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To cite this article: Auns Q Alneami et al 2018 J. Phys.: Conf. Ser. 1003 012112

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Effect of Electrical Current Stimulation on Pseudomonas Aeruginosa Growth

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Abstract. The present study evaluates the effect of electrical current with different frequencies stimulation to kill pathogenic Pseudomonas aeruginosa (PA) bacteria in vitro using human safe level of electricity controlled by function generator. A wide range of frequencies has been used from 0.5 Hz-1.2 MHz to stimulate the bacteria at a voltage of 20 p-p volt for different periods of time (5 to 30) minutes. The culture of bacteria used Nickel, Nichrome, or Titanium electrode using agarose in phosphate buffer saline (PBS) and mixed with bacterial stock activated by trypticase soy broth (TSB). The results of frequencies between 0.5-1 KHz show the inhibition zone diameter of 20 mm in average at 30 minutes of stimulation. At frequencies between 3-60 KHz the inhibition zone diameter was only 10mm for 30 minutes of stimulation. While the average of inhibition zone diameter increased to more than 30mm for 30 minutes of stimulation at frequencies between 80-120 KHz. From this study we conclude that at specific frequency (resonance frequency) (frequencies between 0.5-1 KHz) there was relatively large inhibition zone because the inductive reactance effect is equal to the value of capacitive reactance effect (XC = XL). At frequencies over than 60 KHz, maximum inhibition zone noticed because the capacitance impedance becomes negligible (only the small resistivity of the bacterial internal organs).

Keywords: bacteria, electrical stimulation, frequency effect, Pseudomonas aeruginosa.

1. Introduction

Recently scientists began to depend on developing a new techniques to treat infections caused by pathogenic bacteria or other microorganisms rather than antibiotics, that is belong to their harmful side effect or bacterial resistance to antibiotics [1] [2].

A lot of suggested techniques included and subjected to scientific research include using electrical stimulation (ES), laser, ultrasound, nano technology, electromagnetic radiations, etc. Electrical stimulation influence has a multiple variations that could change its ability to interact with living cells, these variables include voltage; current; frequency; polarization; and wave shape [3].

It is clear that high power electrical current more than 6 mA will be harm for human beings and could be fatal [4]. So the ability of electrical current lower than the effective value needs to be focused on to kill pathogenic bacteria. Pseudomonas aeruginosa is one of the most harmful bacteria which increase patients mortality rate in the hospitals because it is one of the antibiotic - resistant bacteria [5].

When alternating current (AC) pass through living body it will be resisted by extracellular matrix and intracellular matrix, where living cell such as bacteria act as capacitance (C) depending on the size...
of the cell membrane[6][7]. Bacteria is a living cell that contain cell membrane which acts as capacitance too. The total impedance of the AC inside media containing living cells could be represented by the resistance (R) and reactance (XC) where the cell represented by R1 & C in the series and the extracellular matrix represents pure resistance (R2) parallel to the cell impedance as shown in 'figure (1)'

![Figure 1. Pathway of intracellular (path₁) and extracellular (path₂) matrices](image)

If the current frequency is zero, then the electrical current will pass totally through path2 because of higher impedance at path1 compare to path2 (i.e. reactance proportional inversely with frequency and equal to infinity, open circuit, at zero frequency according to equation 1), if the frequency increased then gradually part of AC will pass through path1 as the reactance effect decrease. At very high frequency total AC will pass through path1, according to the difference in the impedance between path1 and path2, there are three equations:

$$X_C = \frac{1}{2\pi fc}$$  \hspace{1cm} (1)

$$Z_1 = \sqrt{R_1^2 + X_C^2}$$  \hspace{1cm} (2)

$$Z_2 = R_2$$  \hspace{1cm} (3)

Where:
- $Z_1$: impedance of path₁,
- $Z_2$: impedance of path₂, and
- $X_C$: Reactance of path ₁

f: is the frequency in hertz.
c: is the capacitance in micro farad

According to equations (2) and (3) if pathogenic PA treated by a suitable frequency of AC current that could kill bacteria avoiding human cell depending on the differences in reactance properties between the human cell membrane and bacterial cell membrane according to the differences in the composition and size of the cell membrane [6][7].

