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# Comparison of Pulsed Laser and Continuous Wave UV Light in Inactivation of *Escherichia coli* and *Listeria monocytogenes*

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**Abstract**

Ultra violet (UV) lasers have some potentials and promising applications in the food industry, medicine and dentistry for inactivation of pathogenic bacteria of various types. The use of laser and other pulsed light systems for phototherapy and other microbial disinfections are based on spectral characteristics and configuration of the light used. In this study, a 355 nm pulsed UV laser was obtained from a passive Q-switched pulsed infrared (IR) laser by non-linear optical conversion. The IR laser and its 2$^{nd}$ and 3$^{rd}$ harmonics were used to irradiate samples of *Escherichia coli* and *Listeria monocytogene* at various energy doses and for different sample volumes. A 350nm continuous wave flash lamb, with output configuration close to that of the 355 nm pulsed UV laser, was also used for the purpose of comparison.The result indicates a statistically significant difference between the mean values of log reduction due to treatments with the pulsed UV laser and continuous wave UV light. However, there was no significant difference between log reductions for the different sample volumes used.

**Keywords:** Pulsed Laser; Continuous Wave Light; Laser Wavelength; Pulsed Frequency; *Escherichia coli* and *Listeria monocytogenes.*

## Introduction

The use of light to inactivate microbes has continued to attract the attention of researchers for some obvious reasons. Traditional methods of pasteurization or sterilization using thermal or chemical treatments have generally been effective. However, they have certain shortcomings. Pasteurization of food materials and other methods of thermal sterilization results into rise in temperature in the bulk of the material, which may be undesirable. Pasteurization also leads to the emergence of pasteurization-resistant bacteria. Thus, there is considerable interest in developing alternative methods for the control of harmful microorganisms; methods which will be effective in deactivating the microbes and yet have less or minimal damaging effect on the material being processed. Studies, aimed at exploring the nature and expanding the applicability of various spectral ranges of light for this purpose has been promoted in recent times.

Pulsed light is one such emerging technology that has the potential to inactivate microorganisms very rapidly on exposed surfaces. It is a relatively novel technology which could be an effective alternative to traditional thermal treatment in order to assure the microbial quality and safety of food products (Artíguez *et al.*, 2011). Previous studies conducted (Bohrerova *et al.*, 2008; Cheigh *et al.*, 2012; Farrell *et al.*, 2011; Hierro *et al.*, 2011) indicate the potentiality of pulsed light for inactivation of bacteria. These studies suggested pulsed light to be a more efficient non-thermal technology for decontamination and sterilization. The pulsed light disinfection systems can therefore be used to destroy dangerous microorganisms in water treatment systems, in air and on other contaminated surfaces.

Pulsed lasers also play some role in microbial inactivation. Recently, the use of laser for phototherapy in medicine and dentistry has featured prominently in various studies (Asnaashari and Safavi, 2013; Guffey *et al.*, 2013; Maden *et al.*, 2013; Schoenly *et al.*, 2012). A major advantage in the use of laser is that lasers, particularly, pulsed lasers, can provide narrow-band emissions of high-intensity, with desirable spectral width and penetration depth in samples (Azar Daryany *et al.*,

2008; Falkenstein, 2001). Another advantage is the fact that laser light makes it easier to provide for easy manipulations and automation (Brygo *et al.*, 2006).

In this study, the microbial inactivation effectiveness of pulsed laser was compared to that of a continuous wave light of comparable output configuration. Pulsed lasers at the infrared, visible and ultraviolet (UV) regions were used in comparison with a continuous wave light UV light to inactivate *Escherichia coli* and *Listeria monocytogenes.*

## Materials and Methods

### *Media and sample preparation*

Four liquid media - Nutrient broth (NB), Nutrient agar (NA), Tryptic soy agar (TSA) and Saline (0.85% NaCl) – all manufactured by Merck, Germany, were first prepared in accordance with manufacturer's instruction and autoclaved (see Table 1). About 20 ml of either NA or TSA were poured into 90 mm petri dishes for plating. The NA plates were used for plating *E. coli* samples while the TSA plates were used for *L. Monocytogenes* samples.

