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Laser Sterilisation of Bacterial and Fungal Spores

Graham N Armstrong

Submitted for the Degree of Ph.D. in the Department of Mechanical Engineering at the University of Glasgow

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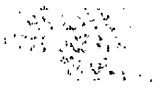

Abstract

Abstract

This work investigated the sterilisation efficiency of the Nd:YAG and CO_2 laser. This work centred on micro-organisms that were important to the food industry and resistant to sterilisation. The efficiency of laser sterilisation was ascertained and quantified. The efficiency of incorporating laser, UV irradiation and heating of bactericidal chemicals was also investigated. These results will aid the future development of novel sterilisation systems. The Nd:YAG laser was found capable of sterilising stainless steel. The addition of UV gave an added effect. Nd:YAG laser heating of hydrogen peroxide gave a marked improvement in the chemical's killing efficiency.

Nd:YAG irradiation completely sterilised *B. cereus* spores on discs of stainless steel (mass 5g) with an energy density of 1800Jcm^{-2}. By decreasing the beam diameter, the percentage of the beam in contact with the spores increased from 43% to 72% but killing remained the same. Calorimetry showed that after exposing steel discs to 1800Jcm^{-2} with a beam diameter of 16 and 8mm the temperature of the steel was the same. Possible killing mechanisms were looked into and it was found that for the Nd:YAG laser it was primarily thermal, and not due to direct photonic interaction. This hypothesis was supported by the killing of spores on 2mm thin steel discs (mass 0.8g) with an energy density 120Jcm^{-2}. This was due to the thin steel heating up rapidly with the relatively low energy density. Electron microscopy showed that the spores responded cytologically in the same way after heating by Nd:YAG laser irradiation and water-bath heating.

It was attempted to increase the laser's killing effect by combining the laser with UV irradiation from 2, 8W UV lamps (254nm wavelength). Lawns of *B. cereus* spores were made and exposed to Nd:YAG laser irradiation (ED 3000Jcm^{-2}). No clearing was observed. By adding UV irradiation (sequentially) the diameter of clearance caused by the laser irradiation increased. To quantify the killing, aliquots of spore suspensions were raised in temperature with Nd:YAG laser irradiation to 90°C. There was little effect on the viability of the spores. When the treatment was preceded or followed by 10s of UV irradiation an increased killing effect was shown. To investigate the effect further, a

multifactorial experiment was designed, incorporating laser followed by UV irradiation of spores on stainless steel discs. These results were analysed by the analysis of variance method. It was found that the laser and UV had a significant effect on the spores, and the effect was additive.

Spores were submerged in sub-lethal concentrations of lactic acid and H_2O_2 and heated with Nd:YAG laser irradiation. There was a definite increased killing effect, and the rapid heating by the laser irradiation allowed low chemical concentrations and low temperatures to have a sporicidal effect. These experiments have shown that laser irradiation alone does have an effect on bacterial endospores, although at a reduced rate compared with vegetative cells. This killing affect can be increased using UV. A more efficient method to improve the laser killing effect was to combine the laser treatment with H_2O_2. The spores were then killed by laser irradiation at relatively low temperatures and with low chemical concentrations.

The treatment of spores of fungal species was also examined. These bodies are more resistant than the vegetative cells of bacteria. The aim of this section was to investigate scanning of spores with CO_2 laser irradiation. Lawns of spores on agar plates were scanned with 1kW irradiation at various speeds, good clearing was obtained, but there was a significant difference between the clearance of different fungal species. Artificially contaminated fruit was scanned with the same laser parameters, but no killing was observed, there was no clearing from the surface of contaminated cheese. A low power CO_2 laser was used and clearing of spores on agar with power as low as 6W (also species dependent) was achieved. The effects of this laser were also shown on cheese and 2W scans completely cleared its surface.

In conclusion, spores of both fungal and bacterial origin were inactivated with laser irradiation, and increased killing of bacterial endospores was achieved after laser irradiation was combined with UV irradiation, lactic acid and H_2O_2.

Promising aspects of this work include the success of the small scanning 13W CO_2 laser system, especially the spiral system that could lead into many branches of experimentation e.g. surfaces and food, and the combination of laser heated H_2O_2 treatment. This combination facilitates not only sterilisation at lower energy densities, but also overcome any problems associated with shadowing. These techniques could be elaborated

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List of Abbreviations

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ArF	argon fluoride
B. cereus	*Bacillus cereus*
°C	degrees centigrade
cfu	colony forming units
CO_2	carbon dioxide
cm	centimetre
DNA	deoxyribonucleic acid
ED	energy density
E. coli	*Escherichia coli*
g	gram
GaAlAs	gallium aluminium arsenic
HeNe	helium neon
hr	hours
Hz	Hertz
J	Joules
L	litre
LAF	laminar air flow
min	minutes
mJ	millijoules
mm	millimetre
msec	milliseconds
mW	milliwatts
NaOH	sodium hydroxide
NaCl	sodium chloride
nm	nanometre
nsec	nanoseconds
Nd:YAG	neodymium:yttrium aluminium garnet
PBS	phosphate buffered saline
ppm	parts per million

PRF	pulse repetition frequency
Ps. aeruginosa	*Pseudomonas aeruginosa*
psec	picosecond
p.s.i	pounds per square inch
PTFE	poly-tetra-fluoroethene
P. vulgaris	*Proteus vulgaris*
RNA	ribonucleic acid
S. aureus	*Staphylococcus aureus*
SDS-PAGE	sodium dodecyl sulphate-polyacrylamide gel electrophoresis
s	seconds
SEM	scanning electron microscope
Strep. sanguis	*Streptococcus sanguis*
TEM	Transmission electron microscope
µsec	microseconds
UV	ultra violet
W	Watts
XeCl	xenon chloride

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1.0 Introduction

Chapter 1 Introduction

1.1.0 Sterilisation Methods

1.1.1 Laser sterilisation

The laser was first shown to have a killing effect when Saks and Roth (1963) used a ruby laser on cells of the algae *Spirogyra* (between coverslip and glass slide). Irradiation of 20mJ per pulse caused the cells to disrupt, releasing the cell constituents. Lower levels of irradiation caused lower levels of damage i.e. darkening then coagulation of constituents. Loss of turgor (cell internal pressure) of target cells was also observed. The authors pointed out there was little damage to the cell out with the area directly targeted, so there could be a surgical use. This paper lead to an interest in laser sterilisation that continues today. The work was followed by McGuff and Bell (1966) who investigated the bactericidal effect of ruby (694nm), neodymium-glass (1060nm) and He-Ne (634nm) lasers on bacterial lawns. The ruby laser caused a temporary loss of pigment after a single pulse of 60J in *Ps. aeruginosa.* Increased pigment production in *Ps. aeruginosa* was observed after a single "burst" of 10 megawatt irradiation, this level of irradiation also caused "rough" colony morphology in *P. vulgaris.* The He-Ne laser irradiation had no effect upon *Ps. aeruginosa, P. vulgaris, S. aureus* or *B. cereus* until after a 300min exposure of 0.5milliwatt where *Ps. aeruginosa* pigment production was reduced. The Nd-glass laser (100J) had no effect until "black agar" was used (india ink). Under these circumstances, pigmentation varied from the centre of the exposure to the periphery. The paper showed that laser irradiation could have an effect on the growth of microorganisms. It appeared that rapid pulses (nanosecond range) of high energy laser irradiation had more effect on the microorganisms than longer exposures (5hr) of low level irradiation. It also showed that the wavelength of the irradiation was an important factor in the resulting effect.

The bulk of laser sterilisation papers have centred on the ability of the irradiation to sterilise instruments for the dental profession. Adrian and Gross (1979) gave evidence for the sterilisation of dental scalpel blades after 2min exposure of 10W CO_2 laser irradiation. Spores of *Bacillus* and *Clostridium* were the test organism. The writers placed spores on the blades, exposed them to the irradiation then tested for complete sterility, this was achieved. One important point was that after exposure, a few of the blades became discoloured where the irradiation had come into contact with heavier areas of innoculum. The paper did nt quantify the killing and the laser beam diameter

was not given. The writers also mentioned that the high cost of the laser and a trained operator made the system impractical at the time but that the possibility of a laser sterilisation system was good.

In this study it has become apparent that a lack of given details of the laser parameters is common in papers involving laser sterilisation.

Hooks *et al* (1980) from the same research group, expressed the need for a rapid but consistent means of "chair-side" sterilisation of dental instruments. They also cited their unpublished data claiming the CO_2 laser can be used to sterilise sharp instruments without affecting the cutting efficiency. In this study, *Bacillus* spores were sterilised on steel reamers with a 3s exposure of 10W CO_2 laser irradiation. Details in this paper were again sketchy, the number of spores on the reamers was not given (the stainless steel reamers were dipped in suspension for 20min) and the laser beam diameter was not given. The writers did not mention the previous problem of darkening of the test material. The paper also touched on the problems of practicality at that time.

Other research followed with a dental slant. In 1985, Myers and Myers showed that Nd:YAG laser irradiation could be used to clean pits in teeth without causing damage to the tooth. The pulse speed was 30psec (faster than the 200msec required initiating pain). The energy was 3.4mJ and 350 pulses were required for cleaning. These papers showed that there was diversity in the research ongoing at this time, in terms of laser used and application the system was aimed toward. Zakariasen *et al* (1986) were interested in the use of CO_2 laser to clean root canals, and this led to their interest in bactericidal action of the laser. The oral bacteria, *Actinomyces viscosus, Strep. sanguis, Strep. mutans* as well as *S. aureus, B. cereus and Ps. aeruginosa* were treated on glass slides. The killing ability of the laser varied to obtain a 3 D-value decrease in viability for each species. An energy density of 18.75kJcm^{-2} was required for *Strep. sanguis*, 9.37kJcm^{-2} was required for *S. aureus* and only 6.25 kJcm^{-2} required for the other four strains. The exposure was for up to 1s, the laser beam diameter 1-11mm. The writers concluded that the oral bacteria did not have a specific resistance to the laser irradiation.

In 1990 members of the group associated with Zakarisen (above), examined the efficiency of CO_2 laser killing of seven organisms on glass cover-slips. Dederich *et al*, used the orally associated organisms, *Actinomyces viscous* and four *Streptococcus* strains. *S. aureus* and *Ps. aeruginosa* were also used. The beam diameter was 2mm on a sample size of 1µl. Clinical *Streptococcus* strains were sterilised after 1273Jcm^{-2}. Above 32Jcm^{-2} more than 90% killing was observed in all the species used. The writers

again concluded that that the test organisms that originated from a specific environment i.e. the oral cavity, and may have adaptations for survival (envelopes etc) did not have any laser resistant qualities. It should be noted that in what was basically a reworking of the earlier paper, the levels of irradiation were markedly reduced, over 6kJcm^{-2} had been required previously for a 3 D-value decrease, yet 1273 Jcm^{-2} now gave sterility. No reason for this was given.

In 1991, Powell and Whisenant compared the effectiveness of the Nd:YAG, CO_2 and argon lasers at sterilising endodontic reamers, again citing the need for a novel fast, reliable (practical) system. The three lasers were capable of sterilising reamers dipped in vegetative bacteria, *Candida albicans* (a yeast) and *B. subtilis* spores. The argon laser did so at the lowest power, 1W and 120s exposure (120J). No other information on the laser parameters was given other than the lasers were used at 1, 2 or 3W for 30, 60 or 120s. The beam diameters were not given. This lack of information makes direct comparison of the results from different authors difficult.

In 1992, Cobb *et al* used Nd:YAG laser irradiation with the aim of sterilisation of pockets in teeth. The irradiation (1.75W, 20Hz, exposure 3min) appeared to vaporise sub-gingival plaque. The treated teeth were left for various periods of time. The cleared areas became re-contaminated after seven to twenty one days, presumably from the untreated areas. One drawback of the laser treatment could be that re-solidified globules of material that were produced could irritate the soft tissues of the mouth.

Stabholtz *et al* (1993) showed that the oral bacteria *Strep. mutans* could be sterilised in liquid and on the surface of blood agar plates with a XeCl laser (λ = 308nm). A 5.6Jcm^{-2} (8s exposure) dose gave approximately a 1-D value decrease in viability. On lawned blood agar plates, a 1Jcm^{-2} exposure gave over 40mm^2 of clearance. The writers hypothesised that this wavelength of laser irradiation would be useful against other organisms. Less than a year later, Whitters *et al* (1994) investigated the activity of pulsed Nd:YAG irradiation on oral bacteria. This work measured the effect of 80mJ, 10Hz or 120mJ, 15Hz pulses for up to 3 min on organisms found in dental plaque. The tests were carried out in plastic micro-centrifuge tubes. *Strep. Mutans* was the most resistant of the nine strains tested, in a set-up designed to recreate a root canal. The laser optic fibre was submerged in the bacterial suspension. In the discussion, Whitters criticised the lack of experimental details in previous work in this field. Also in this year, Rooney *et al* in 1994 also looked at Nd:YAG laser sterilisation. The test

organism, *Enterococcus faecalis* was used in the numbers found in root canals (1×10^5) in capillary tubes. The laser irradiation was quantified in terms of energy applied "J = W x t" no viable bacteria survived after 54 J exposure (0.9W x 60s). The addition of suomi ink increased the killing effect of the irradiation, 25J only being required. The writers, acknowledged that this model may not be directly applicable to the situation found in root canals.

Laser sterilisation research has also been aimed toward the possibility of the irradiation being used on skin and wounds. In 1985, Mullarky *et al* investigated CO_2 laser sterilisation on a porcine skin model. *E. coli* and *S. aureus* were effectively sterilised (approximately 7 D-value reduction). The laser parameters were 25W for 3s with a 3mm beam diameter. The skin was shaved before being decontaminated and "devitalised" but the writer felt that the porcine model results were still applicable to a live system. This was a high level of decontamination in a short exposure. The paper did not mention any problems of hair root pits becoming a source of bacterial protection and therefore a site for recontamination, or if indeed this was an anticipated problem. Al-Quattan *et al* (1989) investigated the efficiency of CO_2 laser irradiation and an iodine based disinfectant (betadine) for wound sterilisation. Wounds made on the flanks of rabbits were artificially contaminated with *Ps. aeruginosa*. After three days one flank was treated by laser irradiation, the other with betadine (concentration not divulged). The laser irradiation was 15W for 5 min (no beam diameter was given) until charring occurred. Only 10% of the laser treated wounds were found to contain viable bacteria, whereas 40% of the betadine treated wounds contained viable *Pseudomonas*. This paper showed the efficiency of the laser and also highlighted cases where patients suffered due to hospital infections and the financial implications. The authors did not comment on the discomfort caused (if any) by charring the tissue slowly over a 5min period. Lee *et al* (1993) showed that low levels (76.4 $mJcm^{-2}$) of GaAs laser irradiation (λ = 904nm) caused an increase in the healing rate of wounds in a rat model. The wounds were seeded with *S. aureus*. Not only did the wounds heal more rapidly, but a decrease in swelling was also observed, denoting a decrease in bacterial load as compared with controls that did not receive laser irradiation. This paper showed that the benefits of low-level laser treatment for wound healing could outweigh the possible problem of bacterial biostimulation caused by the irradiation. The authors did point out

though that the laser levels used in this study were below the 337mJcm^{-2} that they had previously found was optimum for biostimulation of bacteria.

Following the work of Al-Quattan *et al*, showing that the CO_2 laser irradiation was more effective than an iodine based disinfectant, Stranc and Yang (also in 1992), investigated the efficiency of the CO_2 laser irradiation compared to cautery. Again wounds in the flanks of rabbits were used as a test model. Cautery was more effective than the laser, but it was concluded that although both methods of sterilisation were more effective than disinfectant sterilisation, the necrosis of tissue caused by thermal effects, could be construed as too much of a problem.

In 1992 three papers were published investigating the effectiveness of laser sterilisation of agar, wounds and on teeth. This showed the considerable variation in possible uses for laser sterilisation.

Schultz *et al* in 1986 investigated the killing of *S, aureus*, *E.coli* and *Ps. Aeruginosa* with laser irradiation. These organisms were chosen as they are involved in osteomyeletis. Aliquots of suspension (0.5ml) received 120W of Nd:YAG laser irradiation of beam diameter 6mm for an exposure of 10s (energy density reaching 3333Jcm^{-2}). Sterility was obtained for the three test species. Congo red and methylene blue dyes were added to the suspension, but this had little effect on the rate of sterilisation.

Research has been carried out on microorganisms on agar, as this is seen as a relatively easy test. The smooth agar surface allows rapid results and ease of (surviving) bacterial growth. This allows comparisons in laser parameters to be made, as well as comparisons in laser types.

Yanagawa *et al* looked at the effect of the argon laser in 1992. The laser used was capable of emitting a beam of mixed wavelengths (458-514nm). The effect on Gram negative and positive rods and cocci was examined. The test suspensions (1ml) were mixed in molten agar and allowed to set. The laser irradiation was then applied at 72, 144 and 216Jcm^{-2} (exposure 30min). A thermocouple in the agar (depth 1mm) recorded a rise in temperature of 1°C. *Ps. aeruginosa* proved the most sensitive of the organisms tested. This was important as this organism can be antibiotic resistant. It appeared in this work that the Gram positive organisms tested were resistant to the argon laser irradiation, this they suggested, was due to the presence of such molecules as teichoic

acid in the Gram positive wall, providing protection. Some of the Gram negative species used i.e. *Shigella* spp. were also insensitive to the irradiation, so some other factors may be involved. It was hypothesised that A + T % content of the DNA, might be important. The amount of ascorbic acid (an antioxidant) implying that photo-oxidation caused by the Ar laser irradiation may also be a factor.

Frucht-Peri *et al* (1993) looked at the effect of the ArF excimer laser at 193nm wavelength upon the yeast *Candida albicans* on agar. The justification for this research was the hope that laser irradiation could be used against keratinitis, the laser was a good candidate for this as chemical treatments can damage the eye, and this laser is used for laser surgery. This study aimed to cause "photoablation" of colonies and to check the resultant effect on viability. Sterilisation was achieved with a power density of $300mJcm^{-2}$ at 10Hz and 300 pulses. The writers found that the power density applied was more important than the frequency. It was suggested that as killing was proportional to the number of pulses, that the outer cells of the colony were killed first.

Semenov *et al* (1996) stressed the importance of a substitute for antibiotics, but pointed out that lower laser irradiation parameters can cause the proliferation of microorganisms. It was for this reason that they used high laser powers. The writers caused sterilisation of *S. aureus* and *E. coli* with an exposure of $1000Wcm^{-2}$ of Nd:YAG laser irradiation. Investigating the antibiotics resistance angle further, plasmid containing strains were used. The antibiotic resistance conferring plasmids were not lost after sub-lethal amounts of laser (the actual levels were not given). Watson *et al* (1996) examined the activity of seven different wavelengths upon lawns of *E. coli*. The wavelengths ranged from 118μm to 355nm. The CO_2 laser (power 600W, energy density $7.88Jcm^{-2}$) was twice as effective (in terms of energy density applied) as the best of the Nd:YAG tests. There was no clearing of the lawns after exposure from irradiation from a laser diode array or argon ion laser (wavelength 0.81 and 0.488 μm respectively). Nd:YAG laser systems were used that were capable of high peak powers. These systems caused the plastic petri dishes to melt, but did not cause inactivation of the microorganisms. The writers compared this result with what was found with Nd:YAG systems that delivered energy density over a longer time period. The longer exposure allowed clearance. This suggested that killing was by a thermal mechanism.

The importance of this work was that it showed that the wavelength chosen for laser sterilisation was very important.

A couple of important points have been raised in relation to laser sterilisation. Frenz *et al* (1988) showed a disadvantage of laser irradiation of bacteria on soft material. An Er:YAG and a CO_2 laser were used ($\lambda = 2.94$ and 10.6μm), these wavelengths are in the infra red range. By photographing gelatin and phosphorous covered agar as it was laser irradiated, it appeared that expanding water vapour carried the phosphorous particles. The transfer of material to the bottom of the laser-produced pits was water content dependent. When the agar contained a high water content (90%), there was more particle transfer than when the water content of the agar was lower (75%). It was shown that liver, with *E. coli* spread on the surface also allowed transfer. The laser produced pits contained viable bacteria. These findings are an important consideration when advocating the use of lasers as a sterilising tool for soft material. Sadoudi *et al* (1997) used a frequency variable Nd:YAG laser (wavelengths 1060nm, 532nm, 350nm – infra-red, green and UV respectively). The work investigated cleaning of *E. coli* films from the surface of stainless steel, and also any advantages gained by the removal of any bioaerosols produced by the treatment. The maximum energy density used was 30 Jcm^{-2}. Five decimal reductions were recorded after treatment with all three wavelengths, but when a vacuum was used to remove airborne material produced during irradiation, complete sterility was recorded. The authors concluded that part of the sterilisation process was a cleaning one (ablation) by the rapid pulses (20 ns duration), and that this was perhaps the most important mechanism, as low energy density levels (9mm beam diameter, 1 Jcm^{-2}) allowed ablation. The vacuum could then remove this material. The paper did not comment on the killing observed without the vacuum. It was not discussed whether ablation allowed the sample to be "fired off" the steel, i.e. giving a false low viable count. What must be taken from the paper is the importance of recontamination of the test surface by bioaerosols, this could be a great disadvantage of laser sterilisation.

More conventional methods to ensure sterility can be placed under four separate categories: heat, chemical, radiation and filtration.
Each has advantages and limitations, depending upon different circumstance and conditions required.