There are many differences between bacterial cells and live human cells, as illustrated in table 1 [8] [9] [10] [11]:

| Differences | Human cell | Bacterial cell |
|-------------|------------|---------------|
| Cell        | Human cells are in a group and not isolated. It is dependent on other cells for survival. | Cell is isolated and Independent. It survives as an individual on its own. |
| Cell wall   | The wall is absent | Thick protective cell wall is present covering the whole cell. |
| Construction of cell membrane | Lipid bilayer with phosphate molecules. It is hydrophilic to external and hydrophobic in the inner wall. | Made of bilayer phospholipid. But the membrane lacks sterols. |
| Cell wall has | Cytoplasmic bridges are present which help in inter-cellular | No cytoplasmic bridges as there is only one cell. |
transport in between neighbouring cells.

| Cell shape | Only spherical or oval | Cells can be of different shapes |
|------------|------------------------|----------------------------------|
| Cell appendages (External parts) | Absent mostly. Except for example ciliated cells in respiratory tract & gut. | Present. Flagella for movement, pili for sexual reproduction. |
| Nucleus | Prominent nucleus with nuclear membrane. So called as eukaryote type. | Nucleus is Absent. Instead nuclear content like DNA are present in cytoplasm. No distinct nucleus, so called as prokaryote |

Pseudomonas aeruginosa is an opportunistic human pathogen, gram negative, rod shaped, asporogenous, and monoflagellated bacterium that has an incredible nutritional versatility. It is a rod about 1-5 μm length and 0.5-1.0 μm wide. PA is an obligate respirer, using aerobic respiration as an optimal metabolism and can respire anaerobically on nitrate or other alternative electron acceptors [12]. PA can catabolize a ubiquitous range of organic molecules, including organic compounds like benzoate. This, makes PA a very ubiquitous microorganism, for it has been found in wide environmental conditions such as soil, water, humans, animals, plants, sewage, and hospitals [13][14]. PA is the predominant inhabitant in all aquatic ecosystems, which contain high dissolved oxygen content, but its existence in low plant nutrients makes it clearly the most abundant organism [14][15][16].

2. Aim
The aim of the present study is to prove that in vitro the pathogenic bacteria can be inhibited by using low power alternative electrical current that is safe to human body.

3. Materials and Methods
3.1. Materials:
PA were isolated from human skin burns and preserved in the bacterial bank in Al Nahrain University/College of Biotechnology.

- A 60 mm plastic petri dish (100 piece) was obtained and two holes of 2.4mm in diameter were borne through the bottom of each, at distance 1cm apart from the centre. Two of 2 cm length, 2.5 mm-gauge Nickel electrodes (ESAB/VacPac electrodes) were fed through each hollow. A 1.1cm of the electrode inside the petri dish was perpendicular to the base of the petri dish, while the outside portion of the electrode was fixed in with epoxy cement parallel to the base of the dish, with a 0.9 cm overhang for the attachment of the function generator wires figure 2. Then petri dishes were sterilized by alcohol 70% and wiped with cotton, then alcohol 70% again and finally exposed to UV light of 9J/cm2 for 1 hour.

**Figure 2.** Petri dish with Nickel electrodes

- A movable wires (Nichrome inoculation loop gauge 24) were used as an electrodes to stimulate the cultured bacteria. A special setting as shown in figure 3 applied by hanging the sterilized
electrodes in the petri dish. Pouring the cultured media with PA, then it was left to solidify at room temperature (RT) and thus become ready to apply the electrical current.

