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Inactivation and Structural Changes of *E. Cloacae* and *B. Subtilis* Endospores during IR Laser Water Treatment

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Abstract

The efficiency of CO_2 infrared (IR) laser irradiation to disinfect water containing *E. cloacae* and *B. subtilis* endospores was determined and compared to ultraviolet (UV) treatment. Cell viability decreased with IR laser treatment for *E. cloacae*; however, increased viable cell concentrations were observed in *B. subtilis* endospores. Fourier Transform Infrared (FTIR) spectroscopy and the chemometric method of Principal Component Analysis (PCA) were used to examine cell changes. Increased carbohydrate content was observed in *E. cloacae* after IR laser treatment.

Keywords: Infrared; Water; Micro-organisms; Carbohydrate; FTIR

Introduction

IR radiation has been studied for micro-organism inactivation of bacterial spores on metal substrates [1] and on metal and paper substrates [2]. A near-point near-infrared laser water treatment apparatus for use in dental hand-pieces was also developed [3]. To date water sterilisation research using a mid-IR laser technique is very rare.

According to the World Health Organisation [4], examinations for faecal indicator bacteria remain the most sensitive and specific way of assessing the hygienic quality of water. Bacteria that fall into this group are *E. coli*, other coliform bacteria (including *E. cloacae*) and to a lesser extent, faecal *streptococci* [5]. Protozoan cysts from organisms which cause giardiasis are the most frequently identified cause of waterborne diseases in developed countries [6,7]. The use of aerobic bacterial endospores to monitor the efficiency of various water treatments has been shown to provide a reliable and simple indicator of overall performance of water treatment [8,9].The efficacy of IR radiation for water disinfection compared to UV treatment has been further investigated in the present study. In addition FTIR spectroscopy in conjunction with Principle Component Analysis was used to characterise structural changes within the bacterial cells and endospores following IR laser treatment. Changes in carbohydrate content of *E. cloacae* following IR laser treatment were observed.

Materials and Methods

Bacterial methodology

E. cloacae (QUT087) was grown on Nutrient Agar (NA) (Oxoid). A lawn inoculation onto sterile NA was created and incubated at 30°C for 15 hours. Half of the cells from the incubated NA plates were removed and resuspended in sterile 9.0 cm^3 0.9% NaCl solution and centrifuged (MSE Centaur 2) at 2000 rpm for 10 minutes. The supernatant was discarded and the cells resuspended in 9.0 cm^3 of sterile 0.9% NaCl solution.

B. subtilis (UQ40) was grown on R2A Agar (Oxoid) by subculturing from an existing culture held in the QUT culture collection. A pure colony grown on R2A was removed and resuspended in sterile 4.5 cm^3 0.9% NaCl solution followed by a lawn inoculation onto sterile R2A and incubated at 35°C for 15 days. Endospores were harvested [10] and the resulting suspension was placed into a water bath at 75°C for 15 minutes and then refrigerated at 4C.

The detection of *E. cloacae* cells and *B. subtilis* endospores before and during IR and UV experiments was performed by plating onto NA medium and counting by visual identification of the number of viable colonies present.

Reactor configurations

IR degradation studies were performed using a glass batch reactor (Figure 1A). IR irradiation was provided using a Series 48 CO_2 laser (Synrad) emitting primarily at 10.6 µm with an out-put at full power of 27 W and applied dose rate of 8.59 W cm^{-2}.

UV irradiation studies were performed using a batch reactor configuration (Figure 1B). The source was a low-pressure mercury vapour lamp with an applied dose rate of 5.4 mW cm^{-2}.

A volume of 10 cm^3 of 18 MΩ cm Milli-Q water with ~ 1.0 x 10^9 CFU cm^{-3} of *E. cloacae* or 1.0 x 10^7 CFU cm^{-3} of *B. subtilis* endospores were added to the reaction vessels. IR treatment consisted of repeated cycles of 10 second laser pulses, followed by mixing for 5 seconds. IR irradiation was applied for a total of 0, 10, 30, 60, 120, 180 and 270 seconds. UV exposure times were 0, 10, 30 60, and 120 seconds of continuous irradiation. Immediately after exposure cells were kept in the dark at 4°C until detection procedures were performed (no greater than 1 hour).