**Table 1:** Media used for experimental study of bacteria inactivation

| **Medium** | **Concentration (g/ml)** |
|---|---|
| Nutrient broth (NB) | $8 \times 10^{-3}$ |
| Nutrient agar (NA) | $2 \times 10^{-2}$ |
| Tryptic soy agar (TSA) | $4 \times 10^{-2}$ |
| Saline (0.85% NaCl) | $8.5 \times 10^{-3}$ |

*E. coli* (ATCC 11775) and *L. monocytogenes* (ATCC 7645), both from UNISEL, were used for the study. In preparing each sample, a colony of bacteria sample, previously grown on NA plate for 24 hours at 37 $^{o}$C, was aseptically inoculated into 100 ml of NB in a sterilised universal bottle and this was also incubated for 24 hours and at 37 $^{o}$C. The 100 ml culture was then centrifuged at 10000 rpm and 37 $^{o}$C for 15 min. The pellets of sediment obtained in the centrifuge tubes were washed with saline and centrifuged again under same condition as previously. Then 50 ml of saline was added to the final sediment from each of the tubes and shaken vigorously to form a supernatant. Final sample used for treatment was obtained by diluting 5 ml of the mixed supernatant with 95 ml of saline. Decimal serial dilution and plating were done on the sample to determine the initial bacteria concentration. The initial concentration was of the order of $10^5$ or $10^6$ cfu/ml, similar to those of previous studies conducted with pulsed light (Bandla *et al.*, 2012; Franz *et al.*, 2009; Gayán *et al.*, 2011; Maktabi *et al.*, 2011).

### *Sources of radiation*

Three laser wavelengths were used in this study. The fundamental wavelength of 1064 nm, a pulsed infra-red laser (PIRLa), was obtained from a passive Q-switched, flash lamp-pumped Nd:YAG laser (Beijing Anchorfree Model XM100). The second harmonic of the IR laser - a pulsed green laser (PGLa) at 532 nm, and the third harmonic - a pulsed UV-A laser (PUVaLa) at 355 nm were obtained by the process of non-linear optical conversion. Operating input voltage of the laser was fixed at 900V. The output pulse energy, $E_p$, of the laser was measured with a power meter (MILLES GRIOT) while the maximum beam diameter, $d$, was specified by the manufacturer as 6 mm. The specified spot diameter was adopted because focusing lenses were not used. Moreover, spot diameters of the output of the harmonics were confirmed to approximate to the value specified for the fundamental beam, as measured by method of the area of burned photographic paper (Tamuri *et al.*, 2008).

Total fluence $F_{total}$ or energy dose delivered to sample was approximated from pulse fluence, $F_{pulse}$, the pulse spot area, $A$, pulse energy, $E_p$, the pulse repetition rate, $R$, treatment time, $t$, using the expression

$$F_{total} = F_{pulse} \times R \times t$$

i.e.

$$F_{total} = \frac{E_p}{A} \times R \times t \quad (1)$$

In order to make comparison with the laser at the UV domain, a continuous wave UV-A light (CWUVaLi) from a flash lamp (UV Tools) operating at 350 nm was also used. The power of the lamp was 4W with spot diameter of 13 mm. Total fluence $F_{total}$ or energy dose delivered to sample for the lamp was approximated as follows:

$$F_{total} = Fluence\ rate \times t$$

i.e.

$$F_{total} = \frac{P}{A} \times t \qquad (2)$$

where P is power of lamp and A is the lamp beam area.

The set parameters used for the laser wavelengths and UV light are summarised in Table 2.

***Preparation of test samples***

Three aliquot parts of each of the prepared samples were poured into three sterilised beakers containing identical magnetic stirrer. One part was used as control while the other two were the treatment samples. All three beakers were wrapped with aluminium foil and placed on three similar stirrer plates, which were set to rotate at same speed. The treatment samples were subjected to laser radiation and continuous wave UV-a light for some appropriate time. The control sample was not irradiated.

