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Near infrared femtosecond laser-induced bacterial inactivation

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ABSTRACT

The use of light to inactivate microbes as an alternative method to the traditional methods of controlling microorganisms continues to draw the attention of researchers. Traditional methods of sterilization and/or pasteurization using chemicals or thermal treatments have certain limitations such as the creation of resistant bacterial strains. The application of pulsed laser irradiation compromises the physiological function of cells, and the degree of destruction is both dose and strain dependent, ranging from reduced cell growth to a complete loss of cell metabolic activity and finally to physical disintegration. This study aimed at using a range of power densities to investigate inactivation of *Escherichia coli* and *Salmonella enteritidis*. A Titanium sapphire pulsed laser at 800 nm wavelength, repetition rate of 76 MHz, pulse duration of 120 fs, output power of 560 mW was used in this study. A fluence range was applied on bacterial cultures in a 16 mm diameter petri with a beam spot area of 2.5 cm^2 (after expansion). The laser killing effectiveness was evaluated by comparing colony forming units (CFUs) with and without irradiation on 10^{-7} dilutions of bacterial cultures. Cytotoxicity was analysed using the lactose dehydrogenase (LDH) assay. The laser killing rate varied with bacteria species or strains and the level of fluence.

Keywords: Near infrared, femtosecond, Gaussian beam, bacteria, colony forming units, *Escherichia coli, Salmonella enteritidis*

1. INTRODUCTION

Inactivation of pathogenic microorganisms such as bacteria and viruses using various antimicrobial methods remains an area of increasing research interest over the years [1]. Numerous methods of microorganism inactivation have been investigated and such includes ultraviolet (UV) irradiation, microwave absorption, gamma-ray, photochemicals, antibacterial treatments, pharmaceutical treatments, and thermal sterilization [1, 2, 3]. The use of these methods however has several disadvantages. The UV light irradiation method which is a well-established light inactivation treatment induces deoxyribonucleic acid (DNA) damage resulting from UV absorption by DNA at wavelengths of 240 nm to 280 nm [1]. This method is also photocarcinogenic to human cells and causes damage to skin tissue and components of the eye [1, 4]. The main disadvantage with these conventional antimicrobial treatment methods is the lack of selectivity accompanying microorganism inactivation, and thus resulting in severe side effects and the development of resistant strains [1, 2]. With conventional pharmaceutical antimicrobial treatments, a new drug is usually required to treat new or mutated strains of microorganisms [2].

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New and effective target-specific antimicrobial treatment methods are needed. Therefore, the use of near-infrared (NIR) ultrashort pulsed (USP) laser treatment for microorganism disinfection is recommended for treatment. The USP laser treatment method is capable of disinfecting undesired microorganisms such as viruses and bacteria while leaving mammalian cells and proteins unharmed [2, 4, 5]. This method has been successfully used to inactivate bacteria such as *E.coli, Salmonella spp, Listeria,* and a variety of viruses including human immunodeficiency virus (HIV), human papillomavirus (HPV), hepatitis A virus (HAV), influenza virus, encephalomyocarditis, tobacco mosaic virus, and M13 bacteriophage [2]. The use of USP laser is capable to inactivate both gram positive and gram negative bacteria pathogens regardless of their mutation status of structural composition [2]. For the inactivation of viruses, the USP laser beam excites and triggers mechanical vibrations of the virus capsid, targets the weak links of the protein coat, subsequently leading to virus loss or infectivity. For bacterial inactivation, the USP laser beam relaxes and unwinds the super-coiled double-stranded DNA (dsDNA) causing damage to the DNA structure and thus leading to bacteria cell death [2]. The efficiency of USP laser irradiation is relatively higher compared to continuous UV irradiation because higher fluence can be applied within a short space of time resulting in effective disinfection of microorganisms. The total fluence delivered on the bacterial cell surface is the most significant determinant factor for microbial killing [6]. Therefore, the main aim of this study was to explore the use of near-infrared pulsed laser-induced inactivation of *Escherichia coli* and *Salmonella enteritidis.* A diode-pumped mode-locked Titanium-sapphire pulsed femtosecond laser was used in this study. Different fluences were used to determine the effects of laser irradiation on bacterial cell viability.