1.1.2 Heat.

Heat is a so called "physical" means of bacterial destruction, and is the method of choice unless of course the material to be sterilised is heat labile, i.e. some plastic tubing, serum, etc. Microorganisms are found and grow in a temperature range from 0°C to 90°C this varies between species and between strains and often within strains too, (with a rate of 1 in every million organisms in a mesophilic strain showing natural mutation, producing thermophilic properties). However most pathogenic bacteria, fungi and virus' are sterilised within a few minutes at 50°C to 70°C. Although some species of *S. aureus* and *S. faecalis* need to be treated for one hour at 60°C so some variability exists. Most spores of pathogenic strains are destroyed at 100°C although some can survive for hours at this temperature. *C. botulinum* spores can last for 5.5 hours at this temperature and are therefore a commonly found food poisoning agent. Heat kills bacteria in accordance with first order kinetics, i.e. higher temperatures means that less time is required for sterilisation, so to quickly kill the more heat resistant spores, a higher temperature is required. This is achieved by heating under pressure, allowing temperatures of over 100°C being obtained. The higher temperatures mean shorter exposure times, this is useful as extended exposure to high temperatures can damage broth ingredients. Cells contain water as depolarised layers so the presence or absence of water in the surrounding medium is very important, this has led to the expressions moist and dry heat.

It has been known for a long time that moist air was more effective than dry air, Koch and Wolfhugel (1881) killed anthrax spores in cloth with moist heat, dry heat was ineffective. Moist heating is shown by the autoclave method. Autoclaves reach temperatures of 121°C and in 15 minutes kill all vegetative cells and spores. This temperature must be reached because absolute sterility is required for surgical apparatus, culture media etc.

Saturated steam at 15 p.s.i. is pumped around the outer jacket of the autoclave while the central chamber is decompressed. This allows condensation inside the autoclave to be evaporated quickly. The steam in the autoclave must replace the existing air completely or the required temperatures may not be reached, and sterility may not be achieved (Rishworth 1938, Spooner and Turnbull 1942). This is because the steam touches the

cooler objects in the autoclave, condenses and gives up its heat energy until an equilibrium is reached. So the sterilisation almost takes place in an aqueous environment. It must be noted however that if the steam is too hot for its pressure i.e. "superheated", this condensation process can not work properly, and the steam acts as dry air.

Ovens typify dry heat sterilisation. Dry heat is less effective at killing bacteria than moist heat and for reliable sterilisation of glassware temperatures of 160°C are required for one to two hours. Total sterilsation of vegetative cells requires 100°C for 1.5hr, and for sterilisation of spores, a 3hr exposure of 140°C is required (Koch and Wolffhugel, 1881).

To explain why there is such a difference in efficacy between dry and moist heat, the destructive action of heat must be understood.

Heat affects every area in the cell, but principal damage is caused by protein denaturation (Sykes, 1958). The presence of energetic water molecules aids disruption so steam at 100°C has the same effect as dry air at 150°C. The water content of the cell is determined by the relative humidity of the surrounding environment, and even small changes in the relative humidity can have a large effect (70-100%) on the water content of the cell (Sykes, 1958). For this reason moist air has a more dramatic effect on the cell's balance than dry air. Koch discovered that wet proteins were more likely to form free SH groups. These groups form complexes of short peptides upon heating, so again moist heating would be more effective.

It is because of the importance of water that spores exhibit their heat resistance, not because of a lack of water but because the water is locked up in a gel-like state because of interactions between calcium ions and dipicolinic acid.

Disruption of the membrane allowing leakage of cell constituents is also important (Sykes, 1958). This causes osmotic imbalances and is a major cause of cell death. It is thought that death by dry heat is due to the sudden increase of electrolyte concentration and the denaturing of proteins. Another important heat target site is the ribosomes. The heat stability of these organelles is directly proportional to the optimum growth temperature of an organism. Ribosomal R.N.A is thirty times more sensitive to the action of heat, than D.N.A is, and this rR.N.A is degraded before the leakage of cell constituents is observed. Cells that survive heat treatment, often show a lag period before growing. In *S. aureus* this lag has been proven to be due to the regeneration of

ribosomes and rR.N.A. As ribosomes are required in the protein formation process any damage to them will have a detrimental effect on the cell. If other proteins of the cell are damaged by heat treatment, they can be regenerated again, assuming the appropriate energy etc. is available, but if RNA polymerase, ribosomal proteins or the protein factors and enzymes are completely eliminated they can not be replaced.
So although the mechanisms of death by heating are not understood fully, the major causes of death are due to: the disruption of membranes, breakage of single strand DNA and the destruction of ribosomes.

Laser sterilisation can be extremely rapid and gives the added benefit of al'owing more control, i.e. specific sections of a metal surface could be sterilised, but most other heating methods e.g. infra-red lamps are less directional.

1.1.3 Sterilization by chemical means.

For a chemical to exhibit a good bactericidal action it must: adsorb to the bacterial cell wall, penetrate into the cell protoplasm, react with the cell constituents.
The chemical's properties, mode of action and effectiveness obviously depend upon its constituent compounds, ionic make up, hydrophobicity etc. The chemical sterilisation method varies, i.e. detergents dissolve lipids in the cell membrane, alkylating agents damage the proteins and nucleic acids. Chemical action, like heat, increases exponentially with concentration, and the time taken for sterilization decreases. It must be remembered that chemicals sometimes have trouble penetrating clumps of cells, aggregations and biofilms where heat has less trouble. Chemicals are often used at five times the lowest concentration that gives sterility after 10 minutes at 20°C.
Chemicals are useful for heat labile products; common chemicals used for decontamination are discussed below,
Ethylene oxide is a cyclic ether and a water soluble gas that is one of the most commonly used chemical sterilising agents. It is non-corrosive, good for delicate laboratory equipment and heat labile instruments. Ethylene oxide can also enter porous material so is good for bedding in a hospital environment. Unfortunately it is more expensive than heat and is relatively slow (Phillips, 1949). In ethylene oxide sterilising chambers, the gas is supplied at an elevated temperature and under pressure in an effort to increase the reaction speed. Ethylene oxide is an **alkylating agent**. This group of

chemicals work by replacing the labile hydrogen atoms on NH_2, OH, COOH and the SH functional groups. This reaction is partly reversible. These agents have the advantage of attacking spores as easily as vegetative cells. This is due to the gas molecules being relatively small, uncharged and do not require water for action. Alkylating agents attack proteins and work by opening the ethylene oxide ring in the presence of a labile hydrogen, forming a hydroxyethyl radical which then attaches onto the protein where the hydrogen was. This blocks the reactive groups on the protein and death follows. It is also thought to attack DNA and RNA by esterification of purines and pyrimidines. Alkylating agents are possibly mutagenic and carcinogenic to humans so care must be taken when using them. The gaseous nature of ethylene oxide gives it advantages over many chemicals, i.e. it can be used to sterilise the inside of heart-lung machines, this process would be more difficult with chemicals in a liquid form.

Surface active disinfectants attack at the interfaces, i.e. between the lipid containing membrane of the cell and the surrounding aqueous environment. The chemicals contain both hydrophilic and hydrophobic groups, and can be; anionic, cationic or non-ionic. Anionic detergents contain soap-like groups and fatty acids, which dissociate to give a negative ion. These chemicals are more active at acidic pH values and are most effective on G -ve bacteria because of their lipopolysacheride (LPS) outer membranes which are disrupted by the detergent. Of the surface acting disinfectants, cationic agents (positively charged) are the most important, and some are effective in concentrations as low as 1 ppm. When these chemicals are exposed to bacteria, the positively charged ion binds to the phosphate groups of the membrane phospholipids. The non-polar region then enters the membrane, causing distortions and a loss of the membrane's semi-permeability. This leads to leakage of nitrogen and phosphorous containing compounds. A disadvantage is that the presence of protein in the surrounding environment can provide protection for the bacteria, by binding to the chemical (Behring 1890, Kronig and Paul 1897). The activity of these compounds is greatest at alkaline pH values, and it has been observed that G +ve organisms are most susceptible to their action. Winslow and Hothkiss (1922) found that mercury ions were more effective than cadmium and that generally metals of higher molecular weight were more effective than those with a lower molecular weight. It was also observed that ionic chemical potency is increased as the number of ions i.e. Fe^{3+} was more effective than Fe^{2+}. The ionic nature of these compounds aids penetration and helps disrupt the

ionic balance in the cell. Some **non-ionic** detergents can actually aid bacterial growth in some species so are rarely used (Dold and Gust, 1957).

Halogens are characterised by iodine and chlorine. Chlorination is the most common method of ensuring microbiological safety in a water supply. In sufficient doses it causes cell death within thirty minutes.

Chlorine forms hypochlorous acid (HOCl) upon reaction with water. This acid is a strong oxidising agent. Andrewes and Orton (1904) killed anthrax spores with a concentration of 0.01% v/v in 30s. Hypochlorite solutions are for sterilisation of "clean" surfaces, primarily in the dairy and food industry. It is used in concentrations of 200 ppm chlorine. It is only used for clean surfaces because chlorine reacts readily with organic matter which inactivates it.

Skin has been prepared for operations using iodine based chemicals Lowbury *et al* 1960), and is still used today.

Acids can also be used to kill microorganisms, the effectiveness depends upon concentration of H^+ ions (electrolytic dissociation) (Winslow and Hotchkiss 1922).

One important disadvantage of chemical sterilisation, is that the disposal of waste by-products can have a damaging effect on the environment. Gaseous agents such as ethylene oxide can pollute the atmosphere, while chlorine based compounds can pollute water ways (as mentioned chlorine forms hypochlorous acid on reaction with water, this is a strong oxidising agent).

Laser sterilisation is a clean process, the beam does not leave a residue. Equally important is the fact that some microorganisms can survive in chemicals designed for sterilisation. *Pseudomonas* has caused many infections in hospitals by surviving in cleaning solutions used for surgical equipment. (Bosi *et al* 1996, Oie and Kamiya 1996, Bloomfield and Scott 1997).

1.1.4 Radiation (non-lasing)

As a molecule absorbs light it receives energy in discrete units termed "quanta". The absorption of a quantum of energy by an electron in an atom results in the activation of the molecule. X and Gamma radiation provide enough energy for the electron to become so excited it is ionised causing the production of an ion and free electron. Some radiation e.g. visible light can not provide enough energy for ionisation so this type of radiation is termed **non-ionising**.

The killing effect of UV was first observed when it was noted that day light caused killing of vegetative bacteria, and more efficiently than with spores (Downes and Blunt 1877, Ducleaux 1887). The most effective wavelength of UV is 254nm that corresponds to the absorption maximum of DNA. Commercially available mercury vapour lamps can provide this wavelength. UV light only destroys the bacteria it makes contact with i.e. on surfaces, not clumps or biofilms. These lamps are very popular and are supplied to the medical, food and electronic industries (BDH lab supplies catologue 1999) and can contain a filter to remove the unwanted wavelengths.

Lethal action occurs in two ways, one is **causation of lethal mutations,** i.e. by alterations of DNA that can block its own replication or transcription. Lethal mutations occur when a particularly sensitive target is attacked by the radiation. These occur only rarely, it is the minor alterations to the DNA, causing reading problems that primarily cause cell death. The ultraviolet radiation causes covalent bonds to be formed between pyrimidine residues adjacent to each other on the same strand. These are called **pyrimidine dimers,** these cause distortions in the DNA strand shape, and interfere with the base pairing systems. This inhibits DNA synthesis and may stop respiration and replication. It must be noted however that by treating the radiated surface with visible white light, (300-400nm) photo-reactivation may occur. This was first shown in coliform bacteria, with a 45-60 min exposure of visible light (Kelner 1947). This process repairs the damaged DNA by repairing the pyrimidine dimerisation. Many species of bacteria have natural target repair mechanisms that can repair the dimers, this is called the S.O.S. response. *M. radiodurans* has such an effective repair system that it has become one of the most radiation resistant organisms. Visible light also has a bactericidal effect, it possibly acts by photosensitising riboflavin and porphyrins. Photodynamic sensitisation was first shown by Raab (1900). These compounds occur naturally in microorganisms. Fluorescent dyes i.e. methylene blue, aid this process by absorbing light and releasing it over a long period of time. Von Tappeiner (1900) killed paramoecium in 2-4hr with green light when in 1/800 solution of eosin.

Ionising radiation

Ionising radiation such as X- rays or gamma rays has more energy than ultraviolet and therefore has a greater capacity for effect. This type of radiation can penetrate surfaces, and its optimum attack area is just below the surface. Gamma and X-rays attack cells in a similar way. X-rays are produced by the radioactive element Cobalt-60. X-rays have **ten thousand** times as much energy as visible light, so their ability to cause excitations

in the electrons of atoms is very high. If UV is used in preference to X-rays then 100 times more energy is required (Lea and Haines, 1940).

Damage caused by ionising radiation and the subsequent cell death, can occur by two methods, **directly** and **indirectly**.

Direct damage is caused by the destruction of a sensitive target area, by a photon of energy. i.e. Gamma rays cause scissions in double stranded D.N.A. Some bacteria are sensitive in more than one of their internal compounds and are termed multi-target cells. These are more susceptible to radiation action. As mentioned earlier this damage is caused by the excitation of electrons of the atom, causing the electron to actually leave the atom. These electrons, carry a lot of excess energy, which is converted into kinetic energy causing them to reach even greater speeds than normal. The electrons travel and collide with further electrons and cause those to gain energy and become excited. This process is then repeated for these electrons and so on to form an avalanche process.

Indirect damage can be caused by the radiolysis of water. This process causes harmful by-products to be formed, i.e. hydrogen, hydroxyl, hydroperoxyl and hydrogen peroxide **radicals**. These radicals contain large amounts of free energy and are very reactive. As there is a large percentage of water in the cell, this indirect damage caused by water by-products is of obvious importance. It is generally accepted that 2.5 Mrad. is required for certain sterility, this kills all vegetative cells and **spores (which are exceptionally radiation resistant)**. This dose is also within safety margins for use. The rate and intensity of the irradiation does not affect the killing effect, this is determined by the total dose administered (function of rate and intensity). It should be noted that radiation treatment often shows an effect known as the D37 effect, (D37 dose). This phenomenon is often observed upon treating enzymes. Sometimes enzymes can react with radiation to form a product that is also able to absorb radicals. This means that a dose that would normally be expected to disable 100% of the target enzyme, only disables 63%. This is due to 37% of the radicals reacting with an already inactivated enzyme product. The stronger radiation such as gamma and X-rays, are less available than ultraviolet and therefore are found less in the laboratory. It also has a very high apparatus cost and requires a lot of safety equipment and procedures, which are not really suited to laboratory conditions. Radiation is useful for many heat labile items i.e. cat gut, plastic-ware. It has also been used for food, especially soft fruit e.g. strawberries. There was however, a public uproar, as people did not trust the concept of irradiated food, worrying that it may be carcinogenic. The ionising effect only lasts for

a short while and these worries were unfounded. It should be noted that irradiation of fruit is carried out as much, if not more, to add to the longevity (shelf-life) of the food, than to ensure the short time sterility. Powell and Bridges used gamma irradiation to sterilise surgical and pharmaceutical products in 1960.

1.1.5 Filtration

Filtration does not generally kill micro-organisms, but it is a method which removes the bacteria, spores, virus' etc. Filtration works by passing the environmental medium, i.e. liquid, gas etc. that the bacteria are in, through a pore, which stops the bacterial cells, but allows the substrate to pass through. It is a combination of electrostatic forces, adsorption and the sieve like property that causes the bacteria to stick to the filter and not pass through with the substratum. The largest micro-organisms are greater than 10 μm in diameter, where-as the smallest are less than 0.3 μm in diameter, so the filter must be less than this size range to be effective. The most common pore sizes found are between 14 and 0.023 μm. A pore size of 0.22 μm is smaller than bacteria and is therefore the most commonly found. Filters are used for ventilation air in air flow cabinets. These cabinets allow work to be carried out in a sterile environment. Filters are used for this purpose as they can be cheap, simple and effective.

There are three general types of filter employed; depth filters, membrane and nucleopore.

Depth filters consist of a fibrous sheet or mat of material. This material is commonly paper or glass fibres. The mats are constructed of a random array of overlapping fibres criss-crossing each other and forming a maze of paths throughout the filter. The micro-organisms become trapped in this winding maze of endless tunnels. This type of filter is especially useful for the filtration of air, and is not as easily blocked as the other types of filter.

The membrane filter type is the most commonly used group of filters. They are composed of biologically inert cellulose esters, i.e. cellulose acetate and cellulose nitrate. These filters act almost like a sieve, trapping all the bacteria on the surface of the filter. There are a huge amount of tiny holes on the filter surface and almost 85% of the surface area can be open space. By using membrane filters, the bacteria can be removed and counted or analysed, as it is excepted that the numbers on the surface is the total count, although there is a little penetration of bacteria into the filter surface.

Nucleopore filters are produced by treating, thin polycarbonate films (10 μm thick) with radiation. This treatment causes isolated areas of damage in the film. Chemicals are then added which degrade the damaged areas of the film further, into holes. The hole characteristics can be precisely controlled, by altering the chemical concentration or the time of exposure. The holes in these filters run vertically throughout the filter like a true sieve, but tend to clog very easily, as all of the bacteria are trapped on the surface of the filter. It is also used for scanning electron microscopy.

All filter apparatus is autoclaved separately from the actual filters, which are sterilised in ovens using dry heat. Filters have an advantage over heat etc. in that they **remove** the bacteria, rather than try to destroy it. This reduces the chance of viable but non-culturable cells remaining, in the sample medium. It must be remembered however that bacterial cells that are in a state of starvation that could lead to this non-culturable state, will often shrink in size, and may pass through the chosen pore size diameter. It should also be noted that the family of bacteria known as *Mycoplasmas*, are pliomorphic as they have no solid cell wall structure, and can pass through pore diameters smaller than their own actual diameter.

1.1.6 Testing sterility

Sterility does not always mean destruction of the cell, so detecting destroyed cells is not always effective, i.e. dyes can penetrate the cell via damaged membranes, but this will not show cells that have been inactivated by scissions in the DNA. For this reason most tests of sterilisation are indirect. This is shown by autoclave tape, that changes colour at high temperatures, dyes are available that change colour at various temperatures.

Testing the target material by swabbing and spreading onto agar plates is a popular and effective method, it should be remembered though that heat treated bacteria often have more exact nutritional needs (auxotrophism) and plate counts may not give an exact count thus producing falsely low counts. Plates with nutrient supplements are more useful for analysis.

In conclusion, it can be seen that although microbiology has progressed immeasurably in the last century, methods of sterilisation still heavily rely on the physical methods first shown in the 17^{th} and 18^{th} centuries. Advancements have been made in the

understanding of sterilisation, but more work must be carried out before a complete understanding of the complex killing processes can be achieved. The only obvious advancement in sterilisation processing is in the use of chemicals, but bacteria are infamous for their ability to evolve resistance and to spread this property throughout a population, so new chemicals are needed as resistance appears.

1.2.0 Bacterial endospores

1.2.1 Spore resistance

The bacterial endospore develops into a vegetative cell via a series of stages. This transition from the dormant, resting stage to the active vegetative state is known as **outgrowth**. This was originally known as germination, but this terminology is now only used to describe the transition from heat resistant cell to a heat-labile non-refractive cell. The stages are known as; 1) resting, 2) germination, 3) swelling, 4) emergence and elongation , 5) division. Although in a population the cells will be in the various stages of development and not synchronous.

Spore DNA is extremely well protected from heat, desiccation, UV, γ radiation and also oxidising agents. It is this protection which aids in the ability of bacterial endospores to survive unfavourable conditions for extended periods of time (Slepecky and Leadbetter, 1983, Kennedy *et al*, 1994). The spore state is brought about when growing cells of some bacterial species are deprived of certain nutrients (even although a rich supply of other nutrients may be readily available). The actual formation of the spore occurs as the cell reaches the end of the growth cycle (when it contains two nucleoids). At this stage, an asymmetric septum then divides the cell into a larger and smaller cell, each containing a full copy of the chromasome. The smaller cell (forespore) is then engulfed by the larger "mother" cell. After many biochemical events, several external layers are added to the forespore. The mother cell then lyses releasing the free spore (Setlow, 1995). The structure of the spore is different from that of the vegetative cell, as it contains novel layers, including proteinaceous **spore coats** and the **cortex** which is composed of peptidoglycan similar to the cell wall. The central core of the spore is the site of the enzymes, ribosomes and DNA, (Gauthier and Tipper 1972 , Gerhardt and Marquis 1989). In order to allow the long term survival of the spore, the DNA must be protected. The spore may lose up to 99% of an enzyme but has often only one copy of the genome and this must be protected, (Bellivue *et al*, 1990) as damage accumulated

during dormancy could prove fatal. Vegetative (growing) cells, contain DNA repair mechanisms. This is not possible in spores because being metabolically dormant, the high energy pathways required are not present. This means that the spore must: a) rapidly repair any damage upon germination and/or b) protect the DNA from damage in the first place. Factors attributing to the DNA protection in spores include, the low free water content in the spore and the presence of a molecule named dipicolinic acid, DPA. This molecule makes up to 5 - 10% of the dry weight of bacterial spores. The membranes and spore coats are also relatively impermeable to molecules which could be potentially toxigenic. The DNA itself is also more condensed than that of the mother cell or in the vegetative cell, (Setlow, B. 1991, Setlow, P. 1988). The DNA also become saturated with a group of small, acid soluble proteins (SASP's). These occur as two distinct forms, α and β. These DNA binding proteins are synthesised only in the forespore and remain on the dormant spore chromasome playing a major role in protection of the DNA until germination whereupon they are degraded by a protease (Setlow 1988). Mutants deficient in the proteins show a 99% reduction under conditions not normally fatal, i.e. 85°C for 30min, (Fairhead *et al,* 1993). Popham (1995) has shown that these SASP's are more important than the spore water content, and are involved in heat, hydrogen peroxide and UV resistance of *B. subtilis* spores. It should be noted that spores are very resistant to UV, and it has been shown that UV damages vegetative cells of *B. subtilis* 50 times more effectively than it does spores, (Setlow 1992). The mechanism of spore killing is open to conjecture, differential scanning calorimetry has shown that the target by heat is a spore protein or membrane (Belliveau 1992), whereas other studies using spores in a wet environment have shown that many mutants are produced by heating (Kadota 1978), thus implying DNA damage, but this was not correlated by Fairhead *et al* (1993). Many reports show that dry spores incur many mutations after a heat treatment, leading to death (Chiasson and Zamenhof, 1966, Northrop and Slepecky, 1967, Zamenhof, 1960). The relative humidity of the surrounding air also affects the heat resistance of the spores (Pfeiffer and Kessler, 1994). Spores also have a repair mechanism against damage caused by UV. In spores, damage occurs between thymine residues on the same strand, this is called the spore photo-product, (SP) (5-thyminyl, 6-dihydrothymine, TDHT) and can cause a potentially lethal effect upon the spore. Spores have a SP-specific repair system that rapidly and efficiently repair the lesion in the first minute of germination (Munakata and Rupert,

1972, Nicholson *et al*, 1990). Although the spores are also resistant to ionising radiation, higher levels, of radiation may induce spore permeability changes, simulating the responses characterising germination (Levinson and Hyatt, 1960).