FTIR and Raman spectroscopy

FTIR spectra were obtained using a Nicolet 870 Nexus Fourier

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Transform IR spectrometer equipped with an ATR objective (Nicolet Instrument Corp., Madison, WI). An Optical Path Difference (OPD) velocity of 0.6329 cm s^{-1}aperture set to 100, and a gain of 8 was used. A Smart Endurance single reflection ATR accessory with a composite diamond internal reflection element (IRE) with a 0.75 mm sampling surface and a ZnSe focusing element was use. Bacterial samples were introduced as1.0 µL samples onto the diamond IRE and allowed air dry for 50 minutes to remove excess water. The spectral range was 4000-525 cm^{-1} with 128 scans at a 4 cm^{-1} resolution, and was corrected for the wavelength dependence of the ATR experiment. All spectra were normalised to the amide I band at 1650 cm^{-1}. Spectra were analysed using GRAMS32*AI*, Galactic Industries Corp., Salem, NH and chemometric performed using Grams32*AI* computer software package with the PSC-IE chemometric add-in (Galactic industries, Salem, NH).

Results and Discussion

Penetration depth of CO_2 laser beam into water

The depth of penetration of IR radiation into the test substrate determines how far into the substrate inactivation effects are expected to occur. According to Lambert-Bouguer law, the calculated absorption depth of 10.6 µm CO_2 laser radiation in water is 64 µm, with 90% of the energy being absorbed by the depth of ~ 20 µm.

Inactivation using IR and UV treatment

Figure 2A shows the percentage inactivation of *E. cloacae* vegetative cells during exposure of the bacterial cells to the IR laser and UV treatment regimes. In both experiments, a similar initial cell concentration was used (~ 1.0 x 10^9 CFU cm^{-3}). Cell inactivation occurred 27 times faster during UV irradiation experiments compared with IR irradiation experiments.

Figure 2B shows the inactivation of *B. subtilis* endospores during exposure to the IR laser and UV treatment regimes. While the reduction profile during UV treatment was similar to that of *E. cloacae*, the cell inactivation rate was slower, due to the increased resistance of the endospores to UV radiation. Despite this, 99.9% inactivation was attained after two minutes UV exposure. IR exposure did not cause any *B. subtilis* endospore inactivation and, in fact, resulted in a 270% increase in cell numbers after 180 seconds of IR exposure.

Both treatment methods follow first order kinetics. Therefore, inactivation rates were calculated using Chick's law [10]. Table 1 displays the inactivation rates for *E. cloacae* and *B. subtilis* endospores experiments during IR laser and UV treatment. The IR laser treatment system was found to be less effective than UV irradiation. In comparison to vegetative cells, the UV treatment inactivation rate of endospores

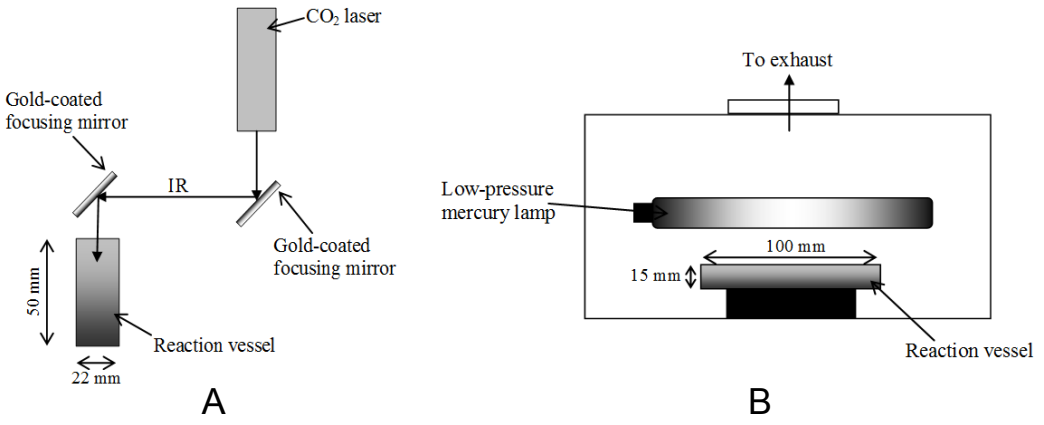

Figure 1: Schematic representations of (A) IR; and (B) UV reactor instrumental set-up (not to scale).