***Pulsed laser versus continuous wave light***

To observe effect of the different laser wavelengths used, 40 ml of the prepared sample was used for both the control and the treatments. The treatment samples were irradiated at approximately same energy dose in sterilised 80 ml beakers, using the pulse infra-red laser and the continuous wave UV-A light. Similar 40 ml of the *E. coli* samples were also treated with the pulse green laser and pulse UV-A laser at approximately same total energy dose as used for the pulse infra-red laser and the continuous wave UV-A light. All three laser irradiations were done at pulse frequency of 5 Hz. The laser parameters were set such that the three laser wavelengths used produce approximately same total energy dose (see Table 2). The above procedure was repeated for three other values of total energy dose.

***Effect of sample size***

To observe any possible effect of sample size, three quantities (40 ml, 50 ml and 80 ml) of both *E. coli* and *L. monocytogens* samples were each subjected to irradiation with PUVaLa at pulse frequency of 5 Hz. Same container size (sterilised 100 ml beaker) was used for the three sample volumes so that three different sample depths were obtained for each of the sample types used. Each sample volume was treated for 15 min, 20 min and 30 min. The same procedure was also repeated for CWUVaLi.

***Serial dilution and colony counting***

After the treatments, decimal serial dilutions of up to the third decimal were performed on each sample. About 0.1 ml of both control and treated samples were plated in duplicates using the automated plating machine (Interscience Easy Spiral). The plates were incubated for 24 hours at 37 $^{o}$C after which viable cell counts were performed using a manual colony counter.

Viable cell densities (VCD) were calculated in accordance with standard plate count rules and guidelines (Sutton, 2011; USFDA, 2001). Measure of inactivation for each sample was estimated using the log reduction (LR) of population (Hamilton, 2011; Wang *et al.*, 2005) which is defined as

$$LR = log\ (VCD)_0 - log(VCD)_{trt} \qquad (3)$$

where $(VCD)_o$ is the viable cell density (in cfu/ml) of the original sample and $(VCD)_{trt}$ is the viable cell density (in cfu/ml) of treated sample.

In order to eliminate systematic error arising from factors such as bacteria death due to ambient conditions and the experimental procedure, the actual log reduction of a sample due to a particular radiation was calculated as follows (Franzen *et al.*, 2011):

$$LR_{rad} = LR_{trt} - LR_{ctr} \qquad (4)$$

where $LR_{rad}$ is the log reduction due to radiation, $LR_{trt}$ is the log reduction of treated sample and $LR_{ctr}$ is the log reduction of control sample.

$$LR_{Rad} = (\log VCD_0 - log\ VCD_{trt}) - (\log VCD_0 - log\ VCD_{ctr}) \quad (5)$$

$$LR_{Rad} = log\ VCD_{ctr} - log\ VCD_{trt} \quad (6)$$

**Result and Discussion**

***Pulsed Laser versus Continuous wave Light***

The log reductions at different values of energy dose supplied for the three laser wavelengths and continuous wave UV light are shown in Table 2. Experiments were performed in triplicates and values of log reduction are given as mean ± standard error (M±SE). A summary of the descriptive statistics of the result for this experiment has been presented in Kundwal *et al.* (2015). In order to discuss the result based on inferential statistics, a null hypothesis was proposed that "*There is no significant difference between the mean values of log reduction due to treatment with the three laser wavelengths used in this study*." The result of the inferential statistics, using a two-way analysis of variance (ANOVA) at 0.05 level of significance, for comparing the mean log reductions as a result of the three laser wavelengths used for the study are presented in Table 3.

The experiments designed to observe the effects of the different laser wavelengths were conducted for 4 levels of dose of radiation. A two-factor (two-way) ANOVA for the data obtained indicates that the probability value (P-value) for the effect of the various wavelengths used is less than the critical value ($P_{crit}$) of 0.05. Thus the null hypothesis was rejected. On the other hand, the P-value for the effect of energy dose was greater than the critical value. Therefore, the null hypothesis was accepted that there is no significant difference between mean values of log reduction due to different energy doses.