2. MATERIALS AND METHODS

2.1 Bacterial cell culture

Strains of *Escherichia coli* and *Salmonella enteritidis* microorganisms were used in this study to explore the effect of NIR pulsed laser irradiation on bacteria. *E.coli* was cultured on MacConkey Agar with Crystal Violet (Merck, batch number 1048589), and *Salmonella enteritidis* was cultured on XLD Agar (Merck, batch number 1047904). A single colony of each of the test strains was inoculated into 2 mL of LB broth (Miller) (Sigma-Aldrich, L3522) and cultured at 37 °C under rotary conditions (at 180 rpm) for 18 hours.

2.2 The optical setup

A homemade optical setup was assembled using a mode-locked Titanium-sapphire pulsed femtosecond laser at 800 nm wavelength which emitted a Gaussian laser beam (Fig. 1). Mirror 1 (M1) and mirror 2 (M1) are NIR coated mirrors and were used to form a periscope elevating the beam. The original beam was expanded using lens 1 (L1) and lens 2 (L2) to fill the 16 mm sample area. The beam was totally reflected using a gold mirror (M3) towards the area containing 10^{-7} diluted bacterial suspension. The moving iris was used to select the incident laser beam diameter.

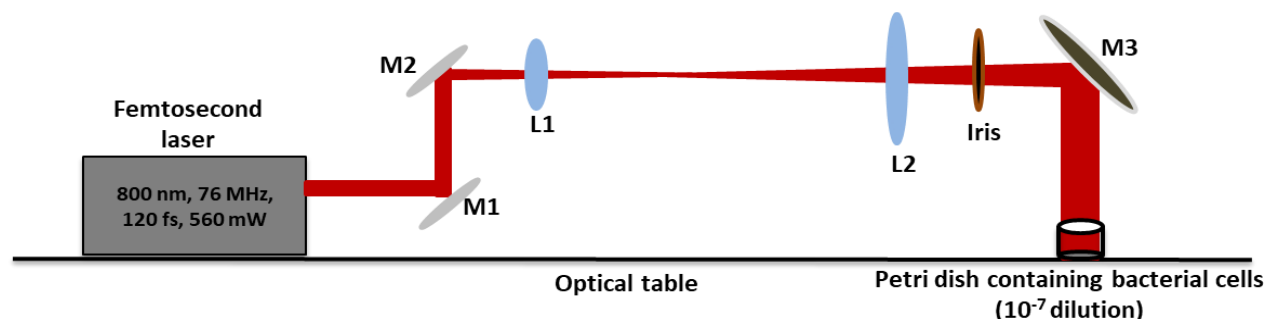

Figure 1: Custom-made optical setup used for irradiation of bacteria. A mode-locked Ti-sapphire pulsed fs-laser emitting a Gaussian laser beam expanded using a two lens telescope (L1 = 35 mm and L2 = 400 mm). The expanded beam was totally reflected towards the direction of the sample area using M3 to fill the surface area of the sample-well containing bacterial cells suspension. Irradiation was performed at 800 nm, 76 MHz repetition rate, 120 fs pulse duration, and 560 mW power output.

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Irradiation of *Escherichia coli* and *Salmonella enteritidis* was performed using laser average power of 560 mW at a repetition rate of 76 MHz with pulse train having a pulse width of FWHM = 120 fs. The typical power used in the experiments was about 410 mW measured using a Coherent Laser power/energy meter, LabMax_TOP.

