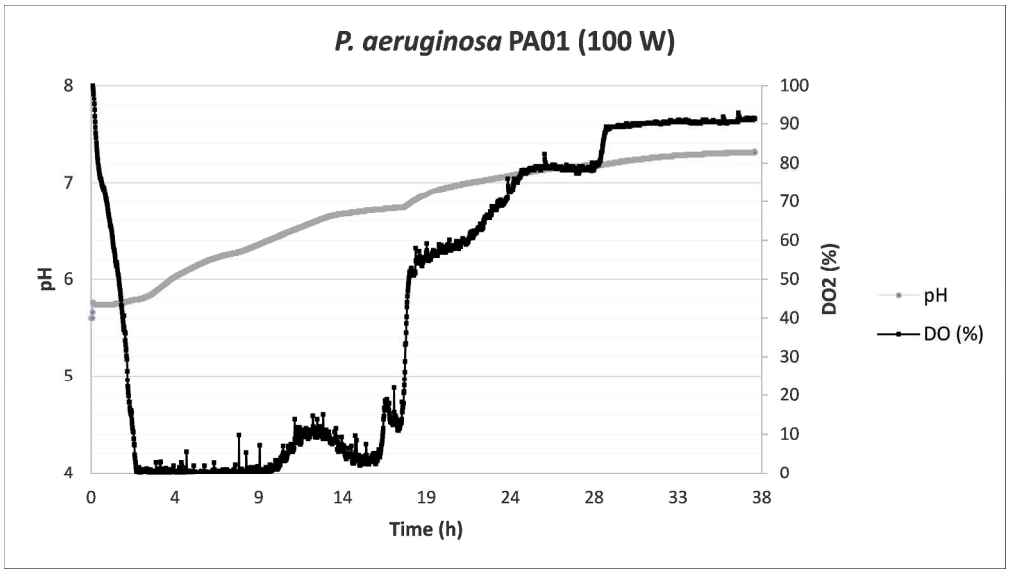


Figure 6 The relationship between dissolved oxygen and pH during microwave exposure.

1032x580mm (96 x 96 DPI)

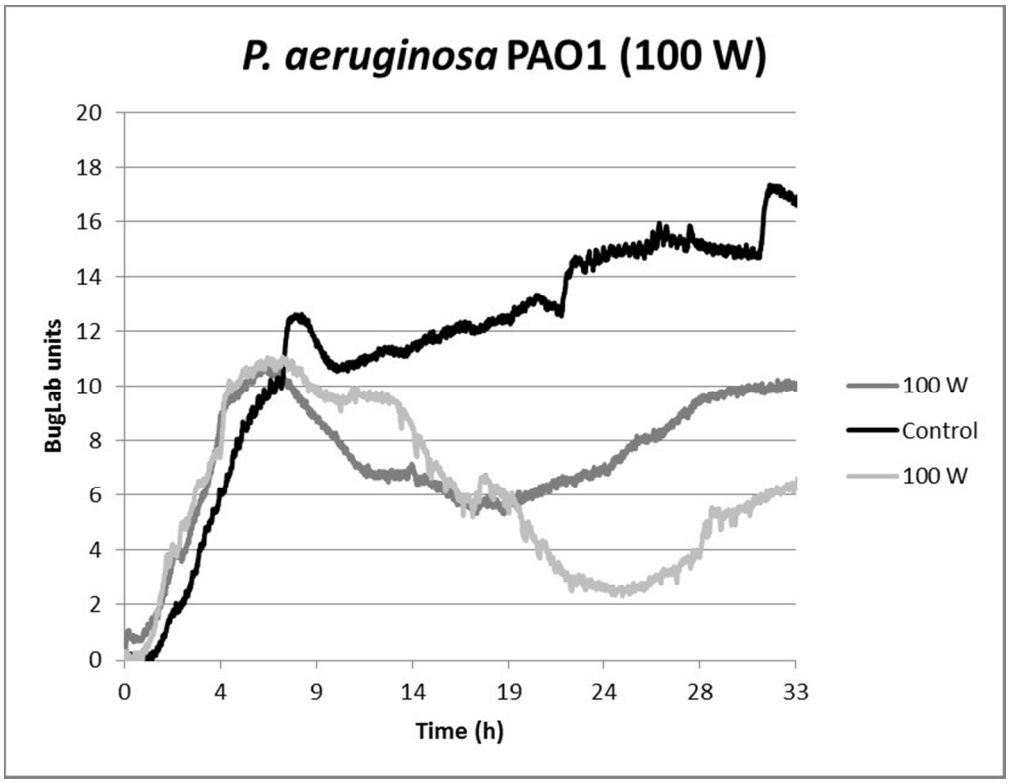


Figure 7 Growth of P. aeruginosa PAO1 during microwave treatment.