**Table 2:** Laser and UV light parameters used for treatment of *E. Coli* and corresponding log reduction obtained

| ***Radiation*** | ***Pulse energy (±0.5 mJ)*** | ***Treatment time (± 0.5s)*** | ***Approximate energy dose ($J/cm^2$)*** | ***Log reduction*** |
|---|---|---|---|---|
| PIRLa | 396.0 | 89.0 | 624.0 ± 3.6 | 0.2912 ± 0.0085 |
| | | 179.0 | 1256.0 ± 3.9 | 0.3548 ± 0.0272 |
| | | 268.0 | 1880.0 ± 4.2 | 0.4506 ± 0.0213 |
| | | 536.0 | 3760.0 ± 5.9 | 0.4729 ± 0.0179 |
| PGLa | 136.0 | 260.0 | 625.0 ± 2.6 | 0.5138 ± 0.0120 |
| | | 521.0 | 1252.0 ± 4.8 | 0.5095 ± 0.0065 |
| | | 782.0 | 1880.0 ± 7.0 | 0.5031 ± 0.0120 |
| | | 1564.0 | 3760 ± 14 | 0.4802 ± 0.0147 |
| PUVa-La | 118.0 | 300.0 | 627.0 ± 2.9 | 0.6385 ± 0.0196 |
| | | 600.0 | 1253.0 ± 5.4 | 0.6614 ± 0.0105 |
| | | 900.0 | 1880.0 ± 8.0 | 0.6874 ± 0.0133 |
| | | 1800.0 | 3759 ± 16 | 0.5091 ± 0.0093 |
| CWUVa-Li | *4.0 | 208.0 | 627.0 ± 1.5 | 0.2916 ± 0.0038 |
| | | 416.0 | 1253.0 ± 1.5 | 0.3342 ± 0.0192 |
| | | 624.0 | 1880.0 ± 1.5 | 0.3562 ± 0.0042 |
| | | 1248.0 | 3759.0 ± 1.6 | 0.2805 ± 0.0172 |

* Power (in Watts)

(Error values for pulse energy and treatment time are instrument precision errors; energy dose errors are propagated errors while errors in log reduction are standard errors)

**Table 3:** Result of two-way ANOVA for log reductions of *E. Coli* at different energy doses for the three laser wavelengths.

| ***Source of Variation*** | ***SS*** | ***df*** | ***MS*** | ***F*** | ***P-value*** | $F_{crit}$ |
|---|---|---|---|---|---|---|
| Energy dose | 0.0080 | 3 | 0.0027 | 0.4814 | 0.7072 | 4.7571 |
| Wavelength | 0.1075 | 2 | 0.0538 | 9.7541 | 0.0130 | 5.1433 |
| Error | 0.0331 | 6 | 0.0055 | | | |
| Total | 0.1485 | 11 | | | | |

**Table 4:** Result for t-test for log reductions of *E. Coli* due to treatments with PUVaLa and CWUVaLi.

| ***Statistics*** | ***PUVaLa*** | ***CWUVaLi*** |
|---|---|---|
| Mean | 0.6241 | 0.3156 |
| Variance | 0.0063 | 0.0013 |
| Observations | 4 | 4 |
| Pooled Variance | 0.0038 | |
| Hypothesized Mean Difference | 0 | |
| df | 6 | |
| t Stat | 7.1030 | |
| P(T<=t) one-tail | 0.0002 | |
| t Critical one-tail | 1.9432 | |
| P(T<=t) two-tail | 0.0004 | |
| t Critical two-tail | 2.4469 | |