2.3 Fluences and irradiation times

Fluence is defined as the energy dose received per unit surface area of a material. For pulsed laser systems, it is the pulse energy over irradiated area and is measured in J/cm^2. It is the combination of pulse radiation intensity and the pulse duration on a material surface (Kundwal et al. 2015). In this study, a range of fluences were pre-selected and the irradiation timed determined as follows:

$$Effective\ focal\ spot\ area = \pi r^2\ (r = \frac{1}{2}D) \qquad (D = 18\ mm) \tag{1}$$

Where r = radius, and D = diameter. Therefore, the effective focal spot area was 2.5 cm^2.

$$Intensity\ (W/cm^2) = \frac{Laser\ average\ power\ (W)}{Effective\ focal\ spot\ area\ (cm^2)} \qquad (Laser\ average\ power = 410\ mW) \tag{2}$$

Where, W = watts. The intensity was determined as 0.16 W/cm^2.

$$Irradiation\ time\ (t) = \frac{Fluence\ (J/cm^2)}{Intensity\ (W/cm^2)} \tag{3}$$

Where t = time in seconds, J = joules, and W = watts. The irradiation times were calculated and listed on table 1 below.

Table 1. Laser irradiation fluences and exposure times used to irradiate strains of *Escherichia coli* and *Salmonella enteritidis* microorganisms.

Fluence (J/cm^2)	Irradiation time
50	5 min
100	10 min
200	21 min
300	31 min
400	42 min
500	52 min
1000	1 hour 44 min
1500	2 hours 36 min

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2.4 Experimental procedure

Post 18 hours bacterial cell culture under rotary conditions, a serial dilution of the bacterial suspension was performed using 1 mL of the suspension added to 9 mL of Hank's Balance Salt Solution (HBSS; Gibco, Life Technologies, 14170-088). The 10^{-7} dilution was used for experiments and non-irradiated negative-control. An amount of 1 mL of the dilution was added into 16 mm diameter wells of a 12-well plate for irradiation and for negative-control. The experiment samples were sham-irradiated at different fluences. Post irradiation, 100 µL of the sample was used to streak each agar plate in duplicates and subsequently incubated overnight for bacterial count (colony forming units), and an additional 500 µL was added to 500 µL LB broth and incubated at 37 °C under rotary conditions (180 rpm) for 18 hours and used for biological assays. The same was done for the negative-control.

2.5 Cell morphology

A bright field inverted light microscope (CKX41, Olympus) with an attached CCD camera was used to study the differences in cell morphology between the three microorganisms of interest and to capture images.

2.6 Bacterial count

Differences in the number of colonies post irradiation in the experiment and negative control was studies using images captures with a cellphone. The number of colonies in each agar plate were counted and compared to the number of colonies in the negative control. To calculate the CFUs, the following formula was used:

$$Colony\ Forming\ Units\ (CFU\ \mathrm{ml}^{-1}) = \frac{Number\ of\ counted\ colonies}{Amount\ plated\ (ml)\ X\ dilution} \quad (4)$$

The colony forming units are expressed as Log_0.

2.7 Adenosine triphosphate assay

A CellTiter-Glo® Luminescent Cell Viability Assay kit (Promega, G7571) was used to study bacterial cell viability. This assay determines the number of viable cells in culture based on quantification of adenosine triphosphate (ATP) which indicates the presence of metabolic active cells. A sample volume of 1 mL from the bacterial sample prepare over 18 hours post irradiation and in the negative-control was centrifuged at 10 000 rpm, supernatant removed and the pellet resuspended with 200 µL of HBSS buffer. 50µl of cell suspension was added to corresponding wells of the 96-well plate (in duplicates), subsequently 50µl of the substrate was added and the mixture was incubated on an orbital shaker for 2 min, followed by incubation at room temperature (dark) for 10 min to stabilise the signal before reading the plate on the GloMax® 96 Microplate Luminometer.

2.8 Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) is a cytosolic enzyme released into culture supernatant during cell membrane/ wall damage. A CellTiter 96® Non-Radioactive Cytotoxicity Assay kit (Promega, G1781) was used to quantitatively measure the amount of released LDH. To perform this assay, 50 µl of the culture supernatant (from Sec. 2.7) was added to wells of the 96-well plate, followed by addition of an equal volume of the substrate mix and further incubated at room temperature (dark) for 30 minutes. Followed by addition of 50 µl of the stop solution and mixing of contents by pipetting. Absorbance at 490 nm was recorded using a 96-well plate reader on the GloMax® 96 Microplate Luminometer.