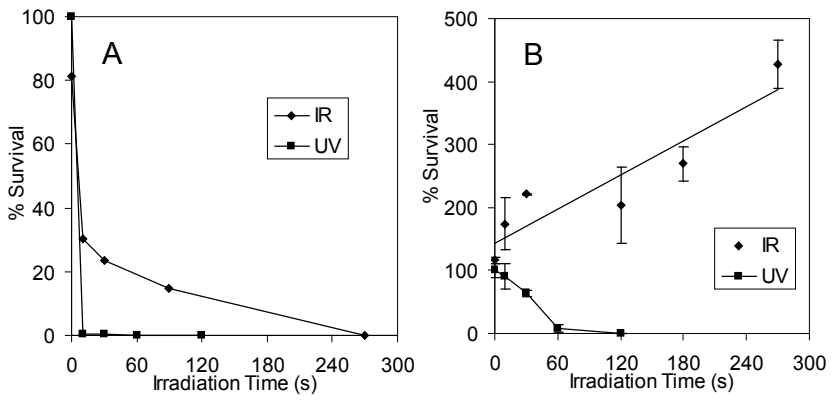

Figure 2: Inactivation of (A) *E. cloacae* and (B) *B. subtilis* endospores as a function of time during IR laser and UV treatment.

was approximately 40% lower than that obtained for vegetative cells, i.e. *E. cloacae*.

Effect of temperature change during IR treatment: Evidence for heat transfer in the present study is two-fold:

1. Increase in *B. subtilis* endospore concentration during IR treatment

B. subtilis endospores have been shown to germinate upon heat treatment at temperatures greater than 50°C [11,12]. Overall water sample temperatures increased from 23 °C to a maximum of 47.5 °C after 270 seconds of IR laser treatment, with localised boiling of the water surface layer also occurring. This could result in germination of *B. subtilis* endospores as witnessed by a 400% increase in endospore numbers after IR laser treatment for 270 seconds. Conversion of endospores into vegetative cells occurs within 30 minutes of germination [13]. Despite samples being kept at 4 C prior to analysis, it is probable that cellular processes were initiated, resulting in the increased viable counts.

2. Localised heating of the *E. cloacae* samples

E. cloacae's optimum temperature range is 30-37°C (1984). Therefore, the sudden increase in temperature beyond the optimum for *E. cloacae* cells most likely resulted in the observed cell death.

FTIR and Raman spectroscopy of bacterial cells and endospores

FTIR spectroscopy has proven a useful technique for investigating cellular changes within micro-organisms. The *B. subtilis* endospore spectra showed negligible changes after IR treatment. However, significant spectral changes occurred in the *E. cloacae* cells during IR treatment (Figure 3).

The υ(NH) region (~3200 cm^{-1}) denoted as the amide A and the υ(CH) region (3000-2800 cm^{-1}) due to fatty acids and methane groups show increased spectral intensity after treatment. Spectral variation within the region 1700-1500 cm^{-1}, dominated by amide vibrations also occurred. These bands are due to υ(C=O) at ~1635-1700 cm^{-1} denoted as amide I, and δ(NH) and υ(CN) at ~1550 -1520 cm^{-1} denoted as amide II. Assignment by curve fitting of these to protein confirmations revealed that proteins of the sample before treatment primarily consisted of β-turn and random coil configurations, while after treatment α-helices and random coils dominated [14]. A reduction in spectral intensity after IR treatment occurred in the mixed region (1450-1220 cm^{-1}) and is composed of two minor groups of variations: proteins, free amino acids and polysaccharide vibrations (1450-1400 cm^{-1}), and the DNA/RNA and phospholipid vibrations (1250-1200 cm^{-1}).

The polysaccharide region (1200-900 cm^{-1}) contains the υ(CO), υ(CH), δ(COH) and δ(COC) modes of vibration and showed the most significant differences during IR treatment. This was of interest as it is in this region that the laser emission will be directly absorbed. The increased carbohydrate content could be a consequence of the breakdown of long chain carbohydrate molecules into shorter chains, or single units, increasing the relative peak intensities within this region. However the optimum temperature range of *E. cloacae* is 30-37°C, and the greatest carbohydrate content was seen in those treated samples whose temperature was brought within this range. At this temperature, bacterial cells have the greatest ability to repair and renew damaged cellular components. It was more likely that the increased carbohydrate content seen within the bacterial samples was due to the repair of damaged carbohydrates and increased production of new carbohydrate molecules.

Micro-organism	*E. cloacae* cells		*B. subtilis* endospores	
Treatment type	IR	UV	IR	UV
% Inactivation after 60 s	81.83	99.99	-200	87.86
Inactivation Rate (CFU cm^{-3} s^{-1} 10^{-3})	25.1	100.25	-4.8	61.1
Inactivation Rate (CFU cm^{-3} kJ^{-1})	0.0154	4.03	-0.00296	2.40
R^2	0.972	0.865	0.995	0.959

Table 1: Inactivation rates for *E. cloacae* cells and *B. subtilis* endospores treated with IR laser and UV treatment.