1.2.2 Spore forming organisms

The species *Bacillus* are mostly harmless saprophytes, but some however i.e. *B. cereus* and *B. subtilis* have been the cause of food poisoning (Hauge 1955), and are also sources of secondary infections (Lázár and Jarcsak 1966). *B. cereus* food poisoning requires the presence of 10^7 - 10^8 vegetative organisms, and the symptoms resemble those of *Cl. perfringens* food poisoning (which requires approximately the same cell numbers). The foods involved vary (Mossel *et al*, 1967), but a common cause is the practice of partially cooking rice in restaurants then leaving it until required then heating it up. The primary heating, heat shocks the spores into germinating, and the second cooking is not sufficient to inactivate any of the enterotoxin produced. *B. cereus* is very like *B. thuringiensis* , an organism which is used as a method of pest control (it produces an insecticidal protein). It is therefore very important to be able to differentiate between the two organisms. DNA probes for this purpose are now available which are based upon variable regions of 16S rRNA, (te Giffel *et al*, 1997). It should be noted that *B. thuringiensis* has also been reported as producing an enterotoxin (Damgaard 1995, Abdel-Hameed and Landén 1994, Jackson *et al* 1995).

Spores are also important in that they can be causative in food spoilage, in that they may lead to spoilage caused by the active vegetative cells, which occur after the inert spores become active and divide.

Few spore forming organisms grow below 10°C or only do so slowly. As it is difficult to remove spores from food, without actually damaging the foodstuff, refrigeration is a good safeguard against infection. Cold tolerant spore formers are also more susceptible to heating.

When studying spores it is important to remember that recovery of spores is affected by pH and NaCL concentration (Graham *et al*, 1997), incubation temperature and the recovery medium used (Gonzalez *et al*, 1994, Cóndón *et al*, 1996). This means that false low results can be obtained by using the wrong conditions and a possible misjudgement of risk obtained.

1.3.0 Fungal spores

Most fungal species are harmless (to man) saprophytes, generally living in soil etc, but some species can become a problem when they infect our bodies or our food.

Most fungi consist of numerous cells of hyphae. Forming a mass known as mycelium. These cells can vary in size, but are generally 5-10μm long, but the mycelial tissue can reach kilograms in weight. The structure of these cells is largely determined by the rigid cell wall. This is mainly composed of an alkali resistant component called chitin (van Wisselingh, 1898). Chitin is also found in the exoskeleton of insects. Cellulose can be found instead of the chitin in some species (Nabel 1939), but both polysacharides are found in *Rhizidiomyces sp.* (Fuller and Barshad, 1960).

Walls known as septa can separate the hyphal cells. The new cells grow out from behind the tip of the mycelium and the hyphal wall grows inwards, forming the septum. Some types have pseudo septa or are aseptate (Hickman 1965). The apical tip – the actively growing region – is longer than the regularly septated regions (Smith 1923) and contains dense protoplasm with many nuclei and mitochondria. The older sections are generally emptier and can also be vacuolated (Robertson 1961). So although the mycelium is very large, most of the material is old or dying. (Schutte (1956) showed with dye that the mycelial tissue absorbs nutrients as the material streams towards the tip. Septa form no barrier to this process.

Fungi contain biological clocks (Bell-Pederson *et al* 1996). These clocks regulate and synchronize many processes, one being the production and release of fungal spores. This synchrony is required due to the requirements for the spores, i.e. light or dark. *Peniccilium sp.* spores are often released at night, to protect the spores from UV and dessication (Carpenter 1949). The regulation is poorly understood, but is genetically controlled.

These asexual spores are produced in order to facilitate spread. The fungal cell becomes dinucleated after meiosis, but formation of the spore wall is poorly understood (Moore, R.E. 1965). It is the spore wall that lends protection to the spore. It is thought that spore formation involves the endoplasmic reticulum, forming a membrane matrix, into which the cell wall material is deposited. The spore wall then becomes thicker (the wall material is deposited in layers). The outer spore coat material passes through the wall and gathers inside the membrane matrix, taking the form of the reticular pattern. As the spore matures, the residual cytoplasmic material breaks down, leaving only a few ribosomes and mitochondria when the spore is released.

Fungal spores are more resistant than most vegetative bacteria (Baggerman and Samson 1971). Pasteurisation at 80°C for 1min will kill most bacteria, but not fungal spores, and this has allowed spoilage of canned fruit (Jensen 1960). Some types i.e. *T. flavus* can produce spores that can survive in fruit products at 100°C for 30min (van der Spuy *et al* 1975). An example of *Fusarium sp.* spores is shown in **Figure 1.**

The lay-out for this thesis incorporates an introduction (chapter 1), then the materials and methods used are described in chapter 2. The main results chapter (chapter 3) is sub-divided into sections, one with results pertaining to bacterial spores, the other fungal spores. The first part of the bacterial spores section investigated the effect of laser irradiation of the spores on surfaces, and how this could be quantified. It was found that the two different laser types used the Nd:YAG and CO_2 lasers were capable of causing killing of spores on stainless steel and PTFE and glass. The CO_2 laser was the more efficient (plastic and glass) but the materials used reached the damage threshold quickly so only a 1D reduction in viability was obtained with this laser type. The Nd:YAG laser was used on stainless steel, and complete sterility was obtained with an energy density of 1800Jcm^{-2}. This first section also showed that the main killing mechanism involved with this was by an indirect heating from the irradiated steel.

The next part of this section showed that under certain circumstances the laser irradiation was not efficient at killing spores, namely in liquid and on the surface of agar. This led on to how the killing could be augmented by incorporating UV irradiation. The addition of UV to the laser irradiation caused an increased killing effect on agar and in suspension. A multifactorial experiment showed that the increased killing was additional and not a synergistic effect. This meant that the amount of laser used could be reduced, but not because of an interaction between the irradiations. It was therefore decided to combine the laser irradiation with different chemicals. Of these hydrogen peroxide proved the most effective. The use of sub-lethal concentrations of chemical meant that heating suspensions to 60°C caused sterilisation of spores that previously could survive 90°C.

The fungal spore results looked mainly at the ability of laser irradiation to inactivate the growth of the spores on agar and foods. It was found that scanned CO_2 laser irradiation was effective at sterilising fungal spores on the surface of cheese, but this could not be repeated on fruit.

Finally in chapter 3, an attempt to investigate the laser mechanism of laser irradiation inactivation of both bacterial and fungal spores was made with electron microscopy. This work showed that the damage observed on fungal and bacterial spores was visually similar to that caused by conventional heating.

Chapter 4 discusses the results presented in more detail, taking each section in turn, and touches upon some of the applications of the findings, this leads somewhat on to chapter 5 where the future work is discussed.

Finally the literature citations are listed in chapter 6.

It is expected that members of both the engineering faculty and also microbiologists would read this thesis. For this reason the appendices contain some relevant information pertaining to lasers and the electromagnetic spectrum. While this information would seem superfluous to a reader from an engineering background, it would prove invaluable to a scientific reader yet to acquire the required background knowledge.

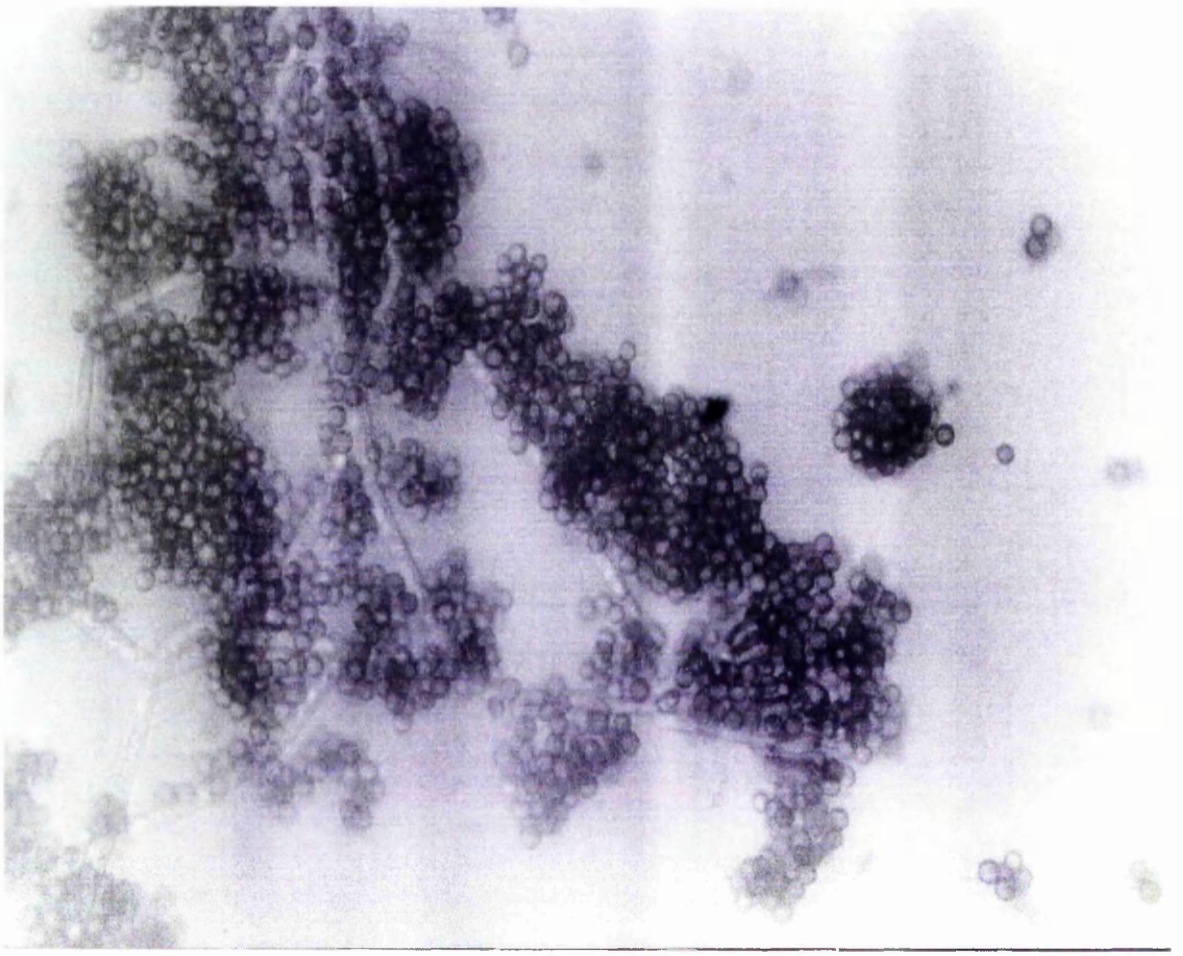


Figure 1 Light microscope picture of *Fusarium sp* spores. (Magnification x1000).

2.0 Materials and Methods

Chapter 2 Materials and methods

2.1.0 Media and Reagents

In this study the reagents bought from Agar scientific were Uranyl acetate (R1260a). The reagents purchased from BDH Ltd. Dorset UK, were; di-sodium hydrogen orthophosphate anhydrous (10249), di-potassium hydrogen orthophosphate anhydrous (29619), Epoxypropane (propylene oxide) (282904W) Glucose (101174Y), Hydrogen peroxide, H_2O_2, (285196H), Lactic acid (101384Q) and Potassium dihydrogen orthophosphate (10203). Bacto® Dextrose (0155-076), was purchased from Difco Laboratories, Detroit USA. From Fisher Scientific UK, Leicestershire, UK, Acetone (A 0560/17), Ethanol (CAS 64-17-5) and Sulphuric acid (CAS 7664-93-9) were purchased. Osmium tetroxide, OsO_4, was purchased from Oxkem Ltd. Oxford UK. The following materials were purchased from Unipath Ltd. (Oxoid) Hampshire UK; Agar technical (agar $n^0 3$), (L13), Czapeks Dox agar, CzA, (CM97), Nutrient agar (CM3), Phosphate buffered saline, PBS, (BR14a), Sabouraud Dextrose agar, SDA, (CM41), Tryptone (L42) and Yeast extract (L21). The following chemicals were bought from Sigma-Aldrich company Ltd. Dorset UK; Bovine serum albumin, BSA, (A-7906), Bromcresol purple (B-5880), Glutaraldehyde (G5882), Lysozyme (L-6876) and Polyoxyethylene sorbitan monolaurate, Tween 20, (P-1379). SPURR resin was purchased from Tabb (S031).

2.2.0 Strains used in study

The strains in this study were chosen for their relevance to the food industry and their high resistance to sterilisation methods. This represented the most difficult scenario for testing a particular system. The bacterial species for this study were all from laboratory stocks; they were: *Bacillus cereus* NCTC 11175, *Bacillus subtilis* 3610, *Escherichia coli* B 10537 and *Staphylococcus aureus* NCTC 6571 (Oxford strain).

The fungal species in this study were either from laboratory stocks, *Fusarium* spp. and *Rhizopus oligosporus*, or isolated from cheese samples, *Penicillium* spp. and *Mucor* spp.

B. cereus is an important causative organism for food poisoning. The reason this organism was chosen though was because it was also capable of forming spores. *B. subtilis* can also produce spores. *E. coli* and *S. aureus* are representatives of the Gram negative and positive groups of vegetative bacteria. The fungal species were chosen

because they have been implemented in the contamination of sliced fruit (a very important economic problem in Spain). The *Penicillium* spp was chosen for its ability to form "blue mould" on cheese.

2.2.1 Growth and maintenance of cultures

Bacterial species (from laboratory stocks) were inoculated onto nutrient agar slopes, and incubated at 37°C for 18hr. These slopes were stored at 4°C and sub-cultured once a month.

The fungal species, *Penicillium* and *Fusarium*, (isolated from food and lab stocks respectively), were inoculated onto slopes of Czapeks Dox agar (CzA) (Oxoid CM97), and incubated at 30°C until confluent growth was observed. These slopes were stored at 4°C and sub-cultured once a month.

The fungal species, *Mucor* spp. and *Rhizopus oligosporus*, (isolated from food and lab stocks respectively), were inoculated onto slopes of Sabouraud Dextrose agar (SDA), and incubated at 30°C until confluent growth was observed. These slopes were stored at 4°C and sub-cultured once a month.

2.3 Lasers used

Nd:YAG laser MS830 (Lumonics Ltd., Rugby, UK.)

This type of laser is termed a doped insulator laser. It has an active medium of neodymium 3^+ ions doped into yttrium aluminium garnet. Neodymium has an infra-red portion at a wavelength of 1.06μm. The laser's maximum power output was 400 W. The laser beam was delivered down a fibre optic and was used focussed or as a collimated beam.

CO_2 lasers

This molecular gas laser has a number of transition wavelengths of irradiation the main being 10.6 μm. Two lasers were available, the first was capable of producing continous wave irradiation of 1 kW (MFKP Laser Ecosse, Dundee, UK.), the second was only capable of producing irradiation of 13W (Synrad, Inc., 48-1'F' version, Seattle, USA). Both laser beams could be scanned via motorised mirror attachments.

These lasers were previously employed in materials processing.

2.4.0 Spores of *Bacillus* species

2.4.1.0 Laser irradiation of spores on surfaces

2.4.1.1 Preparation of spore suspension

Plates containing nutrient agar (Oxoid CM3), 0.5% w/v yeast extract (Oxoid L21) and 0.5% w/v glucose (BDH 101174Y) were prepared. Lawned plates were prepared by inoculating the surface of the plates with 100µl of a 24 hr *B. cereus* culture (37°C) and lawned with a sterile glass spreader. The prepared plates were incubated at 30°C for five days. This temperature was chosen to provide a stimulus for sporulation, i.e. lower than 37°C optimum. The *B. cereus* growth was resuspended with a sterile glass spreader in 5.0 ml of sterile distilled water. The suspension was incubated overnight in a 0.02% w/v lysozyme solution at 4°C for 24 hr. Sterile glass beads were added (1mm diameter) and the suspension was whirlimixed for 30s. The suspension was centrifuged at 3000 x **g**, in a Heraeus Sepatech Megafuge 1.0, for 5 min and washed with ice-cold distilled water. This washing procedure was repeated six times, (adaptation of the method reported by Long *et al* 1958). The spore suspension was placed in an 80°C water bath for 10 min to inactivate any vegetative cells present before use. This was verified by staining techniques. Germination of the spores was monitored by viable counting, with Fortified Dextrose Tryptone Agar (fDTA), (E.E. Sugimoto, 1996) containing (gL^{-1}), tryptone, 10 gL^{-1};dextrose,5 gL^{-1}; yeast extract, 1 gL^{-1}; dipotassium phosphate, 1.25 gL^{-1}; 2ml of a 2% solution of bromcresol purple, and technical agar, 15 gL^{-1}.

2.4.1.2 Correlation of laser beam diameter and distance of the target from the focussing head

The energy density varies as a function of distance from the laser as the beam diverges. Consequently the laser beam diameter was measured to investigate the effect of varying the position of the sample from the laser. A piece of burn paper was placed at a measured distance below the lens of the Nd:YAG laser, and exposed to laser irradiation (10Hz/20J). The beam diameter of the resulting burn print was measured to record the beam diameter of the laser irradiation. This was repeated for varying heights from the focal head.

2.4.1.3 Drying of bacterial suspensions onto stainless steel

The effect of the laser beam on surfaces was examined because of the potential importance of this procedure in the food industry. First it had to be verified that the actual drying of the spores onto the stainless steel did not cause a decrease in viable count. This means that any killing observed can be attributed to the laser and not to the preparation of the sample. Stainless steel discs were sterilised by autoclaving, at 121°C for 15 min at 15 lb pressure, placed in a laminar flow cabinet where 20 µl of 0.1% w/v filter sterilised bovine serum albumin (BSA) (Sigma A-7906) was spread with a plastic pipette tip over the surface and dried at room temperature. A 20µl sample of spores (**protocol 2.4.1.1**) was pipetted a series of discs for: 0.5, 1.0, 30 or 60 min, and left to dry. Each disc was then placed into 20 ml of sterile PBS and shaken on a Sepa-tech cooled orbital shaker at 150 rpm for 3 hr. Viable counts were determined for each disc after each time. A control disc was placed in PBS immediately after the suspension was added to it. The viable count of each disc was compared to determine whether drying had a killing effect.

2.4.1.4 Effect on bacterial endospores by the detergent Tween 20

It was aimed to find out if polyoxyethylene sorbitan monolaurate (Tween 20) (Sigma P-1379) had a detrimental effect on spores. It was planned to use the detergent as a diluent for viable counting to prevent the spores from sticking together and floating at the meniscus due to the hydrophobic exosporium (lipid layer). This experiment aimed to show that such a treatment was not sporicidal. As the Tween 20 was an essential treatment prior to the determination of a viable count of germinated spores it was necessary to check whether this alone would cause a reduction in the count.

Aliquots (200µl) of *B .cereus* of spore suspension (**see protocol 2.4.1.1**), were pipetted into 1.8 ml of 0.1% v/v with Tween 20. The spore/detergent mix was left for varying times and viable counts made for each time, 0 (control), 5, 10, 20, 30 or 60 min. The viability of the spores after each time period was ascertained by a viable count.

The 0min control was an immediate immersion of 200 µl of spores into the Tween 20, the mixture was briefly whirlimixed (2 s) and the viable count immediately ascertained.

2.4.1.5 Effect of storage on spore suspension viability

This experiment was designed to find the optimum temperatures for spore storage so that a spore suspension could be made in bulk and aliquots taken for each experiment. This minimised potential variation between spore batches. Aliquots (1 ml) of the *B. cereus* NCIB 9373 spore suspension were kept at room temperature (20°C), in a fridge (4°C) or in a freezer (-70°C) for varying lengths of time; 7, 14, 28, 38 or 74 days.

At each allotted time, a viable count was made of the spore suspension. These were compared with the viable count of the suspension at time zero (control).

2.4.1.6 Laser treatment of bacteria on solid surfaces

The reduction in viable cells after laser treatment of bacterial suspensions spread on a surface, and the killing curves on different solid surfaces, steel, plastic or glass was determined.