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2.9 Statistical analysis

For statistical analysis, all the experiments were performed in duplicates and repeated three times ($n = 3$). An average of the results was used to plot graphs. Sigma Plot version 14.0 was used for statistical analysis and the mean, standard error and significant differences were calculated. A student t-test and analysis of variance was performed to determine the statistical difference between the negative-control and experimental groups. Results were considered statistically significant at $P < 0.05$. Statistical differences between negative-control and experimental groups are shown in graphs as * = $P < 0.05$, ** = $P < 0.01$, and dispersion bars represent standard error.

3. RESULTS AND DISCUSSION

3.1 Cell morphology

Prior to laser irradiation experiments, *E.coli* and *Salmonella enteritidis* microorganisms were studied under bright field microscope using 40 X microscope objective to confirm viability through bacterial locomotion and analysis of the different shapes of the microorganisms. *E. coli* displayed a rod-shaped morphology and *Salmonella enteritidis* also displayed a rod-shaped structure as documented in literature. However, *E.coli* bacterial cells were larger in size compared to *Salmonella enteritidis* bacterial cells (Fig. 2).

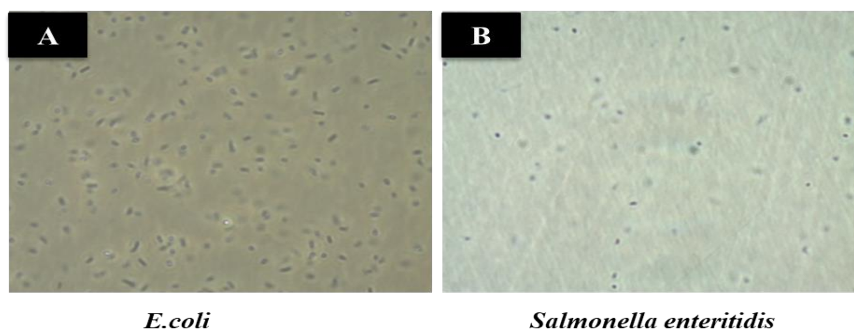

Figure 2: Representation of the morphology of *E.coli* and *Salmonella enteritidis* studied under bright field microscope using 40 X microscope objective. Both bacterial cells displayed a rod-shaped morphology and *E.coli* was larger in size compared to Salmonella enteritidis bacterial cells. Both microorganisms were constantly in motion while under study.

3.2 Colony forming units (Bacterial count)

A comparison of the negative-control and the photoirradiated group was conducted post laser exposure. There was a notable decline in the number of colonies in the irradiated experimental groups for both *E.coli* and *Salmonella enteritidis* (Fig. 3). It is also noted on figure 3 below that *E.coli* forms relatively larger colonies compared to *Salmonella enteritidis*.

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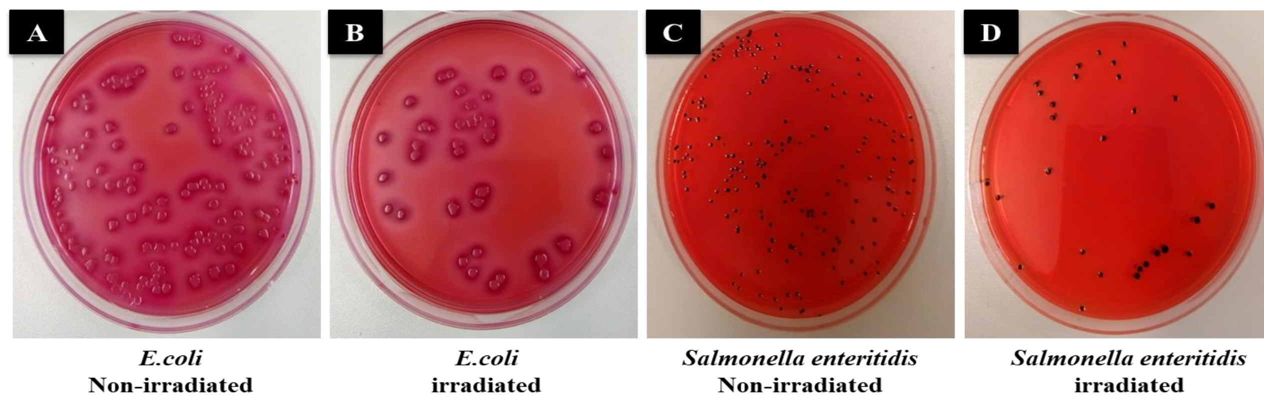