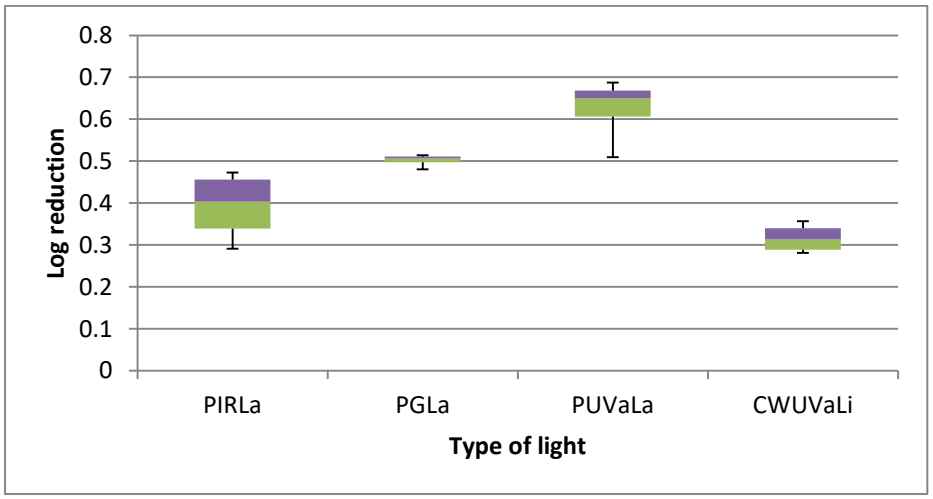


**Figure 1:** Box plots showing the distribution of data generated from treatment of *E. Coli* with laser at three different laser wavelengths and a continuous wave UV-A Light.

Lethal effect of pulsed light is said to depend on the energy dose (or fluence) incident on the sample in addition to the composition of the emitted light spectrum (Artíguez, *et al.*, 2011). The issue of fluence has featured prominently in studies involving interaction of pulse light with microbes (Artíguez and Martínez de Marañón, 2015; Azar Daryany, *et al.*, 2008; Baudelet *et al.*, 2009; Dinu *et al.*, 2002; Hsu and Moraru, 2011). For lasers in particular, consideration of fluence becomes important not only because of the role it plays in the level of inactivation achieved but also in the

limitation it places on the optimum performance of some of the optical components of the laser (Brinkmann *et al.*, 2012; Petrov *et al.*, 2013; Simrock, 2013).

The result from this work indicates that with use of same energy dose, the inactivation of *E. coli* using PUVaLa is slightly higher than those obtained using PIRLa and PGLa. Significant difference might have existed only between treatments with PUVaLa and PGLa as illustrated by the box plots of log reductions for the three laser wavelengths and CWUVaLi in Figure 1. The distribution of data for both PIRLa and PUVaLa overlap with that of PGLa but not with each other, thereby giving higher confidence on significant difference between mean values of data for PIRLa and PUVaLa.

The highest log reduction of approximately 0.7 was obtained with a dose of about 2000J/cm$^3$ of PUVaLa laser. In a similar result reported by Azar Daryany, *et al.* (2008), 16.7 J/cm$^2$ of 355 nm laser, with pulse energy of 60 mJ and pulse repetition rate of 10 Hz, was found sufficient to inactivate *E. coli* by 4 log. However, the measure of inactivation reported by Azar Daryany, *et al.* (2008) was $\log_{10}$ of concentration (in CFU/ml) and the initial concentration of sample used was of the order of $10^4$ CFU/ml whereas in this experiment, the initial concentration of sample used was at $10^6$ CFU/ml.

In comparison to CWUVaLi, a T-test was conducted to test the difference in mean values for the log reductions due to treatments with PUVaLa and CWUVaLi. Result of the T-test (presented in Table 4) gives the P-value to be less than 0.05. This suggests that at the level of significance of 0.05, the difference between the mean values of log reduction due to treatments with PUVaLa and CWUVaLi is not zero. This assertion is confirmed by the non-overlapping distributions of the data for PUVaLa and CWUVaLi from the box plots in Figure 1. The use of laser wavelengths in the IR and visible regions of the spectrum is prevalent, especially in medicine and dentistry, principally for safety reasons. Uncontrolled exposure to UV-C laser could result into serious health consequence for both patients and radiological personnel. The result from this study suggests a promising germicidal effect for PUVaLa in the UV-A region. Since the health risk associated with UV-A is less than that of the UV-C, a careful and well-controlled used of the UV-A could be explored for effective use in the dentistry or medicine.