Aliquots of the bacterial suspension (20µl) were pipetted onto the sterile discs, which were placed in the laminar air-flow cabinet and dried if required. The discs were inserted into a sterile aluminium holder with sterile forceps and exposed in duplicate, to laser irradiation from the CO_2 laser with exposures of 100, 200, 300, 400, 500, 600, 700, 800 and 900 msec. A control disc received no laser irradiation. After exposure to laser irradiation, each disc was placed in a 100 ml Duran bottle containing 20 ml of sterile PBS. The bottles were shaken at 100 r.p.m. for three hr at 25°C in a Gallenkamp cooled orbital shaker to resuspend any surviving organisms on the discs. Serial ten-fold dilutions were made from each shaking solution, and 100 µl of each dilution was plated out on the surface of a nutrient agar plate. At higher exposure times, a 10ml aliquot of the 20 ml sample was taken and filtered through a 0.45µm cellulose nitrate filter (Whatman). The filter was placed on the nutrient agar plate surface. All plates were incubated at 37°C for 24 hr, and the colonies were counted with a Gallenkamp colony counter.

2.4.1.7 High energy, short duration laser irradiation inactivation of bacterial endospores on a solid surface

The reduction in viable cells after laser treatment of bacterial endospores spread on a surface, and the initial shape of the killing curve on different solid surfaces was examined with a high energy, small beam diameter laser. The spore suspension was

prepared as described in **section 2.4.1.1**. Stainless steel discs inserted into a sterile aluminium holder and exposed to a varying numbers of pulses of laser irradiation from the Nd:YAG laser at 48 J, 6 Hz, i.e. 0 pulses (control), 1, 5, 10, 20, 50 and 100 pulses. After exposure to laser irradiation, each disc was placed in a 100 ml Duran bottle containing 10 ml of sterile PBS. The bottles were shaken at 100 rpm. for three hr at room temperature in a Gallenkamp cooled orbital shaker. Serial ten-fold dilutions were made in sterile PBS containing 0.1% (v/v) Tween 20, and plated out on nutrient agar plates. All plates were incubated at 37°C for 24 hr, and the colonies were counted with a Gallenkamp colony counter.

2.4.1.8 Heating of steel coupons for observation of colour change

Coupons of 0.2 mm hard tempered stainless steel were placed in a Carbolite MTF 12/25/250 oven (Carbolite, Sheffield) for 30s at varying temperatures (100, 200, 300, 400, 500, 600, 700, 800, 900 or 1000°C). The steel was then observed for any colour change.

Sections of the 0.2 mm hard tempered stainless steel were placed on PTFE (to minimize heat losses) then exposed to Nd:YAG laser irradiation of frequency 10Hz, energy 10J and beam diameter 16mm. The exposure lengths chosen were 1 pulse (0.1s), 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5.0s.

After the treatments, the coupons were allowed to cool unaided and then observed for colour change.

2.4.1.9 Estimation of the temperature of laser irradiated, stainless steel discs

To estimate the temperature of the discs, laser irradiated discs were allowed to cool for varying lengths of time, and the temperature at each time interval estimated by calorimetry. These results gave a cooling curve, that when extrapolated, would show the temperature at the time when the laser was switched off.

Stainless steel discs of known mass were exposed to 100W Nd:YAG laser irradiation of beam diameter 16mm and immediately placed in 10ml of distilled water. The discs were exposed for 10, 20, 30 or 40s, which was equal to 500, 1000, 1500 or 2000Jcm^{-2}.

Each exposed disc was cooled for 10, 15, 20, 25, 30, 40, 50 or 60s and the temperature estimated for each time period (delay) by measuring the temperature rise in the water.

2.4.1.10 Inactivation of spores on 0.2mm thickness steel coupons

Aliquots (20µl) of *B. cereus* spores were placed on the surface of the 0.2 mm hard tempered stainless steel coupons and allowed to dry. The steel was aseptically placed upon a piece of sterile PTFE plastic and exposed to Nd:YAG laser irradiation of frequency 10Hz, energy 10J and beam diameter 16mm. The exposure lengths chosen were 1 pulse (0.1s), 0.5, 1.0, 2.0 or 2.5s, a control did not receive irradiation and as a further control, non-inoculated steel was used as a test of sterility. After treatment all discs were placed in 5ml of nutrient broth and incubated for 24hr at 37°C. The tests were observed for turbidity or for lack of turbidity (complete sterilisation).

2.4.2.0 Exposure of samples to Nd:YAG laser and Ultra Violet irradiation

The possibility of a synergistic killing effect between laser irradiation and UV irradiation on spores was investigated.

2.4.2.1. Laser treatment of bacterial endospores on agar plates

The rapid clearing of bacterial endospores from the surface of agar plates. Lawns of *B. cereus* spores were prepared by pipetting 100 µl of the spore suspension (**section 2.4.1.1**) onto the surface of a nutrient agar plate, previously dried in a laminar flow hood for 30 min. A sterile glass spreader was used to lawn the spores over the surface.

The spore suspension was only added to the agar surface immediately prior to exposure to the laser beam, to ensure the spores did not revert back to the vegetative state before laser treatment. The petri dishes used for the nutrient agar were divided into six segments, with a marker pen; this allowed six exposures to be made on each plate. The plates used in laser scanning experiments were not sub-divided. The *E. coli* control plates were prepared by adding aliquots of 1.5 ml of a 24 hr *E. coli* culture to the surface of the agar. After the whole surface had been covered by the suspension, the excess was removed and discarded. The plates were dried off in a class III micro biological airflow cabinet (Peteric LTD.) for 30 min. The petri dishes were again sub-divided into six segments.

2.4.2.2 Killing of bacterial endospores on agar surfaces with a combination of UV and laser irradiation

The possible synergistic effect between laser irradiation and UV irradiation was examined. Lawns of spores on agar plates were prepared as described previously. The prepared plates were placed under the UV source for various time intervals and then immediately exposed to laser irradiation. As controls, plates did not receive laser and/or UV irradiation. The plates were incubated at 37°C for 24 hr and any clear area of inactivation was measured (diameter size).

2.4.2.3 Laser treatment of liquid samples

The temperature of a test suspension was increased with laser irradiation, and the subsequent killing of micro-organisms it contained was monitored. A spore suspension (**section 2.4.1.1**) was diluted to an optical density (OD) of 2.0 that corresponded to a bacterial density of 1 x 10^6 $cfuml^{-1}$. This was a good working quantity as the bacterial suspension was only slightly turbid and allowed penetration of the laser irradiation. Aliquots (2 ml) of this suspension were placed in the test vessel.

Samples were laser treated, with the temperature in the vessel raised to; 40, 45, 50, 55, 60, 65, 70, 75 and 80°C. A control was sham treated without laser light. Viable counts were determined to obtain a killing curve against temperature. The Nd:YAG laser was used at 10J, 10 Hz with an 8 msec pulse width and a beam diameter of 14 mm.

2.4.2.4 UV exposure to liquid suspensions

Aliquots (2.0 ml) of spore suspension (**section 2.4.1.1**) were placed in sterile petri dishes and exposed to UV irradiation for 10 and 20 s, 18 cm below the UV lamp. The UV treated samples were placed in the test vessel of the Nd:YAG laser and the temperature was raised to 50, 60 and 70°C by computer controlled laser light. The laser parameters were 10 J, 20 Hz. Further 2.0 ml aliquots of the spore suspension as controls, were exposed to UV irradiation for 10 and 20 s but these were not exposed to laser light. Similarly, further aliquots were laser treated but did not receive UV irradiation. As negative controls, 2.0 ml aliquots of the spore were left untreated. Viable counts were made for each test aliquot spore suspension either UV irradiated, or laser exposed, or UV and laser treated or untreated.

2.4.2.5 Exposure of samples to Nd:YAG laser and UV irradiation

The reduction in viable cells after the combined UV and laser treatment of bacterial endospores was determined on solid surfaces. A 200 µl amount of spore suspension (**see 2.4.1.1**) was pipetted onto the surface of stainless steel discs (**see 2.4.1.3**). The discs were inserted into a sterile aluminium holder and exposed to UV irradiation, for 10 and 20 s, a control did not receive irradiation. For laser treatment, the discs were inserted into a sterile aluminium holder and exposed to the Nd:YAG laser irradiation at variable energy densities 1000, 1500 and 2000 Jcm^{-2} in duplicate. After exposure to laser irradiation, each disc was placed in a 100 ml Duran bottle containing 20 ml of sterile PBS and were shaken at 100 rpm. for three hr at room temperature in a Gallenkamp cooled orbital shaker. Serial 10-fold dilutions were made, 200 µl into 1.8 ml, and 100 µl of sample was placed into 10 ml of nutrient agar (molten pour plate). All plates were incubated at 37°C for 24 hr, and the colonies were counted with a Gallenkamp colony counter.

2.4.2.6 A multifactorial analysis of the effect of laser and UV irradiation and the delay between each treatment, on bacterial endospores.

The effect of laser irradiation treatment followed by UV irradiation and UV irradiation followed by laser irradiation with appropriate controls was examined.

The spore suspension was prepared and 10µl was placed on the surface of stainless steel discs as described previously.

Multifactorial parameters

Laser pre-treatment	5 or 10 s of 10 J, 10 Hz, Nd:YAG laser light.
UV post treatment	20 or 40 s of 2 x 8 W UV lamp irradiation.
Delay in between	10 or 20 min.

Multifactorial experiments require separate test discs to receive a high or low laser exposure, high or low UV exposure, and with a high or low delay in between. Discs received all possible combinations of the parameters above. **Tables 1 and 2.** UV and laser irradiation of the discs was performed as described previously.

Table 1 Combinations of factors for multifactorial experiment.

Laser exposure (s)	UV exposure (s)	Delay (min)
5	20	10
5	20	20
5	40	10
5	40	20
10	20	10
10	20	20
10	40	10
10	40	20

Table 2 Table of controls for multifactorial experiment

Laser exposure (s)	UV exposure (s)	Delay (min)
0	0	Not Applicable
5	0	Not Applicable
10	0	Not Applicable
0	20	Not Applicable
0	40	Not Applicable

2.4.3.0 Increased bactericidal action of laser irradiation and chemicals

2.4.3.1 Monitoring the absorbency spectra of chemicals at 1060 nm wavelength

The absorbency spectra of lactic acid, hydrogen peroxide and distilled water were compared. Aliquots (2 ml) of test chemical were placed in a cuvette (transparant to UV light). The cuvette was placed in a UV-310IPC UV-VIS-NIR scanning spectrophotometer (Shimadzu, Japan). The spectrophotometer measured the absorbency of each chemical across the wavelength range 800 - 1300nm. The chemical was either blanked with distilled water or with air (empty cuvette). A computer printout was produced of the results showing a graph with an absorbency value between -1 and 1 at each wavelength.

2.4.3.2 Hydrogen peroxide treatment of bacterial endospores over time

The reduction in viability after treatment of bacterial endospores with different concentrations of H_2O_2 was determined. Aliquots (200 µl) of spore suspension (**see 2.4.1.1**) were placed in 1.8 ml of various hydrogen peroxide concentrations, 1%, 0.1%, 0.01% (v/v) and a control of 1.8 ml of sterile water, for 10 min. The viable counts were immediately determined for each test concentration after the 10 min had elapsed.

2.4.3.3 The effect of hydrogen peroxide on bacterial endospores, raised from 20 to 60°C.

The reduction in viability of bacterial endospores that were treated at 60°C with sub-lethal concentrations of H_2O_2, was determined. Spores were either exposed to laser light or pre-heated chemical (water-bath). The decrease in viability was monitored for each concentration of H_2O_2. Aliquots (200 µl) of spore suspension (see 3.4.1.1) were placed in 1.8 ml of hydrogen peroxide at sub-lethal concentrations, the spore/H_2O_2 mixture was raised in temperature to 30, 40, 50 or 60°C with laser irradiation of 10 J and 10 Hz. The control was not exposed to laser irradiation. The viability of the spore/H_2O_2 mixture was immediately made for each test after exposure. For the water-bath test, aliquots of the spore suspension (200µl) were placed in 1.8 ml of pre-heated H_2O_2 (at each test concentration) for 45s and a viable count of the suspension was immediately made.

2.4.3.4 Lactic acid treatment of bacterial endospores as a function of time

The reduction in viability of the spores after treatment with different concentrations of lactic acid was monitored. Aliquots (200 µl) of spore suspension (**see 2.4.1.1**) were placed in 1.8 ml lactic acid concentrations of either 1.34 or 2.68 M or in 1.8 ml of sterile water. After 10 min, the viability of each test was monitored and the effect of concentration ascertained.

2.4.3.5 The effect of lactic acid, on bacterial endospores at 20 to 60°C

The reduction in viability of the spores after treatment with sub-lethal concentrations of acid, was monitored after the spore/acid mixture had been raised in temperature to 30, 40, 50 and 60°C with 10J, 10Hz Nd:YAG laser irradiation. The method followed was similar to that in **2.4.3.3**, except 1.34 and 2.68M lactic acid were used instead of hydrogen peroxide.

2.5.0 Spores of fungal species

2.5.1 Exposure of fungal spores to laser irradiation

2.5.1.1 Preparation of fungal spores

A slope of Czapek dox agar, CzA, (Oxoid CM97) was inoculated with *Penicillium* spp. and incubated at 30°C for four days. The spores from this slope were suspended in 10 ml of sterile PBS containing 0.1% (v/v) Tween 20 (Sigma P-1379). The mycelial growth was removed by filtering through a sterile Kleenex tissue.

The spore numbers were counted with a counting chamber (WSI Ltd. England) and diluted to 1 x 10^{-6} spores ml^{-1}.

2.5.1.2 Laser irradiation treatment of fungal suspensions

The decrease in the viability of spore suspensions after raising the temperature of the test aliquots with Nd:YAG laser irradiation was monitored. A 2 ml aliquot fungal spore suspension (**see 2.5.1.1**) was pipetted into a glass vessel containing a mounted thermocouple device. The vessel was aligned with the laser beam and exposed to laser irradiation until the required temperature had been reached. A sample (200µl) was immediately removed and a viable count was made. This was repeated for various

temperatures. A control of fungal spores at room temperature was also prepared and a count made.

2.5.1.3 Laser irradiation treatment of fungal spores on agar surfaces

The inactivation of fungal spores on an agar surface caused by a 13 W, 100 mms^{-1} laser scanning system was monitored. A 1 ml amount of fungal spore suspension (**see 2.5.1.1**) was spread over the surface of a CzA plate and dryied in a laminar air flow hood to produce lawned plates. The prepared plates were laser treated, and incubated until growth was observed. Any zone of inhibition was measured.

2.5.1.4 Laser sterilisation of unwrapped cheese

These experiments aimed to discern whether sterilisation of artificially contaminated cheese was possible by application of laser irradiation. A spore suspension (**see 2.5.1.1**) was used to inoculate individual unwrapped cheese, aseptically from it's vacuum packing. A glass spreader was used to apply 100µl of the prepared spore suspension onto the upper surface. The treated cheese was then i) laser scanned along the test area, with 13 W CO_2 laser irradiation, scan speed 100mms^{-1} or ii) left to grow as a control. The cheeses were left for signs of fungal growth and any zones of inhibition were measured.

2.5.1.5 Laser sterilisation of sliced fruit

The surface of artificially contaminated slices of fruit was exposed with laser irradiation. Species of fruit were aseptically sliced with a knife that had been flamed in alcohol. The fruit was only handled whilst wearing latex gloves cleaned with 70% (v/v) ethanol. Each slice was placed in a sterile petri-dish. Aliquots (200 µl) of a fungal spore suspension (**see 2.5.1.1**) was spread over the surface of the fruit with a sterile glass spreader, and was allowed to dry for 30 min in a safety cabinet. The fruit were exposed to 1kW CO_2 laser irradiation of various scan speeds and then incubated at 30°C until growth was observed, any zones of inhibition were measured. The control did not receive laser irradiation.

2.5.1.6 UV sterilisation of unwrapped cheese

Artificially contaminated cheese was exposed to UV irradiation, and the effect on fungal growth monitored. A 100 µl aliquot of spore suspension (**see 2.5.1.1**) was used to inoculate individual unwrapped cheese (**see 2.5.1.4**). The fungally contaminated cheese was then either i) Exposed to UV irradiation (2 x 8 W lamps) upon the test area, or ii) left to grow as a control. The cheese was left for signs of fungal growth or inactivation.

2.6.0 Mechanisms of laser action

2.6.1 Electron microscopy

2.6.1.1 Transmission Electron Microscopy (TEM) of heat-treated spore suspensions

The structural changes of bacterial endospores after laser treatment was monitored and compared with the effects of conventional heating. Aliquots (2 ml) of a spore suspension (**see 2.4.1.1**) were either exposed to laser light, heated in a water bath, or left untreated as a control. The heat-treated spore suspensions (laser and water bath) were raised in temperature to 70, 80, 90 and 100°C. Each test spore suspension sample was spun down and fixed with 2.5% (v/v) glutaraldehyde (1 hr). The tests were washed 3 times in 0.1M phosphate buffer (Disodium hydrogen orthophosphate anhydrous, BDH 10249 and Potassium dihydrogen orthophosphate, BDH 10203), i.e. in the buffer for 10 min then spun. The supernate was removed and an equal volume (to the pellet) of 2 % (v/v) OsO_4 was added and left for 1 hr. This helps remove all the glutaraldehyde. The OsO_4 was removed after the pellet had been washed in dH_2O 3 times (same method as per buffer). Uranyl acetate 0.5% aq. was added for 1 hr in the dark, and washed 3 times in water. The samples received subsequent 10 min treatment with 30, 50, 70 and 90% (w/v) alcohol then 2 x 10 min treatment with absolute alcohol and 10 min with "dried" alcohol. To the samples was added propylene oxide 3 x 5 min (epoxypropane) (BDH 282904W) and a 1:1 epoxypropane/SPURR resin (TABB, S031) mix overnight. The samples were placed in a rotator for this overnight treatment. The samples were freshly embedded in 100% resin the next day (polymerised in a fume hood oven at 60°C for 48 hr).

The samples were sectioned with a LKB III ultratone sectioning machine and stained with 2% (v/v) methanolic uranyl acetate for 5 min and Reynold's lead citrate for 5 min. (Gibbons *et al*, 1960 and Reynolds 1963). The prepared samples were examined under a Zeiss 902 TEM. apparatus. The images were photographed with Kodak electron microscope film 4489 (Agar scientific P974).

2.6.1.2 Scanning Electron Microscopy (SEM) of heat-treated fungal spore suspensions

The structural changes of bacterial endospores after laser treatment was monitored and compared with the effects of conventional heating. Aliquots (2 ml) of a fungal spore suspension (**see 2.4.1.1**) were either exposed to laser irradiation, heated in a water bath, or left untreated as a control. The heat-treated spore suspensions (laser and water bath) were raised in temperature to 70, 80, 90 and 100°C. Each test spore suspension sample was spun down and fixed with 2.5% (v/v) glutaraldehyde (1 hr). The tests were washed 3 times in 0.1M phosphate buffer (Disodium hydrogen orthophosphate anhydrous, BDH 10249 and Potassium dihydrogen orthophosphate, BDH 10203), i.e. in the buffer for 10 min then spun. The supernate was removed and an equal volume (to the pellet) of 2% (v/v) OsO_4 was added and left for 1 hr. This helps remove all the glutaraldehyde. The OsO_4 was removed after the pellet had been washed in dH_2O 3 times (same method as per buffer). Uranyl acetate, 0.5% (aq) was added for 1 hr in the dark, and washed 3 times in water. The test samples were placed (with a pasteur pipette) onto a 0.2 μm polycarbonate membrane filter. A rubber ring the same diameter as the filter was placed on top and another filter was place upon the ring. The filter/sample sandwich was placed inside a filter chamber. The chamber received subsequent 10min treatment with 30, 50, 70 and 90% (w/v) acetone then 2 x 10min treatment with absolute acetone and 10 min with "dried" acetone. The samples were then critical point dried. The samples were removed from the filter chamber, they were now on the surface of a single filter. The sample was mounted on a stub and gold coated with a Polaron SC515 S.E.M coating system. The prepared samples were examined under a Philip's 500 S.E.M. apparatus. The images were photographed with Kodak PLUS-X 125 pro black and white film. (H. A. West, CAT 352 2265).

3.0 Results

Chapter 3 Results

3.1.0 Spores of *Bacillus* sp.

3.1.1 Laser killing of bacterial endospores on solid surfaces

One of the main uses for a laser sterilisation system would be the rapid clearance of a bacterial load from surfaces. Discs of steel, glass and plastic were used in conjunction with irradiation from either an Nd:YAG or CO_2 laser source.

3.1.2 Beam diameter test.

Initially the beam diameter of a focussed beam from the Nd:YAG laser was measured with previously exposed photographic paper at varying distances from the focal head. **Figure 2** shows the laser beam diameter as a function of distance from the focussing lens. The beam diameter of the laser beam initially decreased as the distance from the focal head was increased, until the focal point was reached, where the beam diameter is at a minimum. The focus point for this laser was 5.0-5.5 cm from the focal head. The beam diameter increased again as the beam passed the focal point and began to diverge. This calibration curve meant that for subsequent experiments the desired beam diameter could be quickly obtained.