Figure 3. Images of *E.coli* and *Salmonella enteritidis* captured with a cellphone. There is a notable decrease in colony number in the irradiated groups (B and D) compared to the non-irradiated negative-control groups (A and C) for both microorganism. *E.coli* forms larger colonies compared to *Salmonella enteritidis.*

The colony numbers of the experimental groups irradiated using a range of fluences (50 J/cm^2, 100 J/cm^2, 200 J/cm^2, 300 J/cm^2, 400 J/cm^2, 500 J/cm^2, 1000 J/cm^2, and 1500 J/cm^2) were counted and bacterial cell count (CFUs) was calculated using equation-4 listed in section 2.6 above. The negative-control (non-irradiated) was compared to each of the experimental groups to determine the effect of different levels of fluence on bacterial cells. Results were considered statistically significant at $P < 0.05$. Statistical differences between negative-control and experimental groups are shown in graphs as * = $P < 0.05$, ** = $P < 0.01$, and dispersion bars represent standard error (Fig. 4)

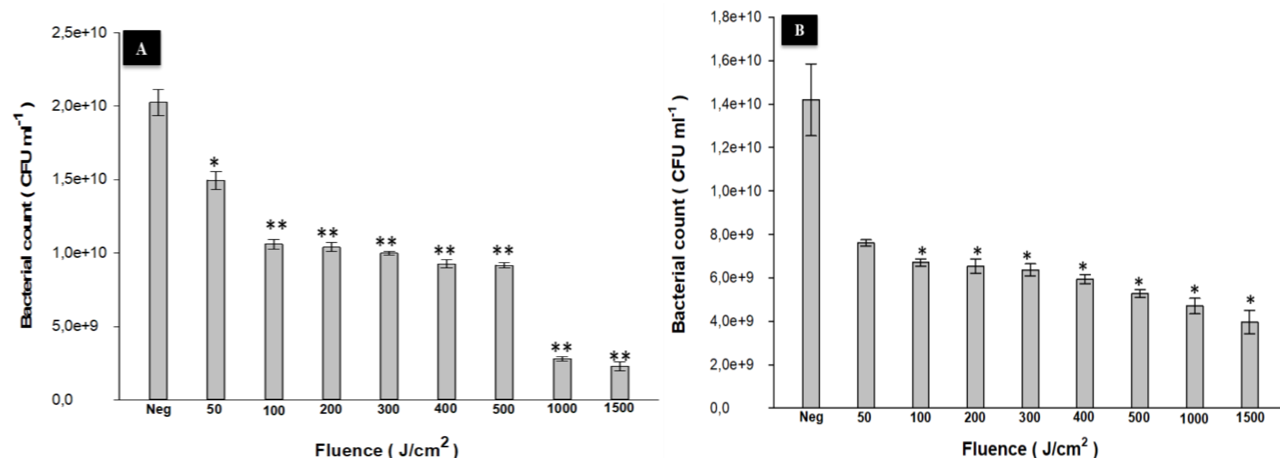

Figure 4: Graph representation of bacterial count (CFUs) for both microorganisms. Graph-A = *E.coli* bacterial count and Graph-B = *Salmonella enteritidis* bacterial count. Both microorganisms were sensitive to the NIR pulsed femtosecond laser irradiation from as little as 50 J/cm^2 fluence level. There is a dose-response decline in the experimental groups compared to the non-irradiated bacterial cells.