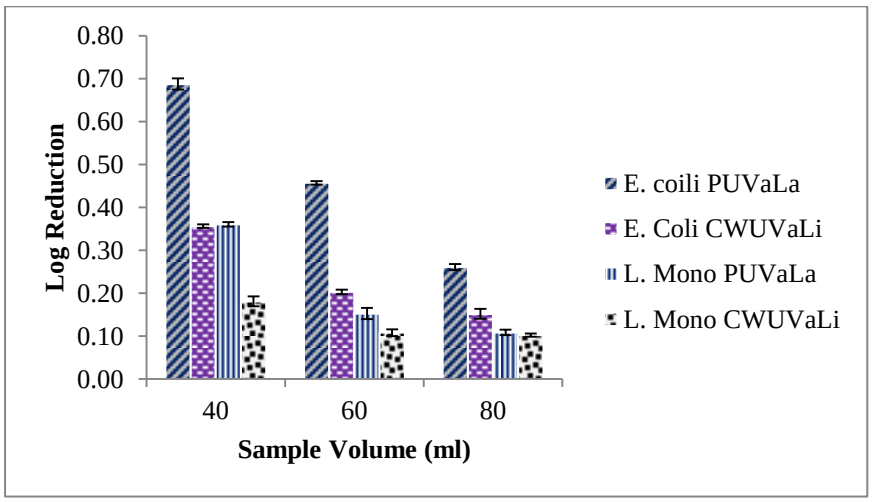


**Figure 2:** Column chart for *E. Coli* and *L. Monocytogenes* showing log reduction versus Sample depth using pulsed UV-A Laser (PUVaLa).

***Effect of Sample Volume***

Three sample volumes were irradiated at same treatment time in order to observe the effect of sample volume on log reduction. The column chart for the log reductions obtained after irradiation of three different sample volumes of both *E. Coli* and *L. Monocytogenes* with PUVaLa and CWUVaLi, each for 15 min, are shown in Figure 2. It was

hypothesize that "*There is no significant difference between the mean values of log reductions for the three sample volumes used in the study*." The ANOVA results for the data relating to this experiment are presented in Table 5.

**Table 5:** Result for two-way ANOVA for log reductions of *E. Coli* and *L. Monocytogenes* treated with PUVaLa and CWUVaLi at different pulse frequencies for different treatment times.

| ***Source of Variation*** | ***SS*** | ***df*** | ***MS*** | ***F*** | ***P-value*** | ***F crit*** |
|---|---|---|---|---|---|---|
| Type of treatment | 0.0712 | 1 | 0.0712 | 3.2290 | 0.1225 | 5.9874 |
| Volumes | 0.1210 | 2 | 0.0605 | 2.7429 | 0.1425 | 5.1433 |
| Interaction | 0.0196 | 2 | 0.0098 | 0.4436 | 0.6612 | 5.1433 |
| Within | 0.1324 | 6 | 0.0221 | | | |
| Total | 0.3442 | 11 | 0.3442 | | | |

Generally, the log reductions due to treatment with both PUVaLa and CWUVaLi tend to vary by almost same ratio with sample volume depth, with the shallower sample exhibiting higher log reduction. Although PUVaLa gave a higher log reduction for each of the three sample volumes, there seems to be no significant advantage in using the PUVaLa over CWUVaLi in relation to sample size. The P-values obtained for effects of the two factors tested and the interaction between the factors are all higher than the critical value. Therefore, the null hypothesis is accepted. However, further investigation may be required.

## Conclusion

Inactivation of bacterial samples with pulsed laser and continuous wave UV light has been attempted. Although the measure of inactivation obtained was very low (<1 LR), it was interesting to find that pulsed UV laser exhibits higher inactivation when compared to continuous wave UV light of close wavelength and beam profile. This could have some implication for some UV-phototherapies where small samples are involved. The result obtained in determination of effect of sample size on the two types of light was inconclusive and requires further investigation. It is therefore recommended that further studies carried out, using different experimental design, to ascertain the actual effect of sample depth on bacterial inactivation.

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