3.2. Instrumentation for electrical current application:
A function generator (FeelTech Model: FY3200S) as illustrated in figure 4.a was used in this study to stimulate bacteria by a customized range of frequency as follows (0.5, 1, 30, 70, 100, 300, 700, 1K, 3K, 7K, 10K, 30K, 120K, 1.1M, 1.2M) Hz at a constant value of continues sine wave 20 Vp-p AC current as shown in figure 4.b and for 10, 20, and 30 minutes. Two cables with alligator clips were used to deliver current to two petri dishes at the same time (two channels of the function generator were used simultaneously each in different frequency and each petri dish stimulated by only one of the mentioned frequency).

4. Procedure:
The cultured PA were grown overnight to reach mid-exponential growth phase at 37°C in trypticase soy broth with a shaking incubator at speed of 200rpm. Next day 2gm agarose were added to 200ml of 0.1M phosphate-buffered saline, molded by using microwave for 140 sec., then was cooled to suitable temperature nearly (37°C) within (20 to 30) minutes. A 20 ml of TSB containing 10⁶ colony-forming units (cfu) of PA / milliliter were added to the mixture and mixed well manually. Fifteen ml of the final mixture were added to each (13) sterile (60mm) petri dish using 20ml syringe [17]. Cultured petri dishes were used immediately after solidification at RT.

Two petri dishes cultured with PA were each stimulated with one of the customized electrical current stimulation mentioned previously, then another two petri dishes stimulated using two new different electrical current frequencies and the procedure was repeated to cover all the 12 values of the chosen frequencies. Each ES frequency was applied to a petri dish for 30 minute at room temperature (20-25 °C) inside sterile hood cabinet. Then each electrical stimulated petri dishes were incubated for another (20) hour at (37 °C), the diameter of the inhibition zone (absence of bacterial growth) surrounding each

**Figure 3.** Petri dish and Nichrome wire electrodes

**Figure 4.** (a): Function Generator / FeelTech / Model: FY3200S and (b): Sine wave 20 Vp-p AC current
 electrode was measured using ruler. Two measurements of each inhibition zone were taken perpendicular to each other, and both of them perpendicular to the electrode. This procedure repeated four times for each frequency (i.e. totally four samples subjected to the same value of frequency).

These measurements were then averaged to obtain a value for the inhibition zone. Inter-tester reliability was determined using 1×3 repeated measures analysis of variance (ANOVA). No electrode corrosion, gas formation, and media discoloration were noticed except 2mm burning trace around positive and negative Nichrome electrodes. A set of 24 cultured petri dishes was incubated as mentioned previously, but without ES, considered as a control group.

All the procedure above was repeated again for 20minute and 10minute electrical stimulation for chosen samples at specific frequencies. Finally (96) samples were used including (24) control.

All samples passes through the following procedure to measure their growth inhibition zones:

- **Step 1:** This is mean that a quantity of TSB prepared and cultured by 10 percent of previously TSB cultured PA from refrigerator to activate bacteria then the sample incubated for 20-22 hours at 37°C shaking incubator at 200 rpm.
- **Step 2:** This mean that preparing agarose by adding 1gm of agarose to each 100 ml of 0.1 M PBS. Then solve the mixture totally by hand then microwave. After slight cooling to almost 37°C a 10 percent of activated bacteria from step 1 is added to the mixture and mixed well manually then 15 ml of the mixture poured in 60 mm petri dish with built in electrodes. After solidification the sample stimulated by AC current of specific frequency for specific time and incubate the stimulated samples and the control (not stimulated) for 20-22 hours at 37°C incubator.
- **Step 3:** checking the inhibition zone of the stimulated samples from step 2.

All samples pass through steps 1, 2, and 3 then finally the inhibition zone measured and recorded in a table.

5. Statistical Analysis
A 1×24×4 repeated measures analysis of variance ANOVA, p < 0.05, was performed to determine a possible main effect for (1) electrode polarity between two levels, (2) sixty types of ES based on (15 frequencies with 4 stimulation times), (3) number of samples stimulated for each type of stimulation (13×3 using Nichrome wire electrode, 8×3 by using built-in Nickel electrodes, and 3×3 using Titanium electrodes) while the fourth used as control.