Samples for laser irradiation were prepared by picking a single colony to prepare a bacterial suspension over 18 hours at 37° C under rotary conditions (180 rpm), subsequently using 10^{-7} dilution to conduct experiments. It is noted on figure 4-A (*E.coli*) that the negative-control consists of high copy number of bacteria compared to figure 4-B (*Salmonella enteritidis*). This could be as a result of *E.coli* forming larger colonies than *Salmonella enteritidis* observed on figure 3 above. There was a significant decline in *E.coli* bacterial count when the negative-control was compared to all the experimental groups. *E.coli* bacterial cells irradiated at 50 J/cm^2,

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100 J/cm^2, 200 J/cm^2, 300 J/cm^2, 400 J/cm^2, 500 J/cm^2, 1000 J/cm^2, and 1500 J/cm^2 displayed a dose-dependent decline in bacterial count ($P < 0.05$ for 50 J/cm^2 and $P < 0.01$ for 100 J/cm^2 to 1500 J/cm^2). These results suggest that an increase in fluence in this study resulted to a further decline in bacterial count. The same was observed in *Salmonella Enteritidis* (Fig. 4-B). There was a decrease in bacterial count when the negative control was compared to bacterial cells irradiated at 50 J/cm^2; however the decline was not statistically significant. There was dose-dependent decline when the negative control was compared to bacterial cells irradiated at 100 J/cm^2 up to 1500 J/cm^2 ($P < 0.05$). Strains of *E.coli* were much sensitive to 1000 and 1500 J/cm^2, however a major decline in bacterial count when *Salmonella enteritidis* bacterial cells were exposed to fluence of a little as 50 J/cm^2 and then followed by a gradual dose response decline. These finding suggests that the *Salmonella enteritidis* bacterial cells were much sensitive to laser irradiation than *E.coli* bacteria used in this study.

3.3 Adenosine triphosphate (ATP) assay

In ATP assay, the amount of quantified ATP secreted by metabolic active cells is used as an indication of viable cells present in culture. Therefore this assay is used to assess the viability of cells (Fig. 5).

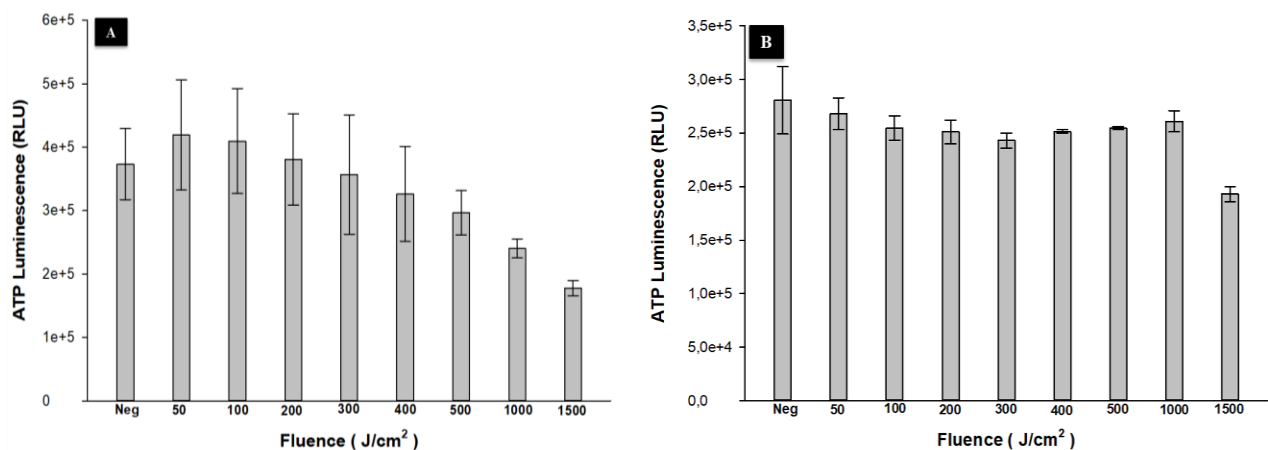

Figure 5: Graph representation in ATP release with respect to an increase in fluence. Graph-A = *E.coli* ATP assay and Graph-B = *Salmonella enteritidis* ATP assay.

From the findings in ATP assay, there was no statistical significance in cellular viability when the negative-control was compared to the experimental cell groups irradiated at 50 J/cm^2 to 1500 J/cm^2 in both *E.coli* and *Salmonella enteritidis* microorganisms. However, a dose-response decrease in ATP release was noted in *E.coli* while fluctuations in ATP release were observed in *Salmonella enteritidis* ATP assay. A major decline in ATP release in *E.coli* and *Salmonella enteritidis* was observed when bacterial cells were irradiated at 1500 J/cm^2; however it was not statistically significant.