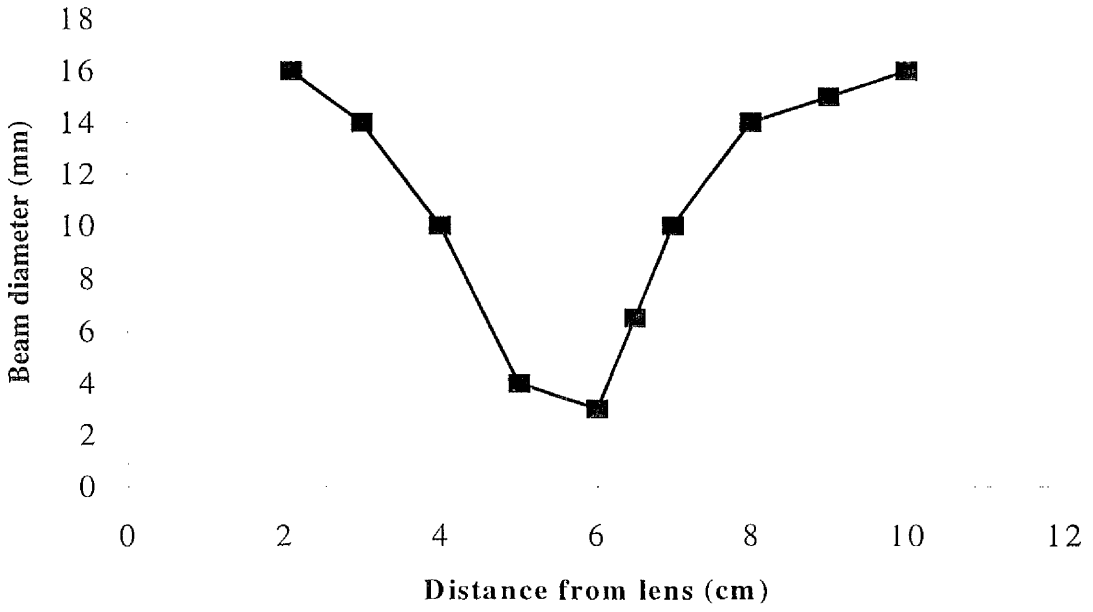


Figure 2 Laser beam diameter test. The graph showed that the laser irradiation beam diameter varied with the distance from the focal head, reaching a focal point 5.0-5.5 cm from the source. After this point was reached, the beam diameter diverged again, hence the shape of the graph.

3.1.3 Effect of storage on spore suspension viability

This experiment was designed to find the optimum temperatures for spore storage so that a spore suspension could be made in bulk and aliquots taken for each experiment. This minimised potential variation between spore batches. Aliquots (1 ml) of the *B. cereus* NCIB 9373 spore suspension were kept at different temperatures for varying lengths of time; 7, 14, 28, 38 or 74 days.

At each allotted time, a viable count was made of the spore suspension. These were compared with the viable count of the suspension at time zero (control) and the results are shown in **Figure 3.**

There was minimal decrease in spore viability at the three different temperatures. For all tests (4, 20, -70°C) there was less than half a D-value decrease after the 74 days had elapsed and indicated that a stock bulk spore suspension could be prepared beforehand and used as required. It was decided to store the spores at -70°C. Before use, the stored spore suspension was placed in an 80°C water-bath and washed in cold distilled water twice.

In addition, the viability at the start and end of an experiment would be the same at the end. This meant that any decrease in spore viability was due to the actual experimental conditions.

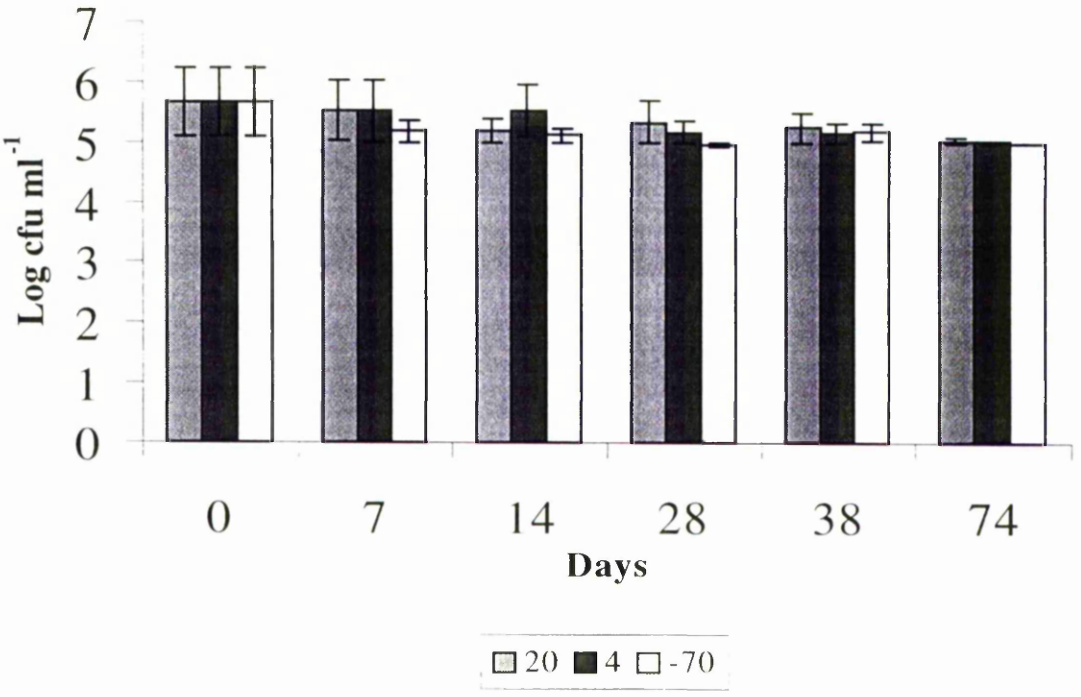


Figure 3 Decrease in spore viability after various lengths of time at different temperatures (see legend, 20, 4 or –70°C). There was very little decrease even after 74 days. The experiment was done in triplicate, the error bars are the standard error of the mean.

3.1.4 Effect on bacterial endospores by the detergent polyoxyethylene sorbitan monolaurate, Tween 20, as a function of time.

Bacillus cereus spores contain a lipid layer called the exosporium. This causes the spores to float in water, thus it was difficult to obtain a reliable distribution of spores in a suspension when spore numbers were estimated by viable counting. It was therefore decided that a detergent, Tween 20, would be added to the diluent, distilled water.

Polyoxyethylene sorbitan monolaurate, Tween 20, was tested for sporicidal action. As mentioned above, it was planned to use Tween 20 as a diluent for viable counting, but it was important to ensure that the detergent at a concentration of 0.1% (v/v) did not have an effect on spore viability.

Aliquots, 200µl of 4.0 x 10^7 cfu ml^{-1} *B. cereus* spore suspension were placed in 1.8ml of 0.1% (v/v) Tween 20 for increasing time periods. The viability of the spores after each time period was ascertained. The results are shown in **Table 3**.

B. cereus spores were placed in 0.1% (v/v) Tween 20, and left at room temperature for time periods between 0 and 60min. The 0min control was an immediate immersion of 200 µl of spores into the Tween 20, the mixture was briefly whirlimixed (2 s) and the viable count immediately ascertained. This immediate immersion and counting gave an average log viable count ml^{-1} of 6.9. After one hour the log viable countml^{-1} showed a slight decrease in viability to 6.53.

For subsequent experiments, during the viable counting process, the spores were only in contact with the Tween 20 for a few seconds before being plated out. It was therefore decided that this concentration of Tween 20, 0.1% (v/v), would be acceptable for the diluent in forthcoming experiments.

3.1.5 Survival of bacterial endospores dried on stainless steel discs

The main surface to be investigated in this section was stainless steel and both wet and dried aliquots of spores would be the target for laser irradiation. It was important to show that drying the spores onto the steel surface would not cause a large decrease in spore viability.

Aliquots, 10µl of 2 x 10^8 cfu ml^{-1} *B. cereus* spores were placed on the surface of stainless steel discs. The samples were dried at room temperature for various time periods, 0 - 60 min in a laminar airflow hood and subsequently the viability was determined for each test by viable plate counts as shown in **Table 4**. These experiments showed that there was no significant decrease in the viability of the spore suspension, after it was allowed to dry onto the surface of a stainless steel disc. Only a 0.5 D-value loss in viability with the disc dried for 60 min. Consequently, any loss in cell viability can be attributed to the test and not the process of drying the organisms on the discs or the recovery technique. For the subsequent experiments, there was only 20 min between the start of the drying process and the start of the experiment.

Table 3. Activity of spores in the presence of Tween 20. Prolonged exposure (60 min) to Tween 20 was required before a slight decrease in spore viability was observed (0.5D decrease). The experiment was carried out in triplicate.

Time (min)	Logcfuml^{-1}	Standard Error of mean (N =3)
0	6.9	0.07
5	6.98	0.03
10	6.82	0.02
20	6.71	0.02
30	6.78	0.01
60	6.53	0.33

Table 4 Spore survival on steel. After one hour on the disc, there was little loss of viability. It should be noted that at time 20 min, the suspension dried. The experiment was in triplicate and the error bars visible are the standard error of the mean.

Drying Time (min)	Log surviving counts ml^{-1}	Standard Error of Mean (N=3)
0 (control)	6.52	0.03
0.5	6.47	0.01
1.0	6.39	0.04
30.0	6.51	0
60.0	6.44	0.04

3.1.6 Laser treatment of bacteria on solid surfaces

3.1.6.1 CO_2 laser treatment

B. subtilis spores (10µl of 1.0 x 10^7cfu ml^{-1}) were placed on the surface of plastic discs and allowed to dry at room temperature. The prepared samples were exposed to 1kW power CO_2 laser irradiation, 11mm beam diameter, and the maximum energy density of irradiation applied was 105.28 Jcm^{-2}. The samples received a single pulse of irradiation of varying length, 0 (control), 4, 6 or 10 ms. The corresponding energy densities are shown in **Table 5**.

The spore suspensions were placed on plastic discs for treatment because the CO_2 laser irradiation wavelength (10.6µm) was more readily absorbed by plastic than by steel. A dried sample was exposed because CO_2 laser irradiation is very readily absorbed by water. This causes the production of steam and the sample can be splattered off the surface of the disc, due to the rapid absorption of energy. This is unsafe as aerosols of bacteria could be generated and this would effect the results in terms of artificially low viable counts due to the sample being lost. Bacterial aerosols may also contaminate the air above the disc and then fall on the cleaned surface, and recontaminate it. This would be an important consideration for an industrial set-up.

The results of these experiments showed that exposure to CO_2 laser irradiation gave a decrease in viability, a 1.4D value decrease was observed, after an exposure of only 10ms, an energy density of 105.28 Jcm^{-2}. The results are shown in **Figure 4a**.

The results indicated that this procedure was more effective than the Nd:YAG laser irradiation on stainless steel discs (**see 3.1.6.3 and 3.1.6.4**), in terms of killing in relation to energy density applied. The Nd:YAG laser irradiation gave a 0.66D decrease after an energy density of 157.3 Jcm^{-2} had been applied to the spores. The length of exposure time with the CO_2 laser system was only 10 msec as opposed to 1 sec with the Nd:YAG, this means a large high-powered system with CO_2 laser irradiation would be more effective than the 300W Nd:YAG system. The killing effect may have been greater after a longer exposure but the plastic was reaching its tolerance limits to damage caused by the laser at this value, due to the high absorbency of the material for this laser wavelength.

Table 5 Energy density of the CO_2 laser irradiation applied to the discs

Exposure (ms)	Energy Density (Jcm^{-2}) Plastic	Energy Density (Jcm^{-2}) Glass
0 (control)	0	0
4	4.2	-
6	6.3	-
10	10.5	6.7
15	-	10.1
20	-	13.5

Note that the energy density values vary between the tests after the same exposure, due to 1 kW or 640W being used.

The experiment was repeated with glass discs and CO_2 laser irradiation of 640W (this allowed exposure of 20ms). The energy densities are also shown in **Table 5**, the reduction in viability caused by the laser exposures is graphed in **Figure 4b**.

Lower power CO_2 laser irradiation was used in these experiments (640 W not 1 kW). This allowed the exposure length applied to the discs to be increased to 20 msec without them being damaged.

The CO_2 laser irradiation was also effective on glass discs, even at the lower power setting. After a 15msec exposure, a 3.24D value decrease was observed. This was obtained with an energy density value of only 10.1 Jcm^{-2}. This energy density was lower than the average energy applied to the *B. subtilis* spores on the plastic discs, but a greater inactivation effect was observed. It should be noted however, that after 20ms exposure, there was a high count. This was due to one trial giving higher counts than the others, note the higher standard error of the mean. It is thought that this was due to fluctuations in the beam profile (see discussion).

3.1.6.2 CO_2 laser scanning of spores on steel

A smaller CO_2 laser was also available, with a maximum power of 13Wand a scanning mirror. Strips of stainless steel were produced with a groove set in the centre to contain the sample. *B. cereus* spores (10µl of 6.0 x 10^5 cfu ml^{-1}) were placed on the surface of the stainless steel and allowed to dry at room temperature. The sample was exposed to CO_2 laser irradiation in the form of a 100mm s^{-1} scan, with a beam diameter of 2.5 mm. The laser power applied varied from 0 (control) to 3, 5, 7, 9, 11 or 13W. The results are in **Figure 5**. The result of these experiments showed that the scanning CO_2 laser was ineffective on the spores on the surface of stainless steel even at the full 13 W power setting. It is apparent that the amount of irradiation given to the spores was too low to achieve a significant reduction in the viable count, either in terms of power setting, or the scanning speed was too fast.

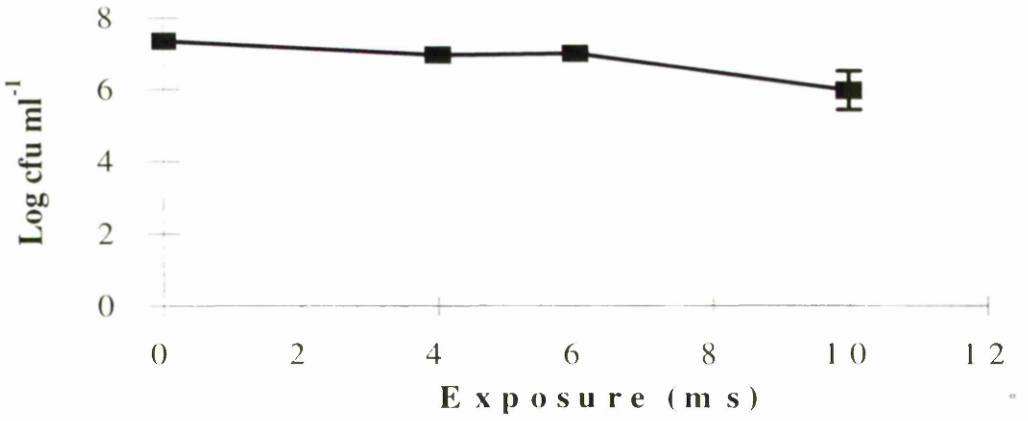


Figure 4a The decrease in viable numbers of *B. subtilis* spores on plastic discs caused by different exposure times to a pulse of CO_2 laser irradiation (msec). A 1D decrease was observed after 10 ms. The error bars are the standard error of the mean.

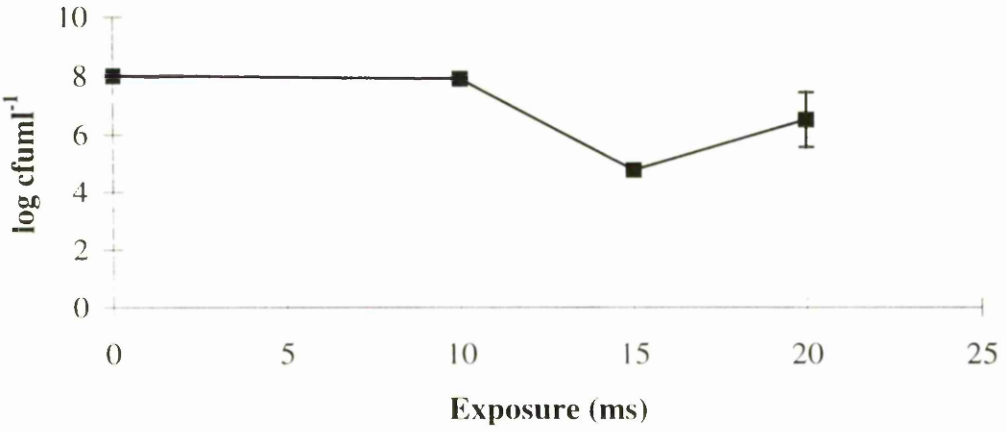


Figure 4b Decrease in viability of *B. cereus* spores on glass discs after exposure to CO_2 laser light 640W for different lengths of time (ms). A 1.5D decrease was observed after 20ms. The fluctuations observed were due to irregularities present in the laser beam profile. The experiment was carried out in triplicate and the error bars shown are for the standard error of the mean.

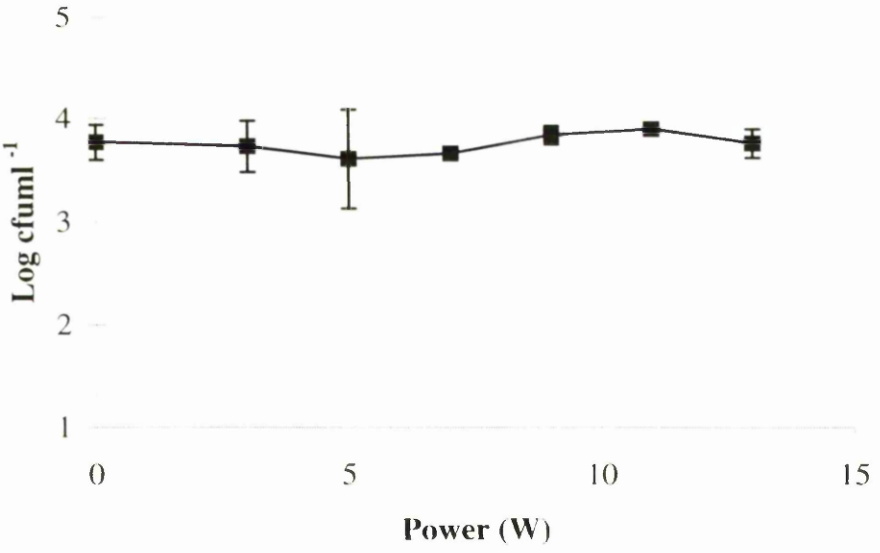


Figure 5 The action of scanned low power CO_2 laser irradiation on *B. cereus* spores on stainless steel. There was no significant loss of viability caused by the scanning laser irradiation. The experiment was done in triplicate, the error bars are the standard error of the mean (n=3).

3.1.6.3 Nd:YAG laser treatment - High energy, short duration laser irradiation killing of bacterial endospores on a solid surface

B. cereus spores, (10µl of 3.3 x 10^8cfu ml^{-1}) were placed on the surface of stainless steel discs. Both wet and dry samples were tested, i.e. the dry samples were allowed to dry at room temperature for 20 min. The samples were exposed to a single pulse of Nd:YAG laser irradiation (pulse width 8ms), beam diameter 8 mm. The pulse energy was varied: 0 (control), 5, 10, 15 or 35 J. The decrease in viability at each test parameter is shown in **Figure 6**. It was apparent that there was very little killing caused by these pulses of laser irradiation, in either the wet or dry samples. The energy density was delivered in a single rapid pulse (8 msec) but it was not sufficiently high to facilitate the desired killing effect. The results were analysed statistically with a 2 sample t-Test. The difference in killing between the wet and dry treatments proved statistically insignificant, $p = 0.96$ and 0.34 respectively (95% confidence limits). Under the same limits there was also no significant difference between the treatments at 0-35J, $p = 0.32$.

To test the efficiency of this method further, aliquots of the non-sporulating organism, *S. aureus* (10µl of 6.8 x 10^{10}cfuml^{-1}) were placed on the surface of stainless steel discs. The samples were immediately exposed to a single pulse of Nd:YAG laser irradiation (pulse width 4 msec), beam diameter 7mm. The energy of the pulse was varied: 0 (control), 10, 20, or 30J. The energy densities applied were 0, 157.9, 315.8 or 473.7 Jcm^{-1}. There was no sporicidal action. The results are in **Figure 7.**

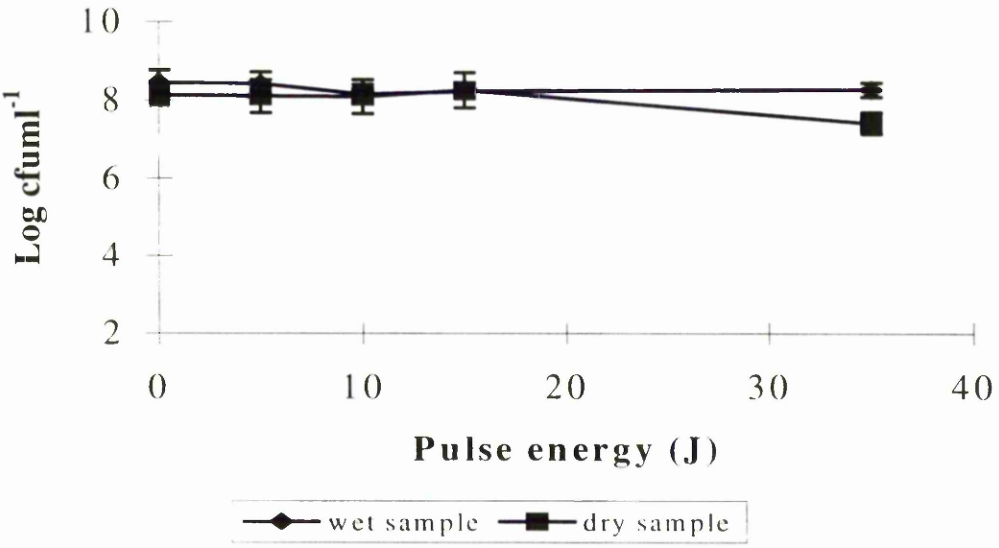


Figure 6 The effect of a single pulse of Nd:YAG laser irradiation of different pulse energy, on *B. cereus* spores on stainless steel discs. There was very little reduction in viability with both wet and dried test suspensions. The experiment was done in triplicate and the error bars are shown as standard error of the mean.