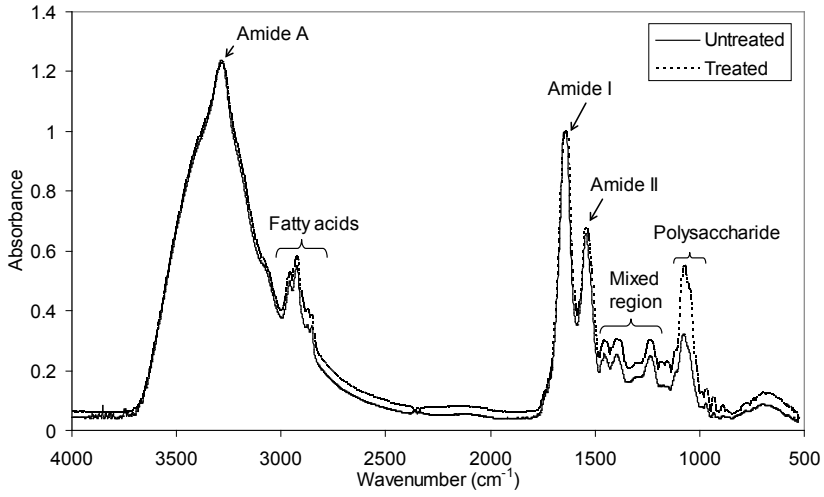

Figure 3: FTIR-ATR-corrected, normalised (to Amide I band at 1640 cm^{-1}) absorbance spectra of *E. cloacae* before and after IR laser treatment for 30 s.

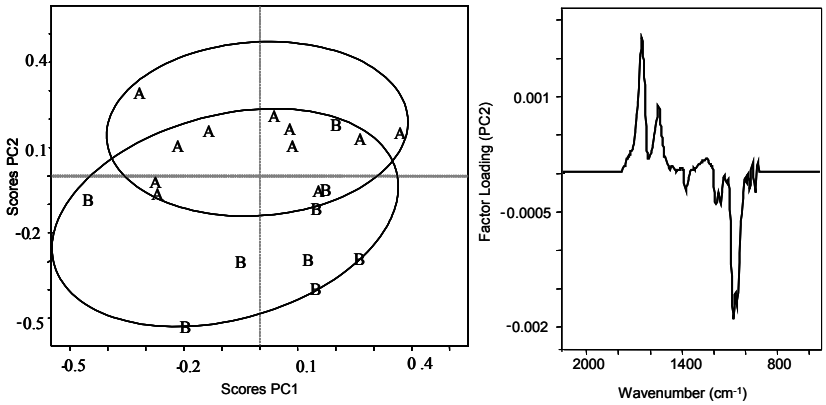

Figure 4: PC1 vs PC2 scores and loadings plots of *E. cloacae* IR treatment spectral data set from 1760 – 850 cm^{-1}.

Chemometric Principal Component Analysis (PCA) was applied to the FTIR-ATR spectra obtained for both *E. cloacae* and *B. subtilis* endospores using the IR laser and UV treatment regimes. The only experimental data set which showed cluster separation was the IR laser treated *E. cloacae* samples; however, cluster overlap was still seen. The entire spectral region was investigated, but it was the region from 1760-850 cm^{-1} that showed separation (Figure 4). Here, the before and after samples were separated by the PC 2 axis, predominately governed by positive factor loadings peaks at 1625 cm^{-1} (amide I), 1520 cm^{-1} (amide II) and 1240 cm^{-1} (phosphodiesters). Negative peaks at 1060 cm^{-1} (carbohydrate) indicated that untreated samples contained more protein, due to the increased amide I and II intensities, and that treated samples possessed a higher carbohydrate content than their untreated counterparts [15].

Conclusions

CO_2 laser irradiation was investigated for inactivation efficiency, and compared to that of UV irradiation, of *E. cloacae* and *B. subtilis* endospores. *E. cloacae* inactivation was achieved using IR laser treatment; however, this was most likely due to thermal heating of the sample. The more resistant *B. subtilis* endospores were not inactivated using IR laser treatment.

FTIR-ATR spectroscopy exposed dramatic increases in the polysaccharide content of the *E. cloacae*. Chemometric analysis applied to the FTIR-ATR spectra showed cluster separation for the IR laser treated *E. cloacae* samples indicating cellular changes occurred during the IR laser treatment. The changes identified using chemometric analysis were a decrease in protein content and an increase in total polysaccharide. As the IR laser used emitted within the polysaccharide region it was possible that these molecules within the bacterial cell absorbed sufficient energy to be damaged, which in turn could cause the cell to replace or repair the lost polysaccharides.

Although inactivation after exposure of *E. cloacae* to IR irradiation did occur, the IR laser treatment method failed to inactivate resistant micro-organisms, i.e. *B. subtilis* endospores, and may limit its utility for water disinfection purposes.

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