3.4 Lactate dehydrogenase (LDH) assay

The LDH assay was performed to quantitatively measure the amount of released LDH as a result of cell membrane damage. Lactate dehydrogenase is a cytosolic enzyme which is released by cells when the cell membranes has been damaged, hence making it a crucial indicator or marker for necrosis (Fig. 6)

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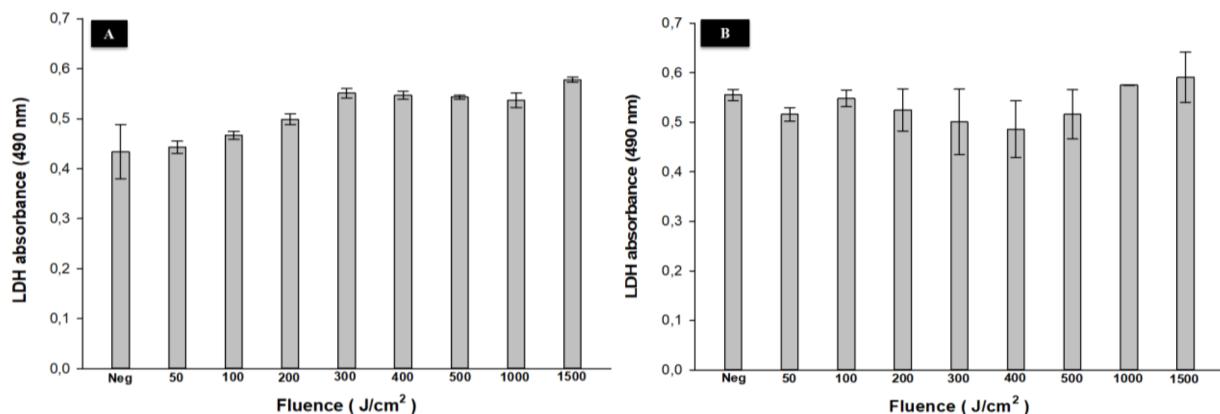

Figure 6: Representation of cell membrane damage by measuring the amount of LDH release into culture supernatant. Graph-A = *E.coli* LDH assay and Graph-B = *Salmonella enteritidis.* There was no statistical significance when the negative-control was compared to the experimental groups of both microorganisms under study.

In the *E.coli* LDH assay, there was a dose-dependent increase in LDH release with respect to an increase in fluence. Interestingly, there was a dose-dependent decrease in ATP release in *E.coli* observed on figure 5 above. A similar finding of fluctuations in LDH release was observed in *Salmonella enteritidis* such as it was observed in ATP fluctuations on figure 5 above. There was no statistical significance in the amount of release LDH for both *E.coli* and *Salmonella enteritidis* when the negative-control was compared to the experimental groups.

4. CONCLUSION

The finding in this study suggest that, NIR pulsed femtosecond lasers are suitable for inactivation of microorganisms. From the reported experimental results it was discovered that *Salmonella enteritidis* is much sensitive to fluences as little as 50 J/cm^2 and *E.coli* responded much positively to 1000 and 1500 J/cm^2 fluence as much as there was a significant decline from 50 J/cm^2. These findings suggest that different microorganisms may not respond similarly to laser conditions and doses. It was also observed that *E.coli* follows a dose-dependent decrease in ATP release and a dose-dependent increase in LDH release with respect to an increase in fluence, while fluctuations in ATP and LDH release were detected in response to an increase in fluence. Various studies have also conducted microorganisms inactivation using visible light especially blue light, commonly using 405 nm and 435 nm wavelength and have had similar findings of successful microorganisms inactivation. Therefore, future prospects of this study will focus on detailed profiling of various microorganisms (both bacteria and virus) for optimum laser parameters for inactivation without the use of photosensitizers and causing DNA damage on the irradiated microorganisms.

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