S. aureus was chosen as the test organism, which only occurs as vegetative cells. It was felt that if a trend were to appear, the more easily killed vegetative cells would express it more noticeably than the more resistant spores. This time the energy was applied over a smaller beam area and delivered in 4 msec. The Nd:YAG laser irradiation pulse width was reduced to 4ms, so that the energy density applied would be delivered in a shorter period of time, i.e. total energy density deliverance in a short burst, as opposed to letting it be delivered gradually. The beam diameter was reduced too, in an attempt to increase the energy density. The results of these experiments showed a great deal of variance. There was a 2D-value decrease observed after the 30J pulse, but after allowing for errors, this may not be an accurate estimation. The results obtained from this experiment showed very little killing. The applied energy density would have to be increased to allow killing of the organisms.

3.1.6.4 Nd:YAG irradiation administered over longer time periods

Aliquots, 10µl of 4.65 x 10^8 cfuml^{-1}, of *B. cereus* vegetative cells were placed on the surface of a stainless steel disc. The wet samples were exposed to Nd:YAG laser irradiation at 10J and 30 Hz, beam diameter 1.55 cm. The sample received exposures of varying time length, from 0 (control) to 5 s. The corresponding energy densities are shown below in **Table 6**. The bacterial suspensions placed on the steel discs, were exposed to laser irradiation for varying lengths of time i.e. 0 min (control) to 5 s. This corresponded to a maximum energy density of 795.35 Jcm^{-2}, the reduction in viability is shown in **Figure 8**. It is apparent from the graph, that the Nd:YAG laser irradiation was effective in inactivating these vegetative cells on the stainless steel surface. The bacterial cells were completely inactivated after 1.5 s exposure (238.6 Jcm^{-2}). This corresponded to more than a 6D-value decrease in the viability of the cells. After a 1.0 s exposure, the laser irradiation caused a 3D value decrease in viability of the cells. The energy density applied in this case was 159.07 Jcm^{-2}. This showed that the stainless steel inoculated with vegetative cells of *B. cereus* was effectively sterilised.

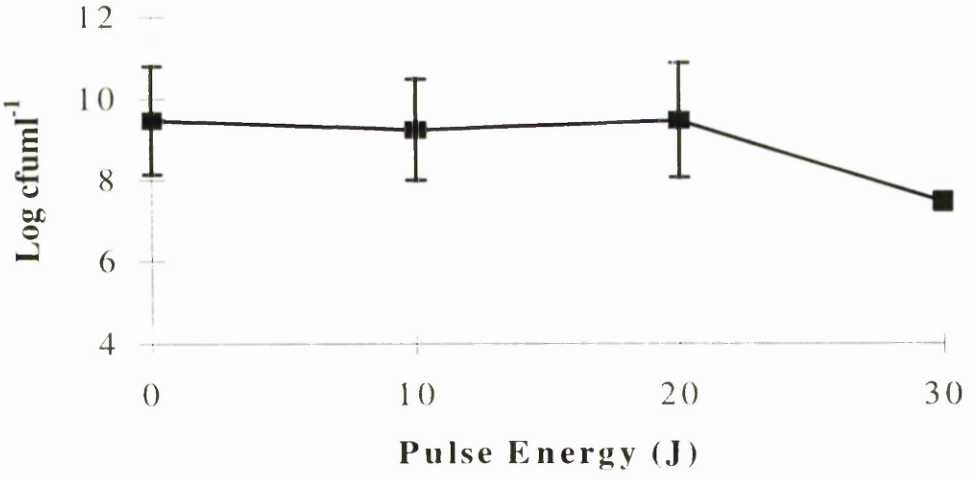


Figure 7 The effect of delivering a pulse of Nd:YAG laser irradiation of different energy levels on *S. aureus* cells on stainless steel discs. The errors shown are the standard error of the mean (n = 3).

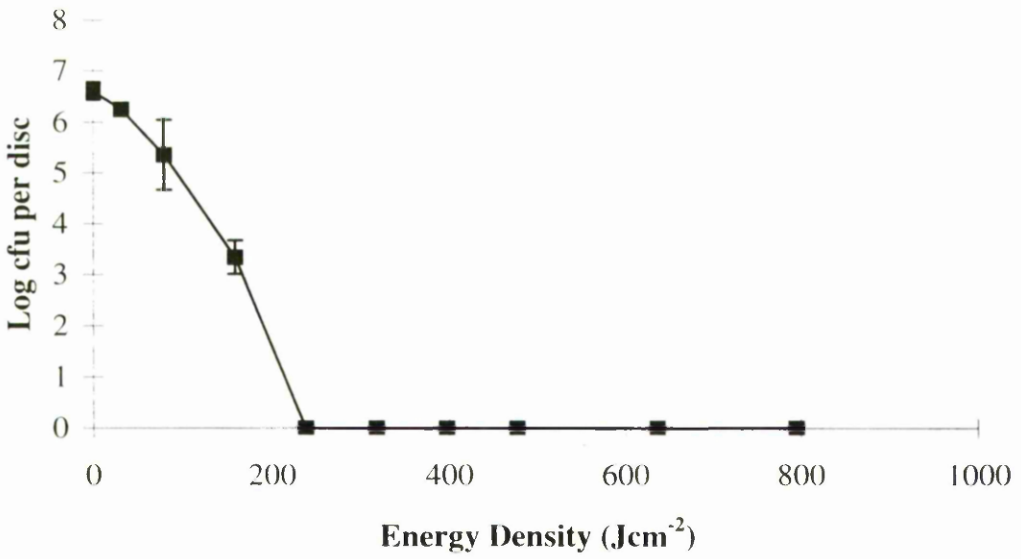


Figure 8 The decrease in viable numbers due to Nd:YAG laser irradiation exposure of vegetative cells of *B. cereus* on stainless steel discs. The vegetative bacilli were inactivated in 1.5s (6-D values). The experiment was in triplicate the error bars where visible, are the standard error of the mean.

Table 6 Nd:YAG laser irradiation energy density applied to vegetative cells of *B. cereus* on steel discs.

Exposure (s)	(control)									
	0	0.2	0.5	1.0	1.5	2.0	2.5	3.0	4.0	5.0
Energy Density (Jcm^{-2})	0	32	80	159	239	318	398	477	636	795

Complete inactivation was achieved at an energy density of 239 Jcm^{-2}.

On the other hand, when aliquots (10µl) of a 2.1 x 10^9 $cfuml^{-1}$ *B. cereus* spore suspension were placed on the surface of stainless steel discs, and exposed to Nd:YAG laser irradiation quite different results were obtained. The laser parameters were 10J, 10 Hz and a 9 mm beam diameter, with an 8 msec pulse width. The test suspensions received varying exposures of irradiation, 0 (control), 1, 2 or 5s. The corresponding energy densities are shown in **Table 7**, the reduction in viability is shown in **Figure 9**.

These results showed that as expected, the bacterial endospores were more resistant to the laser irradiation than the vegetative cells of the same organism. The spores were reduced by 2 D-values after an exposure of 5s (energy density 786.35 Jcm^{-2}) this was attributed to inactivation of germinated spores.

3.1.6.5 Estimation of the temperature of laser irradiated, stainless steel discs

It was felt that to gain a better understanding of the results above, it would be worthwhile to estimate the temperature of the laser irradiated discs. It would help to compare the killing effect of more conventional means, and help to show the efficiency of the laser treatment, i.e. were the temperatures obtained high enough for spore inactivation.

To estimate the temperature of the discs, laser irradiated discs were allowed to cool for varying lengths of time, and the temperature at each time interval estimated by calorimetry. These results gave a cooling curve, that when extrapolated, would show the temperature at the time when the laser was switched off.

Stainless steel discs of known mass were exposed to 100W Nd:YAG laser irradiation of beam diameter 16mm. The discs were exposed for 10, 20, 30 or 40s, which was equal to 500, 1000, 1500 or 2000Jcm^{-2}.

Each exposed disc was cooled for 10, 15, 20, 25, 30, 40, 50 or 60s and the temperature estimated for each time period (delay) by calorimetry. This range was repeated for each energy density, see **Table 8.**

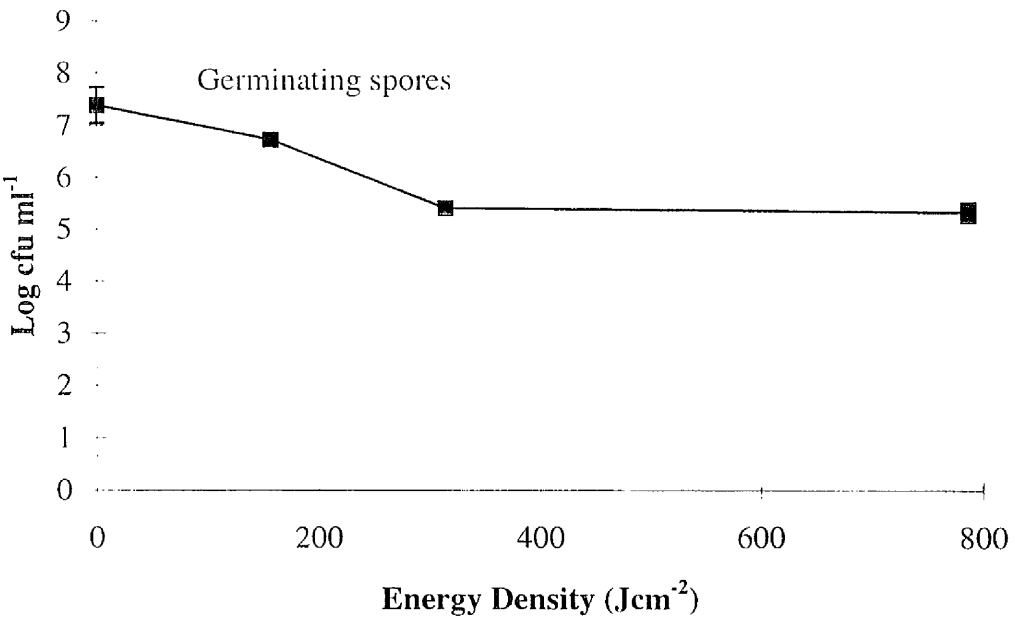


Figure 9 Killing of *B. cereus* spores by Nd:YAG laser on stainless steel discs. There was a 2D decrease caused by exposure to the irradiation probably due to germination. The average laser irradiation energy density applied, was 778Jcm^{-2} after 10s. This was much higher than the 238Jcm^{-2} energy density that caused complete inactivation of vegetative cells. The experiment was carried out in triplicate, and the standard errors of the mean are shown.

Table 7 Nd:YAG laser irradiation energy density applied to wet spores of B. cereus on steel discs.

Exposure (s)	(control)			
	0	1	2	5
Energy Density (Jcm^{-2})	0	157	315	786

Complete inactivation was not observed.

Table 8 Temperatures found for stainless steel discs exposed to Nd:YAG laser irradiation of energy density a) of 500, 1000 and b) 1500 or 2000Jcm^{-2}. The estimated temperature reached after energy density of 500Jcm^{-2} was 79°C. After 1000Jcm^{-2} the temperature reached was 98°C. After 1500Jcm^{-2} the temperature reached was 88°C. (lower than expected) After 2000Jcm^{-2} the found temperature was 128°C. The experiment was in triplicate.

a)

Energy Density (Jcm^{-2})	Time (s)	Temperature (°C)	S.E.M.	Energy Density (Jcm^{-2})	Time (s)	Temperature (°C)	S.E.M.
500	Laser off	78.59	1.4	1000	Laser off	98.16	9.8
	10	67.98	6.0		10	79.9	5.1
	15	62.24	5.5		15	76.48	3.5
	20	52.04	3.3		20	68.3	2.8
	25	45.97	6.1		25	49.34	4.8
	30	40.7	3.6		30	53.51	3.5
	40	32.85	5.0		40	45.81	2.5
	50	31.89	3.0		50	38.04	4.9
	60	28.91	3.0		60	33.12	4.03

b)

Energy Density (Jcm^{-2})	Time (s)	Temperature (°C)	S.E.M.	Energy Density (Jcm^{-2})	Time (s)	Temperature (°C)	S.E.M.
1500	Laser off	87.98	7.4	2000	Laser off	127.96	1.4
	10	95.45	15.9		10	126.39	6.3
	15	84.37	6.7		15	95.07	3.8
	20	65.04	17.7		20	83.9	12.6
	25	63.05	15.3		25	77.09	13.2
	30	53.76	2.2		30	83.72	8.9
	40	49.6	3.8		40	50.04	17.1
	50	48.84	9.7		50	46.74	11.4
	60	44.95	5.6		60	46.58	7.7

This was interesting as it showed that the temperatures being reached by the disc were not really that high i.e. at energy density of 500Jcm^{-2} the disc only reached 76°C and the exposure was 10s. This would be deemed low, as spores can survive in liquids at 100°C. It should be noted however that the temperatures found were the bulk temperature of the disc, the surface temperature would be higher (it is not known by what factor).

3.1.6.6 High energy density killing of spores on steel discs

Once the range of working temperatures was known, it was decided to attempt to kill spores at higher energy density levels, but a slightly different method was employed Aliquots (10µl) of *B. cereus* 9373, spore suspension (6.4 x 10^6 cfu ml^{-1}) were placed on steel discs, and exposed, in either the wet or dry state to 100W Nd:YAG laser irradiation and beam diameter 16mm for 34, 36, 38 or 40s. This was equal to 1700, 1800, 1900 or 2000Jcm^{-2} respectively. Twenty seconds after the laser was switched off, the disc was placed in 10ml of sterile nutrient broth. These were incubated at 37°C and observed for turbidity. As controls, a disc without spores, and an inoculated disc that did not receive laser treatment, was placed in broth. The results show the average number of turbid tubes (out of 5 tests) and are shown in **Figure 10.**

The 2000Jcm^{-2} points were compared with a 2 sample t-test at 95% confidence limits but there was no significant difference ($p = 0.3$).

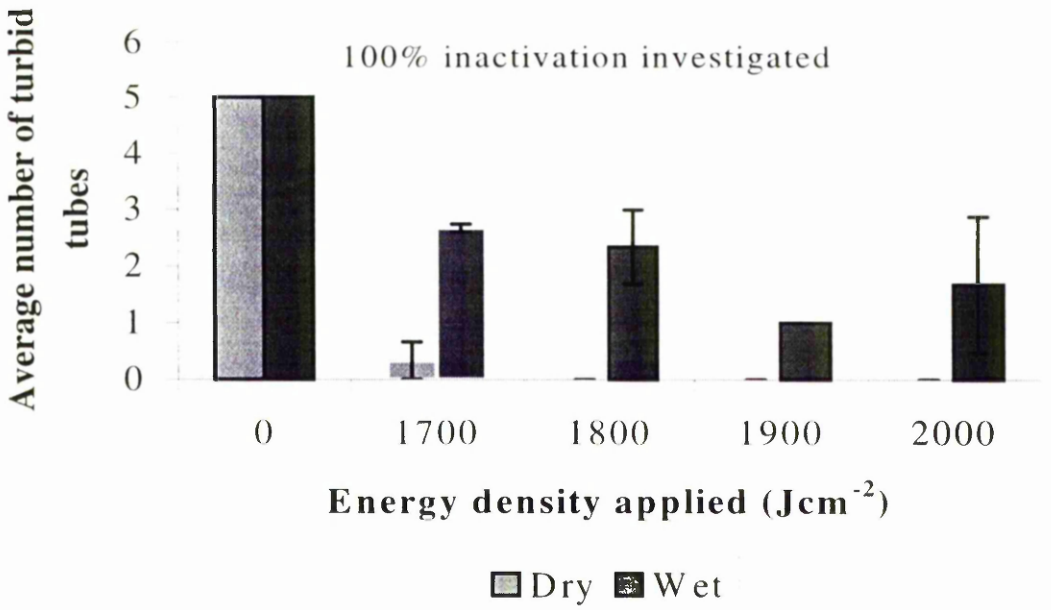


Figure 10 Inactivation of wet and dry spores by Nd:YAG laser irradiation. Note that complete sterilisation of the spores was achieved for the dry sample at 1800Jcm^{-2}. The experiment was carried out five times in triplicate. The error bars shown are the standard error of the mean.

There had been complete inactivation of the spores at 1800 Jcm^{-2} as no test showed any growth from the spores as shown by the turbidity tests. This showed that laser sterilisation of spores was attainable with dry samples.

At 1800 Jcm^{-2} the time was quite high, 36s, so the experiment was repeated with a smaller beam diameter (8mm) this allowed the same energy density to be applied in 9s. The experiment was only attempted with dried samples.

Aliquots (10µl) of a *B. cereus* 9373 spore suspension (1.3 x 10^6 cfu ml^{-1}) were dried on steel discs and exposed to 8mm beam diameter, 100W Nd:YAG laser for 8.5, 9, 9.5 or 10s. This was equal to 1700, 1800, 1900 or 2000Jcm^{-2}. Twenty seconds after the laser was switched off, the disc was placed in 10ml of sterile nutrient broth, incubated at 37°C for 24 hr and observed for turbidity. As controls, a disc without spores and an inoculated disc that was not laser treated, were placed in broth. A negative killing result gave turbidity in the tube. Five tubes were done for each test, the results are shown as the average number of sterile tubes out of five in **Figure 11**.

3.1.6.7 Estimation of the temperature of laser irradiated (8mm beam diameter) stainless steel discs

The calorimetry experiment was repeated for 8mm beam diameter Nd:YAG laser irradiation, as the killing was achieved in a quarter of the time, it was important to see whether this was because the disc was heating to a higher temperature than before. The stainless steel discs of known mass were exposed to Nd:YAG laser irradiation (of beam diameter 8mm). The energy of the irradiation was 10J and the frequency was 10Hz, power 100W, energy density 500, 1000, 1500 or 2000Jcm^{-2}.

Each exposed disc was allowed to cool for 10, 15, 20, 25, 30, 40, 50 or 60s and the temperature estimated for each time period (delay) by calorimetry as described previously. This range was repeated at each energy density.

The results are shown in **Table 9.**

Figure 12 shows the temperatures for the exposed steel discs, for the various energy density levels. The 16mm and 8 mm beam diameter laser irradiation heating could now be compared.

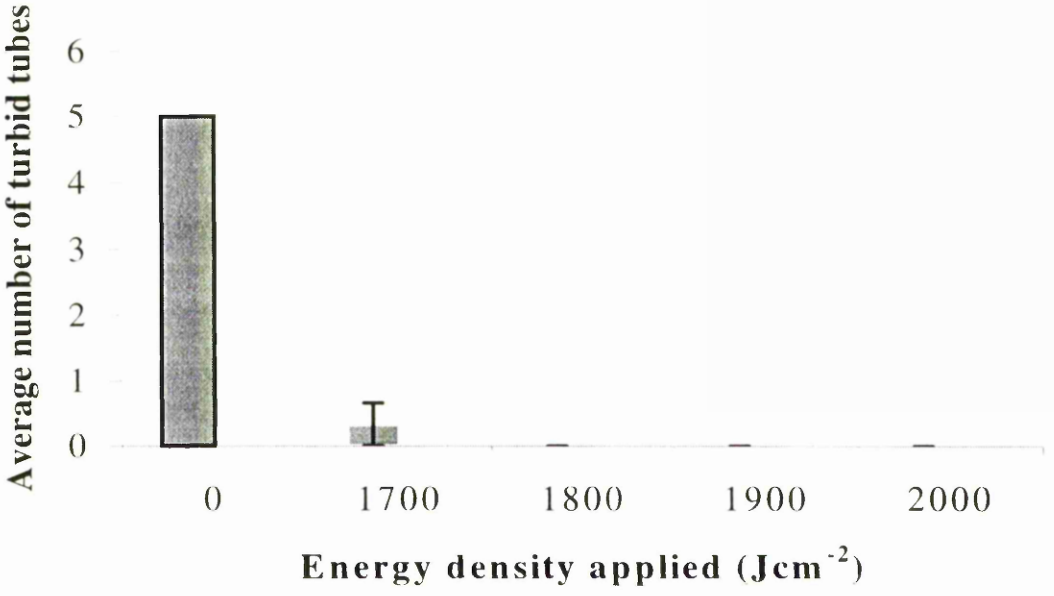


Figure 11 Complete inactivation of spores on steel discs by Nd:YAG laser irradiation of 1800 Jcm^{-2} for an exposure of 9s, 8mm beam diameter. The experiment was carried out five times in triplicate. The error bars are standard error of the mean. Note only dry tests were attempted.