139x107mm (150 x 150 DPI)

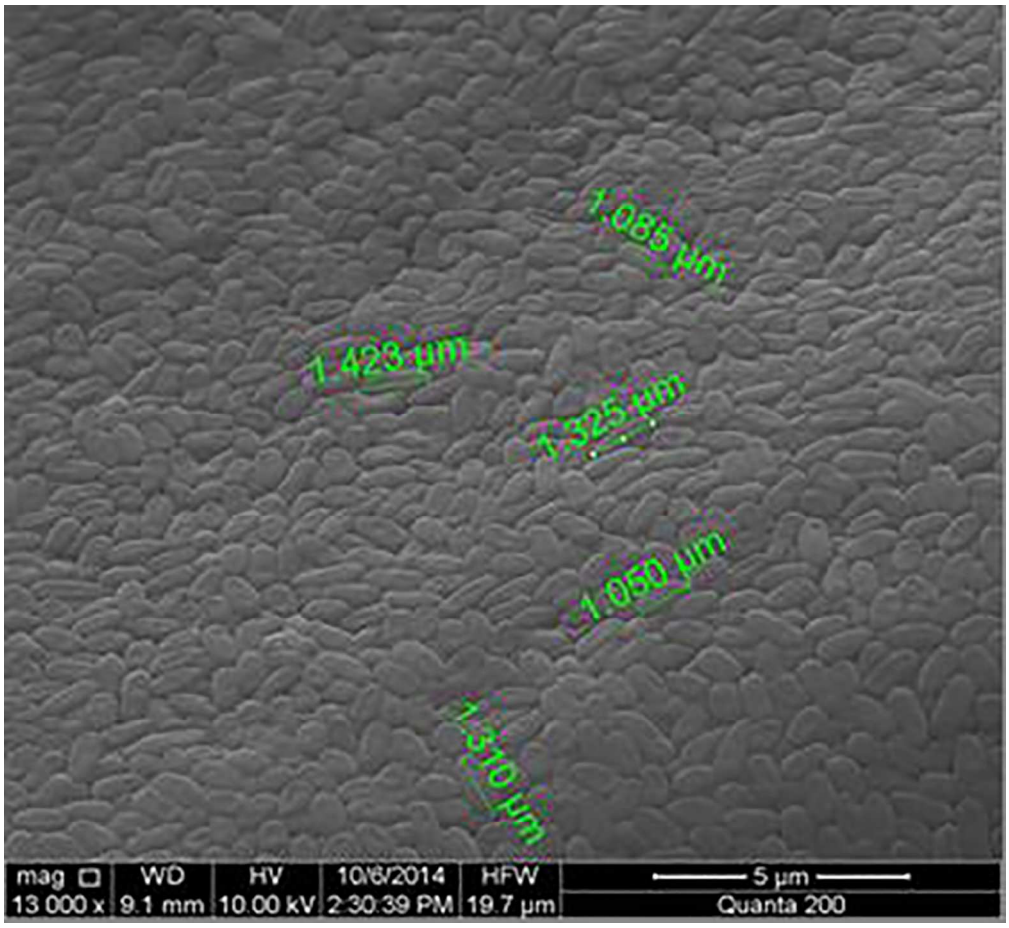


Figure 8 Scanning electron micrograph of P. aeruginosa PAO1 grown without microwave treatment.