6. Results
Tables 2, 3, 4, 5, 6, and 7 and related figures 5, 6, and 7 below show the practical result of the present study clarifying the inhibition zone at each examined frequency and time:

**Table 2. Inhibition zone according to the frequency and time by using Nichrome electrodes**

| NO. | Freq., Hz / Stimulation time (minute) | Inhibition zone, Anode, Cathode, (mm) |
|-----|-------------------------------------|-------------------------------------|
| 1.  | 0.562/30                            | 15, 14                              |
| 2.  | 5.04/30                             | 16,15                               |
| 3.  | 7.04/30                             | 16,15                               |
| 4.  | 30.44/30                            | 16,15                               |
| 5.  | 300/30                              | 10,10                               |
| 6.  | 500/30                              | 10,8                                |
| 7.  | 1K/30                               | 10,10                               |
| 8.  | 3K/10                               | 10.8                                |
| 9.  | 3K/12                               | 6.4                                 |
| 10. | 3K/30                               | 11,10                               |
| 11. | 30.01K/30                           | 15,9                                |
| 12. | 61.8K/12                            | 4,4                                 |
| 13. | 303K/15                             | 12 , (24×16)                        |
**Figure 5.** Shows the AC stimulation of PA by using Nichrome electrodes according to values as shown in Table 3.

**Table 3.** Inhibition zone area related to AC stimulation frequency / Nichrome electrodes

| Freq., Hz | Inhibition zone area, mm² |
|----------|--------------------------|
| 0.5      | 330                      |
| 5        | 377                      |
| 7        | 377                      |
| 30       | 377                      |
| 300      | 157                      |
| 500      | 128                      |
| 1000     | 155                      |
| 3000     | 174                      |
| 30000    | 240                      |
| 300000   | 415                      |

**Figure 6.** Shows the AC stimulation of PA by using Titanium electrodes according to values as shown in Table 5.

**Table 4.** Inhibition zone according to the frequency and time by using Titanium electrodes

| NO. | Freq. , Hz / Stimulation time (minute) | Inhibition zone Anode (mm) |
|-----|---------------------------------------|-----------------------------|
| 1.  | 700/30                                | 0 but Lighter growth        |
| 2.  | 120K/30                               | (35, 30) mm ellipse at one side. Closely total killing. |
| 3.  | 1.1M/20                               | (35, 30) mm ellipse at one side. Closely total killing. |

**Table 5.** Inhibition zone area related to AC stimulation frequency/Titanium electrodes

| Freq. | I.Z. area, (mm²) |
|-------|------------------|
| 700   | 0                |
| 120000| 825              |
| 1100000| 825             |
Table 6. Inhibition zone according to the frequency and time by using Nickel electrodes

| NO. | Freq. , Hz / Stimulation time (minute) | Inhibition zone Anode , Cathode, (mm) |
|-----|--------------------------------------|--------------------------------------|
| 1.  | 0.5/30                               | (20×30), 15.6                        |
| 2.  | 30/30                                | 20, 15                               |
| 3.  | 300/30                               | 28, 15                               |
| 4.  | 80K/20                               | 26, 15                               |
| 5.  | 100K/20                              | 34, 20                               |
| 6.  | 120KHz/20                            | 33, 24                               |
| 7.  | 120KHz/30                            | Nearly total inhibition              |
| 8.  | 1.2M/30                              | 100% inhibition                      |

Table 7. Inhibition zone area related to AC stimulation frequency/Nickel electrodes

| Freq. | I.Z. area (mm²) |
|-------|----------------|
| 0.5   | 660            |
| 300   | 707            |
| 80000 | 707            |
| 100000| 1222           |
| 120000| 2827           |

Figure 7. Shows the AC stimulation of PA by using Nickel electrodes according to values as shown in table 7.

* Da: diameter of inhibition zone at anode
* Dc: diameter of inhibition zone at cathode

The figures (8,9,10,11,12, 13) below show some photos’ results (as clear as possible photos have been chosen, taken into account that glue drops leave its trace around built in Nickel electrodes):

Figure 8. Inhibition zone of 5 mm burning trace at anode side using movable Nichrome electrodes at 3 KHz for 10 minutes.
Figure 9. Inhibition zone of 25×15 mm using built-in Nickel electrodes at 0.5 Hz for 30 minutes.