Table 9 Estimation of temperature for stainless steel discs exposed to Nd:YAG laser irradiation of energy density of a) 500 or 1000Jcm^{-2} b) 1500 or 2000Jcm^{-2}. The estimated temperature reached after energy density of 500Jcm^{-2} was 78°C. After 1000Jcm^{-2} the temperature reached was 90°C. After 1500Jcm^{-2} the temperature reached was 122°C. After 2000Jcm^{-2} was applied the found temperature was 141°C.

a)

Energy Density (Jcm^{-2})	Time (s)	Temperature (°C)	S.E.M.	Energy Density (Jcm^{-2})	Time (s)	Temperature (°C)	S.E.M.
500	Laser off	77.46	12.6	1000	Laser off	89.4	2.3
	10	72.49	9		10	78.4	5.5
	15	64.24	16.9		15	78.24	5.0
	20	41.02	3.8		20	60.66	11.2
	25	48.86	4.7		25	49.3	5.3
	30	39.12	4.5		30	40.31	2.4
	40	24.43	2.2		40	32.11	4.7
	50	28.56	4.6		50	32.32	3.3
	60	24.62	1.3		60	30.27	1.0

b)

Energy Density (Jcm^{-2})	Time (s)	Temperature (°C)	S.E.M.	Energy Density (Jcm^{-2})	Time (s)	Temperature (°C)	S.E.M.
1500	Laser off	121.52	5.8	2000	Laser off	141.29	7.2
	10	110.24	11.6		10	124.11	4.2
	15	75.14	11.4		15	106.94	4.9
	20	77.52	13.5		20	88.15	12.1
	25	51.38	7.4		25	77.05	12.4
	30	59.34	3.5		30	53.43	6.6
	40	33.44	7.0		40	43.02	1.3
	50	31.58	5.2		50	42.64	7.4
	60	27.42	10.4		60	38.43	7.7

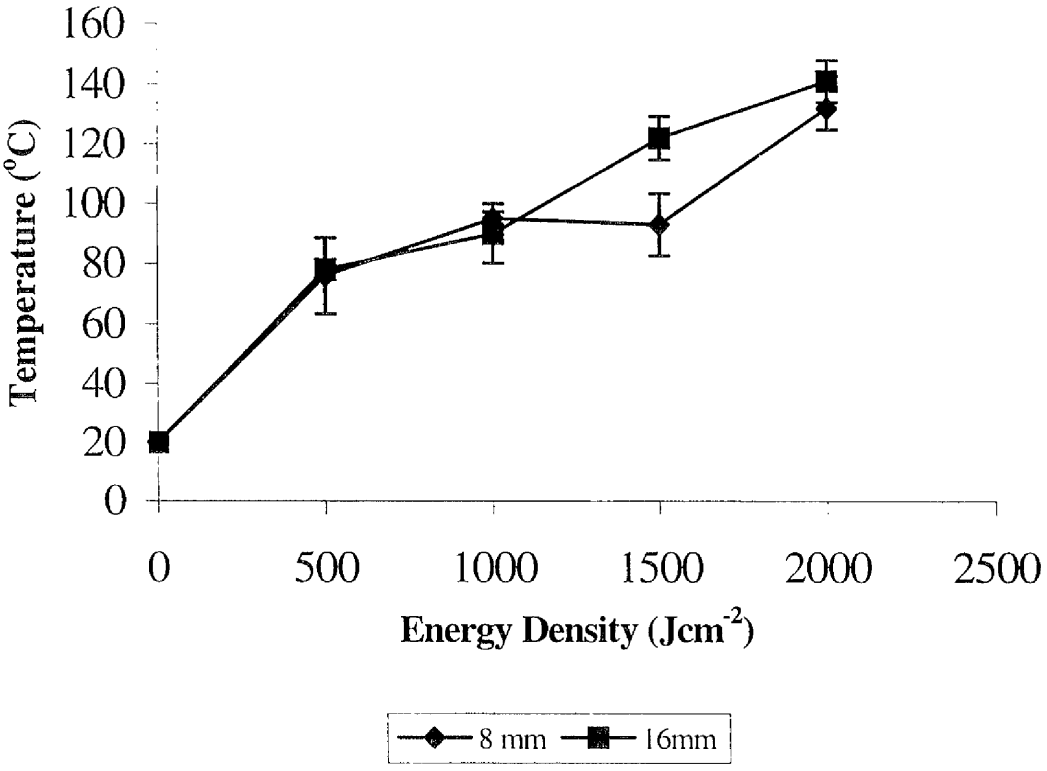


Figure 12 Comparison of found temperatures of stainless steel discs, exposed to Nd:YAG laser irradiation of 8 or 16 mm beam diameter. The bulk temperature of the disc rose as the energy density was increased. The error bars are the standard error of mean (n=3). The difference in the results was not statistically significant, the same energy went into the discs, and the same temperature was found. Note that heat losses would have been into the stainless steel holding block and through the air above the disc.

3.1.6.8 Laser irradiation killing on 0.2mm thick steel

In relation to the laser beam profile, the 8mm beam diameter should have given more effective inactivation than the 16mm beam diameter (see discussion). The spores were inactivated equally well by both beam diameters at 1800J cm^{-2}. The discs did reach the same temperature, and it was hypothesised that this was the important factor. To help investigate this further, it was decided to try exposing thinner pieces of stainless steel to laser irradiation. The thinner material would heat up quicker, and reach a higher bulk temperature than that achieved with the same energy density given to a thicker test disc.

Coupons of 0.2 mm hard tempered stainless steel were placed in an oven for 30s at varying temperatures (100-1000°C in 100° increments). The steel was observed for any colour change. The stainless steel showed no change in appearance until the temperature reached 400°C when a straw colour was observed.
Sections of the 0.2 mm hard tempered stainless steel were also placed upon PTFE (to keep temperature loss down) then exposed to 100W Nd:YAG laser of beam diameter 16mm for varying exposure lengths between 1 pulse (0.1s) and 5.0s. There was no change in the appearance of the steel until the exposure reached 3s, at this point a straw yellow colour was observed. For the laser irradiation treatments, it was decided to keep the exposure length below 3s. This would maintain the steel surface appearance and allow an estimation of the temperature reached, i.e. below 400°C.

Aliquots (20µl) of *B. cereus* spores were dried on the surface of the 0.2 mm hard tempered stainless steel coupons and exposed to 100W Nd:YAG laser irradiation beam diameter 16mm. The exposure lengths chosen were between 0.1s (1 pulse), and 2.5s, a control did not receive irradiation and as a further control, non-inoculated steel was used as a test of sterility. After treatment all discs were placed in 5ml of nutrient broth and incubated for 24hr at 37°C. The tests were observed for turbidity or for lack of turbidity (complete sterilisation). The results are shown in **Figure 13**. Sterilisation was achieved after 2.5s. This exposure was equivalent to 120 Jcm^{-2}. This energy density was lower than in any of the previous experiments this showed again that the bulk temperature (reached after the energy density was applied) was the important killing agent (see discussion).

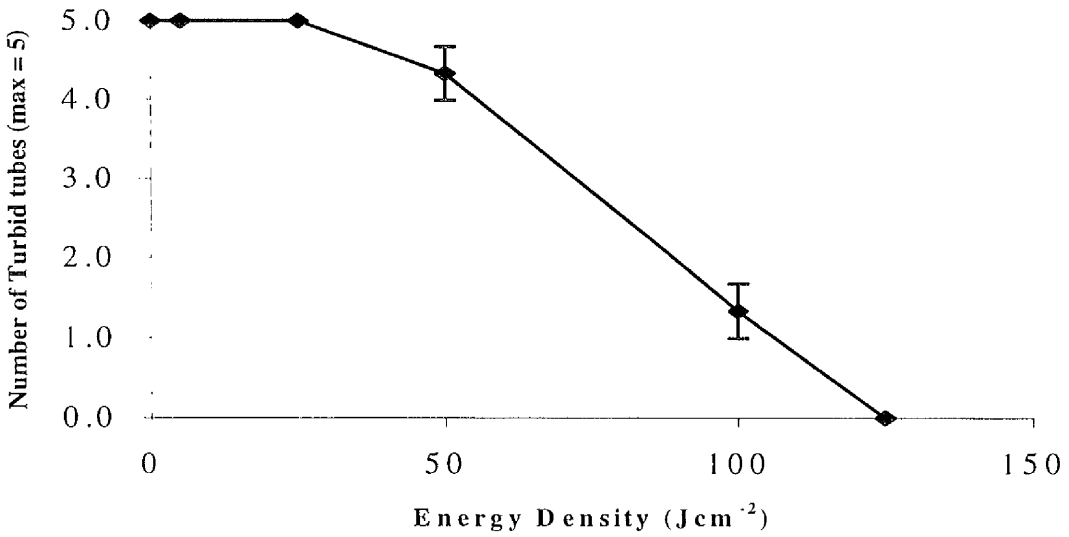


Figure 13 The effect of Nd:YAG laser irradiation on spores on 0.2mm thick stainless steel coupons. There was complete sterilisation after 2.5s at an energy density of 125Jcm^{-2}. The experiment was done five times in triplicate. The errors shown are the standard error of the mean.

3.2.0 Exposure of bacterial endospores to laser irradiation before and after ultra violet irradiation

Although laser irradiation caused inactivation of spores, it was at a reduced efficacy in relation to vegetative cells. The aim was to increase this efficacy by additional exposure of the spores to sub-lethal levels of UV irradiation.

3.2.1 Laser treatment of bacterial endospores on agar plates

3.2.1.1 Nd:YAG treatment

Nutrient agar plates were lawned with 200µl containing 1 x 10^6 cfu ml^{-1} *B. cereus* or *B. subtilis* spores. The lawns were immediately exposed to varying levels of 100W Nd:YAG or 1kW CO_2 laser irradiation. The highest energy density reached was 3000 and 900Jcm^{-2} respectively.

There was very little clearing of the spore lawns, by either type of laser irradiation. Subsequently, the spore lawn was subjected to Nd:YAG laser irradiation of energy density of 3000 Jcm^{-2} and the pulse repetition frequency (PRF) and the pulse energy was varied. This energy density was relatively high (in terms of the values needed for clearing of vegetative cells), and caused an indentation (melting) on the surface of the agar plate. This meant that the agar was absorbing the laser irradiation to such an extent that it was being damaged. The PRF was varied from 5 to 30 Hz and the energy values varied from 10 to 40 J. There were no clearing of any of the spore lawns, of either *B. cereus* or *B. subtilis* which indicated that the spores were very resistant to Nd:YAG laser irradiation on the surface of nutrient agar plates. As controls, lawns of *E. coli* received the same energy density of laser irradiation, at all levels the non-sporulating control organism was inactivated very successfully. In an attempt to increase the energy density applied to the lawns, a piece of aluminium was placed under the nutrient agar to reflect some of the unabsorbed irradiation resulting in a second exposure for the spores. When laser irradiation of 10J, 10Hz and energy density of 3000 Jcm^{-2} was applied to the lawn, there was still no clearing. At 20Hz and 10J,and still with an energy density of 3000 Jcm^{-2} but the plastic petri dish holding the agar melted and blackened.

3.2.1.2 CO_2 laser

Spore lawns were also exposed to irradiation from the CO_2 laser. Single pulses of 1 kW power were applied to the spore lawns for various lengths of time, 200 msec to 500 msec, the energy density ranged from 176-442 Jcm^{-2}. These exposures caused great indentations of the agar (melting of agar and/or evaporation of water), the diameter of the area of damage was also greater than the laser beam diameter of 11mm, this was due to lateral diffusion of heat and evaporation of the water in the agar. The deep indentations were due to the high water content of agar, and the high absorption of the CO_2 laser wavelength (10.6 μm) by water. The agar was therefore absorbing the laser irradiation, but again the spores were not being inactivated during the process. Lower power CO_2 laser irradiation was applied to the lawned plates to achieve longer exposure times. Energy densities were 660-880 Jcm^{-2}. These exposures caused considerable stress on the tests, and the damage threshold of the petri dishes themselves was reached. There was again no clearing of the lawns. A laser scan was attempted with 500W CO_2 laser irradiation, scan speed 10 $mmin^{-1}$. This scan speed allowed the beam diameters to overlap by one third of their diameter. The exposure caused a furrow along the previously smooth surface, but again there was no clearing of the lawn. The results showed that the CO_2 laser irradiation should not be chosen for sterilisation of this surface.

3.2.2 Killing of bacterial endospores on agar surfaces with UV irradiation and laser irradiation

Nutrient agar plates were lawned with 200 μl of 1 x 10^6 $cfuml^{-1}$ *B. cereus* spores. The lawns were immediately exposed to UV irradiation from 2 x 8W lamps (distance from lamps was 24cm) for various lengths of time, namely, 0 (positive control), 2, 4, 5, 6, 8, or 10 min. Immediately after each UV exposure, the plate received Nd:YAG laser irradiation, (beam diameter 14 mm). The laser parameter chosen was an energy density of 3000Jcm^{-2} achieved with a) 10J 10Hz and b) 20J 10Hz laser irradiation. Note this level of exposure only damaged the plates when aluminium was below. A section of each plate did not receive laser irradiation as a further control.

No clearing of the lawn was observed until a UV exposure time of 2-4 min was administered, as shown in **Figure 14.** Notice that the zone of clearing increased with the UV and laser exposure. The rise in clearance with 200W was more pronounced than for 100W. A relatively constant zone of inhibition was apparent after 4 min of UV exposure. The laser irradiation negative control gave no observable clearance of lawn. An example of exposed plates is shown in **Figure 15.** The average diameters of clearance and percentage of maximum diameters of clearance are shown in **Figure 16.** The experiment was done in triplicate and the error bars shown are the standard error of the mean.

When no UV was applied, there was very little, if any, clearance observed. When the lawn was subjected to an exposure of UV irradiation however, clearance was obvious after laser irradiation. As the UV irradiation exposure was increased, the zone of inhibition observed after laser treatment increased. The maximum amount of clearing was achieved after four min of UV irradiation. The increased killing effect was reached because of the nature of the laser energy distribution in the beam. The centre of the beam is most intense so enough energy was available to cause some inactivation, the periphery of the beam did not, but when combined with UV, the lower energy levels of the beam were able to cause clearing, therefore, an increased killing effect was registered.

For a power level of 100 and 200W, the exposures were varied so as to administer the same energy density for each power level. The diameter of inhibition obtained for each exposure remained the same. The maximum diameter of clearance was 9 – 10 mm when 100W was applied to the lawn. This increased to approximately 11 mm with 200W. The only statistically significant difference between the results for each power was when the laser was combined with 2 min of UV they gave a probability value of 0.11 (95% confidence limits).

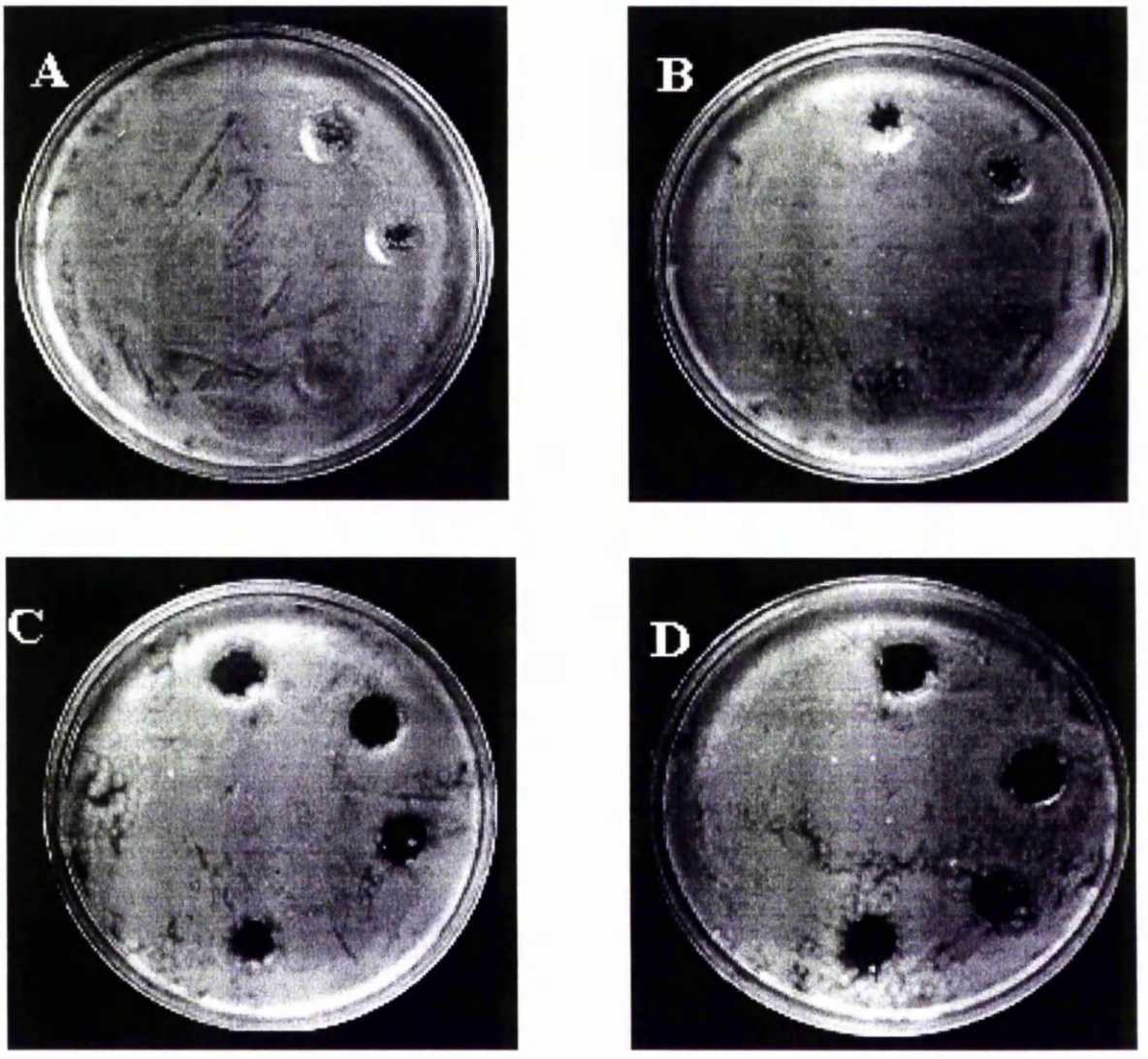


Figure 14 Examples of agar plates lawned with *B. cereus* spores with increasing zones of inhibition caused by laser light as the UV applied was increased. Each plate shown received various exposures of UV irradiation, followed by exposure to laser irradiation (constant for all plates, 3000 Jcm^{-2}). Key, A = control (No UV), B = 2 min UV, C = 4 min UV, D = 5 min UV. The laser exposures were, clockwise from top of dish, 200W, 200W, 100W, 100W, the remaining area of the lawn did not receive laser irradiation. The laser/UV combination caused clear areas on the spore lawn, the resultant increased effect was clearly observed in comparison to the control dish A.

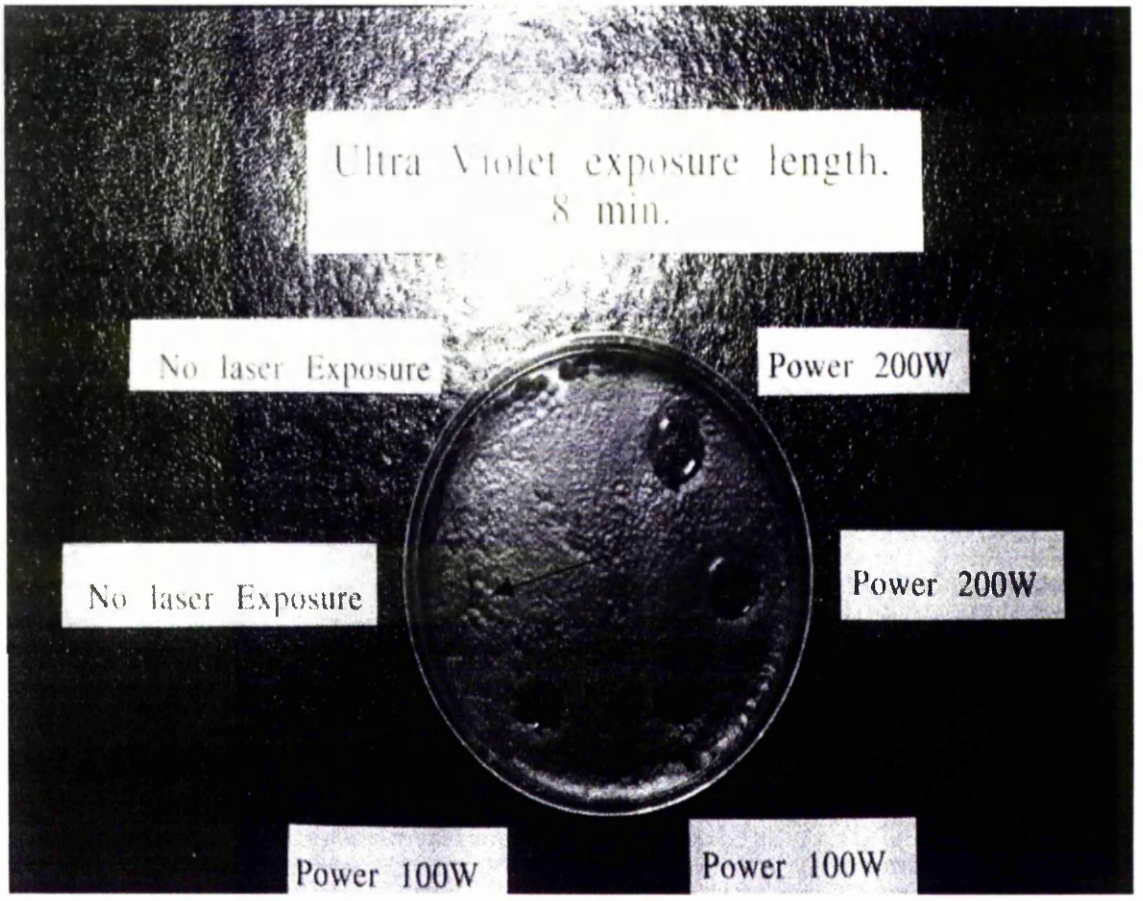


Figure 15 Example of spores exposed to 8min of UV then laser radiation. The growth of the *B. cereus* was stopped in some areas (marked in figure with an arrow) where only UV was applied.