468x429mm (72 x 72 DPI)

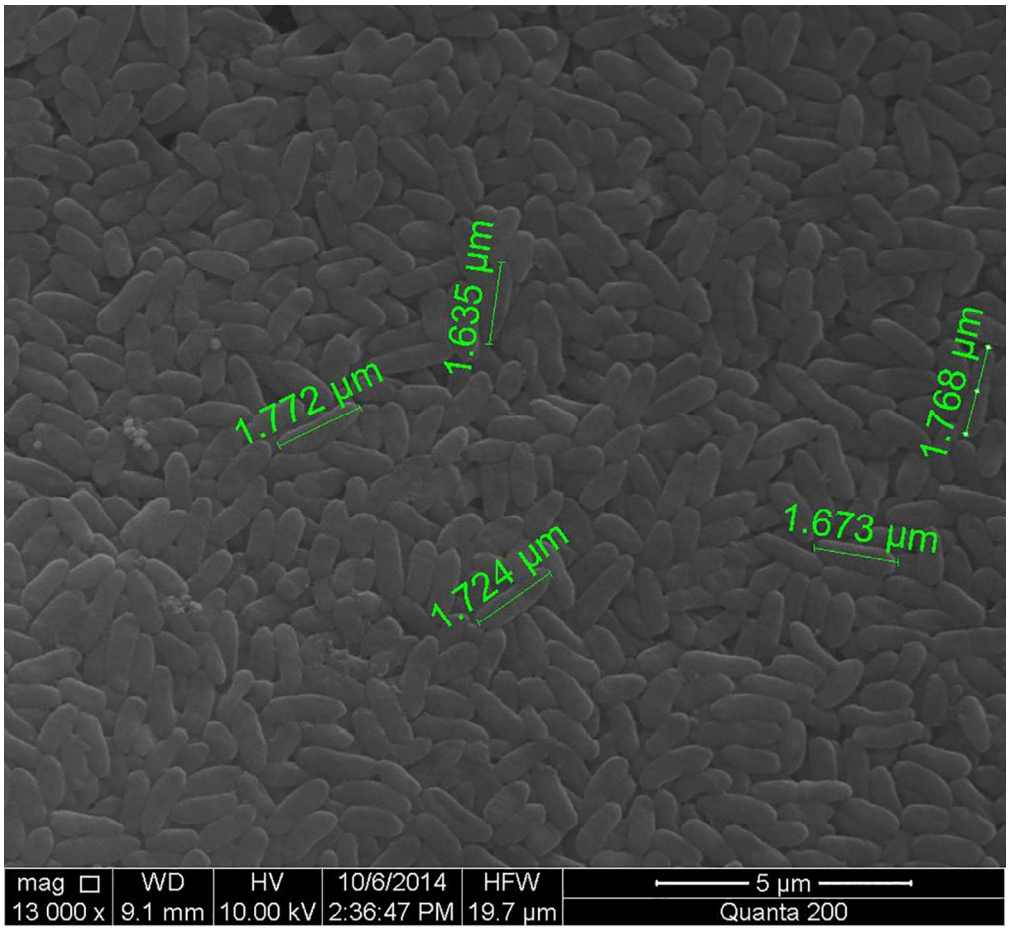


Figure 9 A scanning electron micrograph of the P. aeruginosa PAO1 persisters.

468x431mm (72 x 72 DPI)