Figure 10. Inhibition zone of 20×15 mm, using built-in Nickel electrodes at 30 Hz for 30 minutes.

Figure 11. Inhibition zone of 28×15 mm using built-in Nickel electrodes at 300 Hz for 30 minutes.
Figure 12. Inhibition zone of 33×24 mm using built-in Nickel electrodes at 100 KHz for 30 minutes.

Figure 13. Inhibition zone of 35×30 mm, closely total bactericidal effect using built-in Titanium electrodes at 120 KHz for 30 minutes.

7. Discussion
The various values of AC current frequencies can inhibit PA growth in different inhibition levels. A significant inhibition zone diameter observed when low frequencies were applied (between 0.5 Hz and 1 KHz), in comparison with higher frequencies (between 3 Hz and 60 KHz), but there is no significant difference in their effect between each other (similar inhibition zone) which is agree with Fadel M. Ali et al. in [2013] whose results indicated that exposure to positive square pulsed electric fields can inhibit bacterial growth at particular resonance frequencies 0.7 Hz and 0.5 Hz and clearly act on cellular activity as well as cause changes in molecular structure that affect (inhibition) the cellular division or proliferation [18]. While increasing frequencies between 3 KHz and 60 KHz show smaller inhibition zone. The inhibition zone area increased significantly after stimulation by electrical current of frequencies between 80 KHz and 300 KHz .The inhibition zone increased at all frequencies levels by increasing stimulation power and time. This meant that bacterial membrane acts as capacitive resistance while increasing frequency the capacitance reactance decrease leading to decrease cell impedance and therefore more current (electrons) passes through bacterial cell and cause higher interaction leading to faster inhibition of cells and occurrence of larger inhibition zone.

We clearly notice that at frequencies less than 60 KHz the inhibition zone was less than 5 mm at short stimulation time (20 minutes) while it was 20 mm for samples stimulated by 120 KHz for the same time.

This study confirmed that low power of 20 V_{pp} and less than 5μA was enough to inhibit PA pathogenic bacteria, these values of electrical current is theoretically safe to the human body.

It must be referred to the effect of burning in the cathode and anode sides of the stimulation electrodes which must be avoided in future study by using wider electrodes cross section area. There was no noticeable corrosion and coloration on the Nickel or Nichrome wire electrodes by examined
using naked eye while using stainless steel wire show some physical effects in previous study [19] but more tests needed to find the exact amount of corrosion if any.

It was also clear that Nickel electrodes cause either bacterial attraction towards the electrodes or toxic effect or both depending on frequency (i.e. in some frequencies the bacteria aggregate in areas around electrodes and the inhibition zone was observed in the remaining cultured area of the petri dish even in control sample because of either old PA isolate or may be due to the dual effect of Nickel; toxic and attraction), it is also important to mention that different bacterial isolates shows similar, but not identical, reactions to different electrical frequencies values stimulation.

It was only clear that wide area of Titanium electrodes cause no burning effect on the samples.

8. Conclusion
This study aimed to investigate the effect of changing the frequencies of low voltage and power alternative electrical current (values theoretically safe to humane body) on PA growth and it was found that low electrical current values can inhibit PA growth and the inhibition significantly increased at high frequencies more than 80 KHz and reach maximum at 120 KHz.

9. Recommendations for Future Work:
- This study recommends additional investigation about Nickel effect on PA growth.
- Using wide plates of inert material as an electrode to avoid burning effect.
- Developing a methodology work on to measure the suitable frequency that can effectively kill any species of bacteria and can be programmed to find out if it is safe to human tissues, and can be applied to use it as a therapy in addition to the other therapeutic strategies.
- Additional studies should be established to prove that in general high frequencies have no harmful side effects on human body and especially on organs their functions related to electrical stimulation such as the heart and nervous system.

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