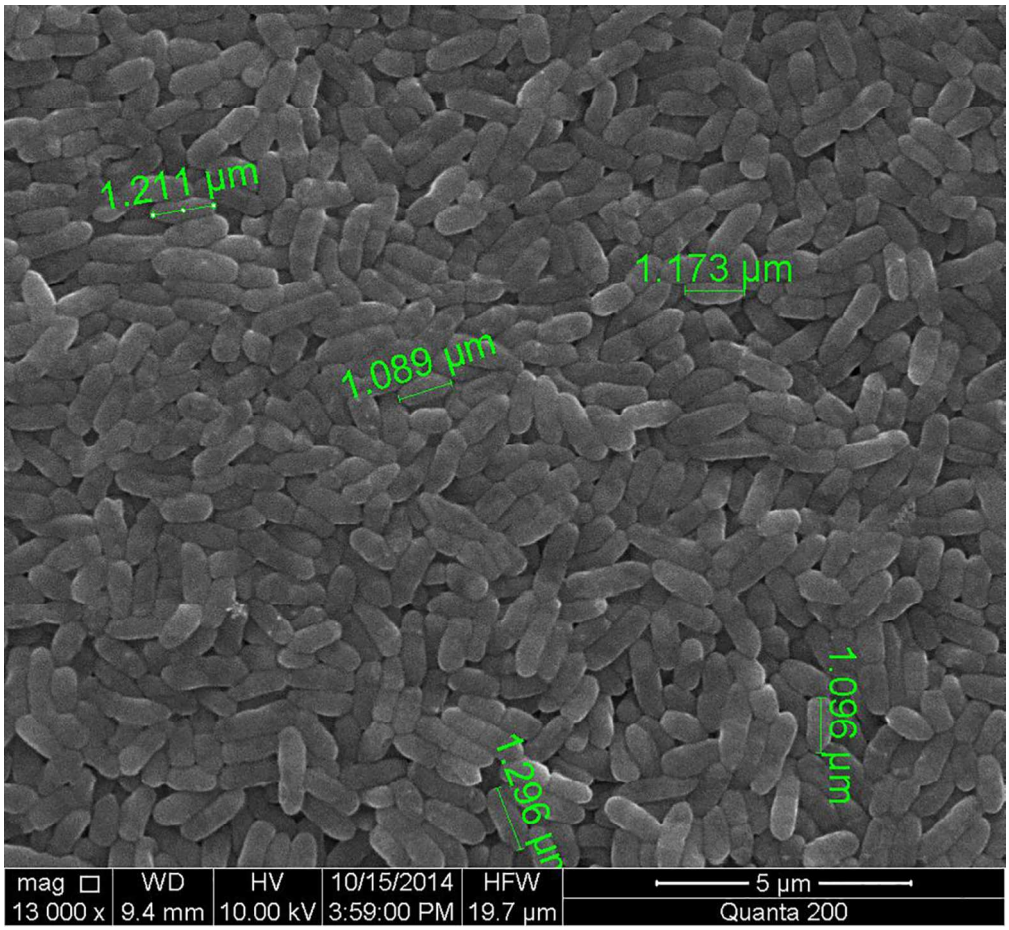
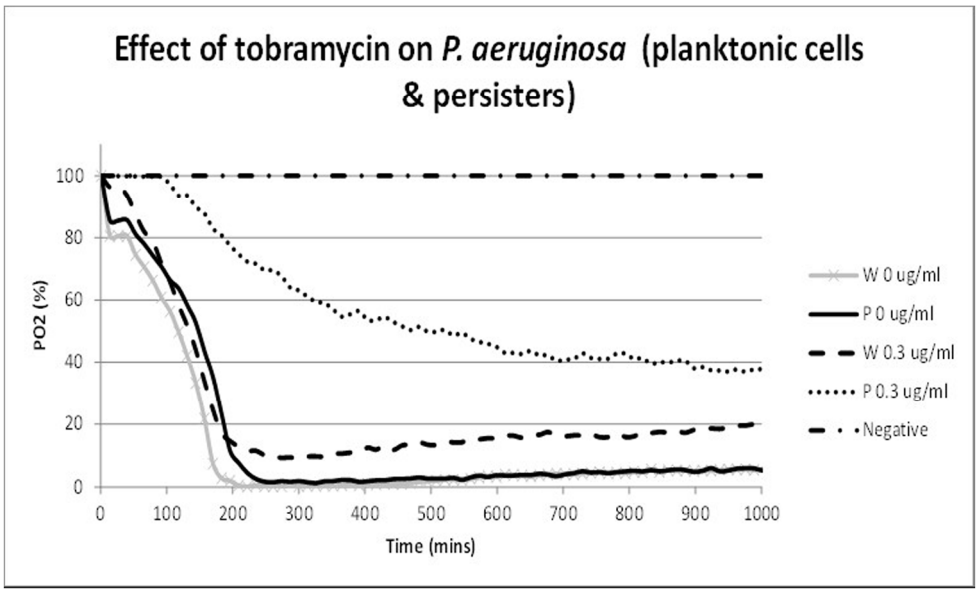


Figure 10 Persisters re-grown.

468x431mm (72 x 72 DPI)



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