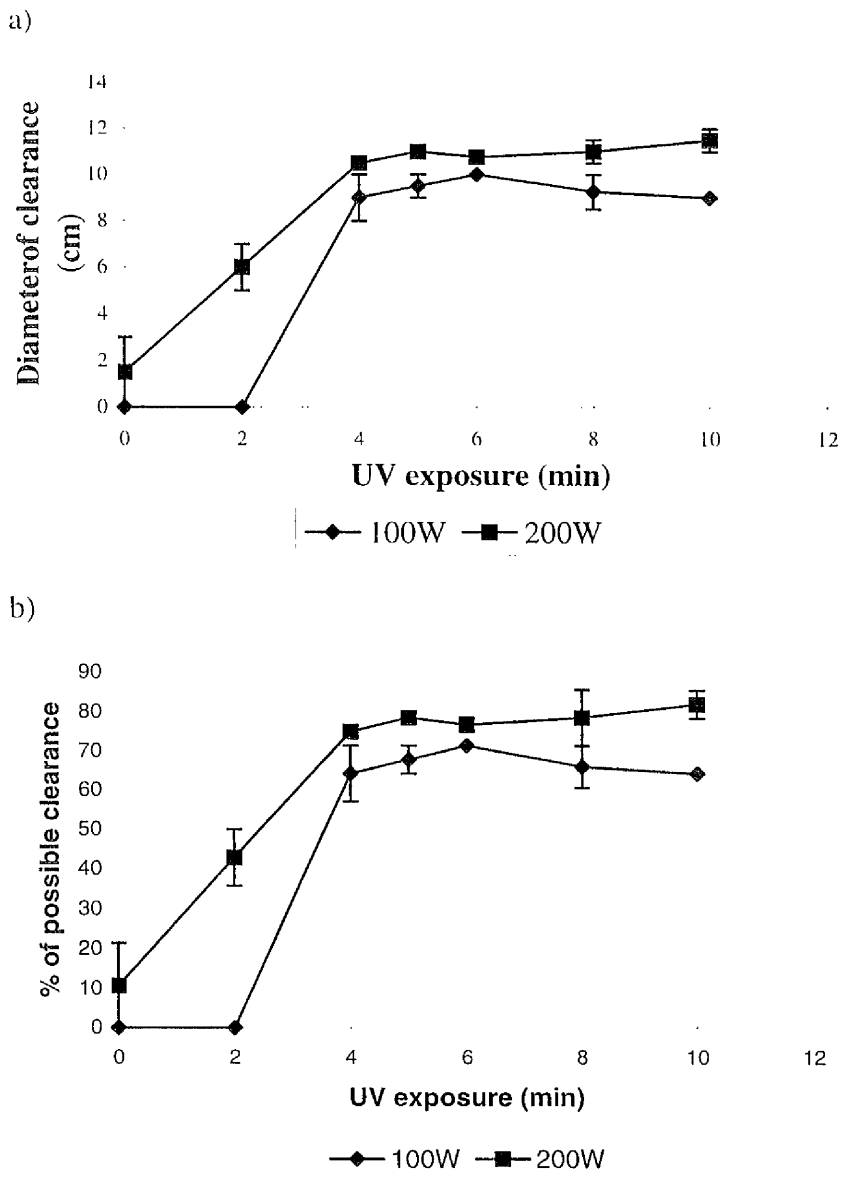


Figure 16 Clearing of spore lawns by combined laser and UV irradiation (power 100W and 200W). Graph a) shows that as the UV exposure was increased to 4min, the diameter of clearance increased. Graph b) shows the percentage diameter of clearance i.e. 16mm of clearance would be 100%, this graph gives an indication of how much of the beam diameter contains a sufficiently high enough energy density to cause spore inactivation. Note that the higher power, 200W, gave a more rapid clearing effect, (although the energy density was kept constant).

It must be noted that as the UV irradiation applied was increased, there was a visible effect on the spore lawn that was exposed to the UV alone. After 6 min of UV irradiation the lawn of spores no longer appeared to be 100% confluent, and some clear areas were observable. This meant that the effect of UV and laser was possibly an additive one with an optimum of 4 min of UV with laser.

The experiment was repeated with 2 x 4W UV lamps (12cm from the tests). These parameters caused an observable decrease in the confluence of spore lawns after only 20s exposure. Rapidity would be more advantageous in a practical setting. After UV exposure, Nd:YAG laser irradiation of less than 3000Jcm^{-2} was administered but no clearance of the lawn was observed.

3.2.3 Laser treatment of liquid samples

B. cereus spores (1 x 10^6 cfu ml^{-1}) were exposed to irradiation from the Nd:YAG laser, 20J, 10Hz, 8ms pulse width and 14mm beam diameter. The rise in temperature was controlled and monitored by computer. This was done by connecting a thermocouple to a computer, via an analogue to digital (A/D) card. The calculated program showed the temperature within the test vessel. The laser shutter could be opened or closed, allowing the laser irradiation to come into contact with the test suspension. The spore suspensions were raised in temperature up to 90°C, this temperature was reached in less than 40s. When the desired temperature was reached the laser shutter was closed. The experiment was done in triplicate. The reduction in viability was observed at each temperature and graphed in **Figure 17.** The corresponding energy densities are shown in **Table 10.** These experiments revealed the difficulty of killing spores in a liquid suspension. Spores proved to be relatively resistant to the rapid heating caused by the absorption of the Nd:YAG laser irradiation by the suspension fluid (distilled water). Vegetative cells were routinely destroyed at 50-55°C, but even after the temperature was raised to 80 or 90°C there was a minimal decrease in viability of spores, (this is also true of other forms of heating). The maximum temperature the computer would monitor was 97°C. The accuracy of temperature measurement was +/- 1°C and 90°C was the highest value used. The *B. cereus* test showed a decrease in viability, this time a 0.5D decrease was observed after the suspension was raised in temperature to 90°C.

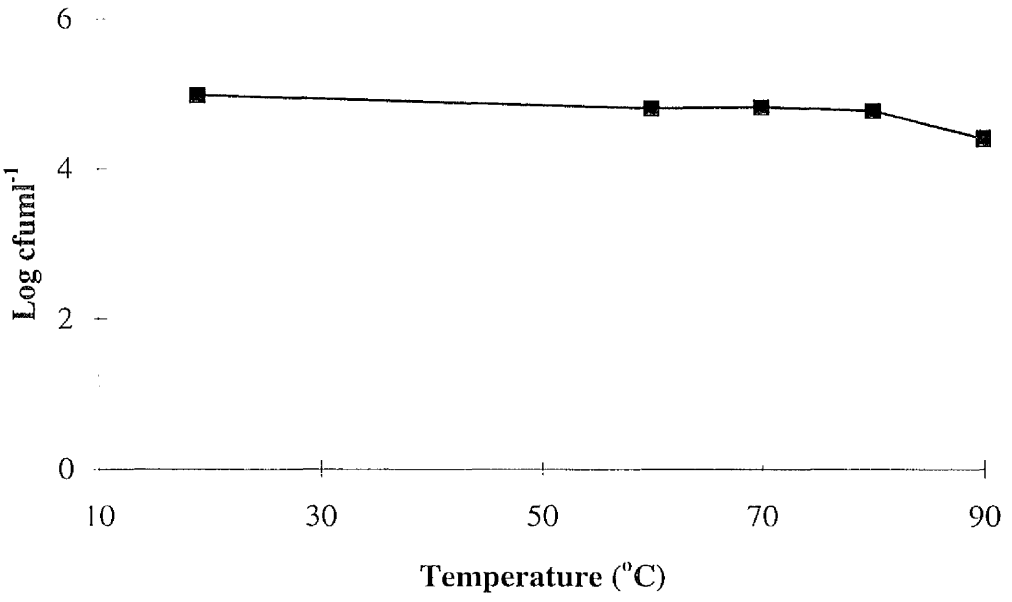


Figure 17 Killing of liquid spore suspensions with laser irradiation. Graph shows inactivation of *B. cereus* spore suspension by raising the temperature with Nd:YAG laser irradiation. It was observed that there was a small decrease in viable spore numbers, 0.5D value decrease after the aliquot was raised in temperature to 90°C. The experiment was triplicated and the error bars (non-visible as they are smaller than the point) are the standard error of the mean.

Table 10 The mean energy density required for the above experiments.

Temperature (°C)	20 (control)	60	70	80	90
Energy Density (Jcm^{-2})	0	1294	1690	1995	2424

3.2.4 Exposure of spore suspensions to Nd:YAG laser irradiation and ultra violet irradiation

The next stage was to test the effect of exposing spore suspensions to laser irradiation followed by UV, and vice versa. Both these methods were compared with a laser alone control.

Aliquots (2ml) of *B. cereus* were exposed to both laser irradiation and UV irradiation. For the laser exposure, Nd:YAG laser irradiation with a beam diameter of 14 mm and 8 msec pulse-width was chosen. The irradiation was applied to the aliquot until the temperature reached 70, 80 or 90°C. The UV irradiation (from 2 x 8W lamps) was applied for 10s. The laser energy density required for each temperature is shown in **Table 11** and the results are shown in terms of the average laser irradiation, the energy density applied is shown in **Figure 18**.

The results of these experiments were interesting in that from the control tests i.e. laser but no UV, it was observed that there was a 2.4D decrease in spore viability after the aliquots were raised in temperature to 90°C, with an applied energy density of 2074.6 Jcm^{-2}. There was a slight increase in sporicidal action when laser heating was followed by an exposure of UV irradiation, a 2.6D decrease was recorded. However when UV irradiation was applied before the laser heating process, the increase in sporicidal action rose further, causing a 3.37D reduction in viable spore numbers. It was interesting to observe that the best results were obtained when the UV was used first. The 90°C tests were analysed with a 2 sample t-test. Neither of the laser and UV tests proved statistically significantly different to the laser alone test, ($p = 0.51$ for laser first, $p = 0.25$ when UV first, 95%confidence limits). The combined tests were also compared (90°C), again there was not a significant difference, $p = 0.4$.

The liquid samples required approximately 30 s to reach 90°C. This means that a 2.45D decrease in spore viability was observed in only 30s, and a subsequent 10s exposure of UV irradiation caused a 3.37D decrease.

Table 11 Average energy densities of laser irradiation applied in laser and UV irradiation combined tests.

UV exposure (s)	Temperature (°C)	Average laser E.D. (Jcm^{-2})	Treatment
none	19	n/a	none
none	70	1481.0	Laser
none	80	1860.1	Laser
none	90	2074.6	Laser
10	19	n/a	none
10	70	1340.0	Laser:UV
10	80	1724.9	Laser:UV
10	90	2062.9	Laser:UV
10	19	n/a	none
10	70	1437.0	UV:Laser
10	80	1710.3	UV:Laser
10	90	1933.6	UV:Laser

n/a = not applicable, as these were controls.

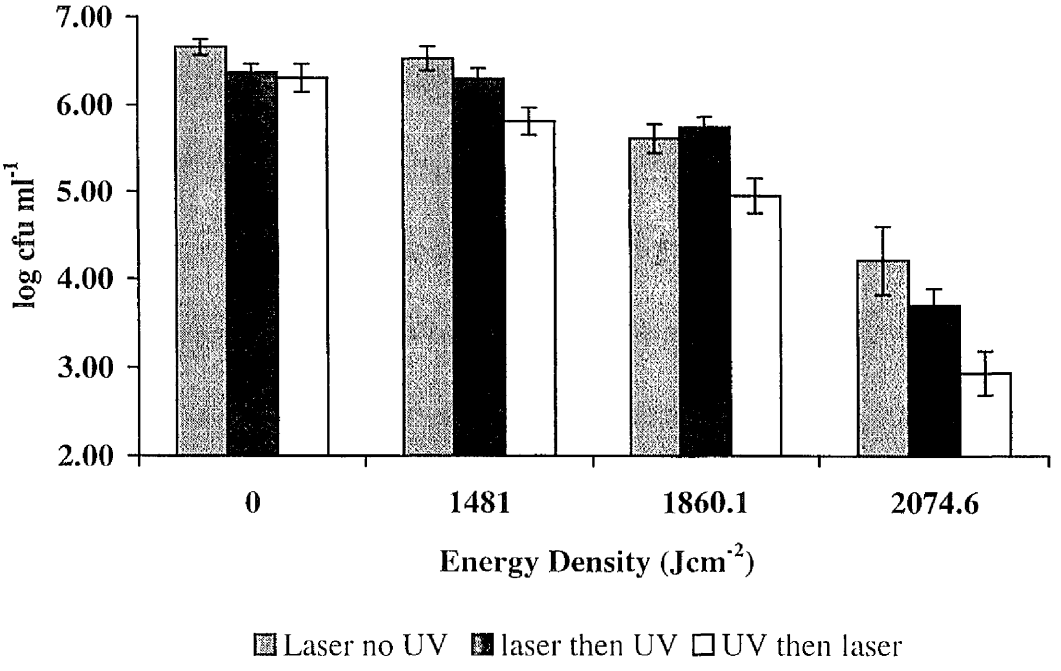


Figure 18 Log_{10} viable counts plotted as a function of energy density for different laser energy densities, administered before and after a UV irradiation treatment. The graph above shows the increased sporicidal effect shown by preceding and following a laser irradiation exposure with UV irradiation. Inactivation of spores by laser irradiation alone caused a 2.4D decrease. With the laser irradiation before UV irradiation, a slight increase in spore killing was observable, 2.6D decrease. On the other hand, with UV before laser irradiation, there was a 3.37D reduction.

3.2.5 Multifactorial analysis of laser, UV, and the delay in between on spores

A multifactorial experiment was designed, which incorporated a high or low laser irradiation test, a high or low UV irradiation test, and a high or low delay between the tests. The laser parameters chosen were: Nd:YAG laser irradiation pre-treatment, 1 or 6s of 10J, 10Hz, 12mm beam diameter and an 8ms pulse-width (ED of 88.46 or 530.79 Jcm^{-2}). The UV parameters chosen were a post treatment of 20 or 40s of 2 x 8W UV irradiation, 26cm from the source. A delay in between the treatments of 10 or 15 min was chosen. All possible combinations of delay, laser and UV were examined. See **Tables 1 and 2**. The experiment was done five times. The decrease in viability caused by the treatments is shown in **Table 12**.

In these experiments, an aliquot of spore suspension received a combination of high or low laser irradiation exposure, high or low UV irradiation exposure, with either a high or low delay between the treatments. Every possible combination was incorporated into the experiment, by looking at the results from the control discs, the effect of each individual factor could be ascertained, i.e. 1s exposure of laser irradiation gave a 0.53D reduction in viability. The higher laser exposure, 6s, caused a 1.75D reduction. The reduction caused by exposing the spores to just UV could also be quantified. The lower exposure, 20s, caused a 0.49D reduction, while the higher exposure, 40s, caused a 1.35D reduction. This gave the expected when the laser or UV level was increased there was a decrease in viability. The exposures in these experiments were chosen because they gave the decimal reductions above. This low level of reduction (incomplete killing meant that any increased killing would still be observable).

This meant that when the UV and laser irradiation were combined, it could reasonably be expected to observe at least an additive effect. In this case, the decimal reduction value observed for a combined test disc i.e. 1s laser, 20s UV, would be the same as the decimal reduction observed in the two control discs. The actual observed results are shown in **Table 12**. In almost all the cases, the combined test disc, did not show an increased reduction, as compared with the two individual factors from the control series. The results were statistically analysed with Minitab for Windows (with the general linear model). The results showed that as a single factor, the individual tests, laser and UV were significant. The results also showed that the laser pre-treatment and the delay had a significant interaction (under the 95% confidence limits chosen).

The results did not suggest however that either the laser and UV interaction or the laser, UV and delay interaction were statistically significant. i.e. they did not give a P value of less than 0.05. The results verified the significance of the laser exposure and the delay however, this relates to the significant increase in killing associated with a longer delay in the high UV, high laser test.

The increased killing observed must be due to an additional effect between the laser and UV irradiation. This is a positive outcome, as a combination of the two types of irradiation would increase the killing effect, or cause a decrease in the exposure time required. This knowledge would aid the production of a working system.

Table 12 Results of multifactorial control treatments. There was no delay in the controls.

Laser exposure (s)	UV exposure (s)	Delay (min)	Log cfuml^{-1}	Standard Error of Mean (N=5)
0	0	N/A	7.42	0.06
1	0	N/A	6.89	0.09
6	0	N/A	5.67	0.05
0	20	N/A	6.93	0.08
0	40	N/A	6.07	0.21
1	20	10	6.69	0.16
1	20	15	6.83	0.30
1	40	10	6.08	0.26
1	40	15	6.17	0.29
6	20	10	5.76	0.12
6	20	15	5.36	0.20
6	40	10	5.36	0.16
6	40	15	3.17	0.89

N/A = not applicable

3.2.6 Microwave irradiation killing of *B. cereus* spores

It was decided that it would be interesting to investigate killing of the spores with microwave irradiation. It was hypothesised that a system could be developed which contained laser, UV and microwave irradiation (Watson personal communication). As a primary experiment, the sporicidal efficiency of the microwave irradiation was investigated.

Aliquots of spore suspension (50µl) were placed on the surface of high-density PFTE discs. The still wet samples were exposed to 800W-microwave irradiation for varying lengths of time. The discs received 7, 8, 9 or 10 min of microwave radiation. A control disc received no irradiation. The treated discs were placed in 20ml of sterile distilled water and shaken for 3 hours. Viable counts were then made, the results are shown in **Figure 19.**

The results showed that there was very little effect on the viability of the spores, a 0.6 D-value decrease was observed after 10 min. This exposure would be too long for a practical system for most applications, even if an increased efficiency of killing was observed.

It was observed that the discs themselves became hotter, and the sample of spores dried. It is assumed that a combination of the lack of free water in the spores, and poor heat transference from the disc to the sample, meant that this medium was ineffectual at killing the spores.

The heating distribution in the microwave cavity was investigated. Temperature sensitive stickers (RS 285-970 clock indicators) were placed on the surface of PTFE discs distributed across the floor of the microwave. These discs showed that there was an irregular distribution throughout the microwave. The centre of the microwave (where the above experiments were done) was one of the coolest areas (lower than 60°C). The front of the microwave was also below 60°C. The back area of the microwave was 60°C or higher and the sides (in line with the magnetron and either side of the centre) was over 82°C. This highlighted a problem of the microwave. While providing a means of containment, the chamber design causes "cold" spots. This could not be tolerated as the samples to be sterilised were at risk of being underexposed.

It was therefore decided that there was little merit in continuing with the microwave irradiation treatments.

3.2.7 Killing of spores with pulsed light

Lawns of spores were prepared and exposed to pulses (1-20) of broad-spectrum white light. The distance from the lawns to the agar was 9.5cm. The lamp voltage was 1kV. After the exposure, the plates were incubated and any areas of inhibition measured. The results are shown in **Figure 20**. Clear zones were apparent after only two pulses. The clearing increased as the number of pulses was increased until a maximum of $25cm^2$ was reached after 15 pulses. The results showed great promise but time restraints meant that this avenue of research could not be pursued.

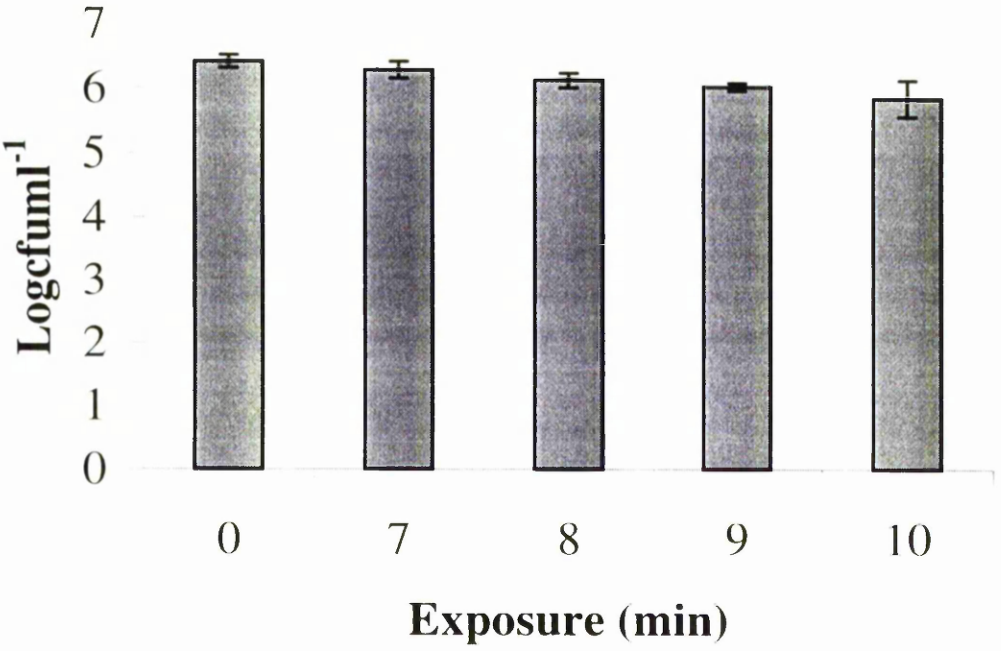


Figure 19 The sporicidal effect of microwave irradiation, on spores on PTFE discs. There was a small decrease in viability (0.6D-value) after 10 min. The experiment was done in triplicate. The errors are the standard error of the mean.

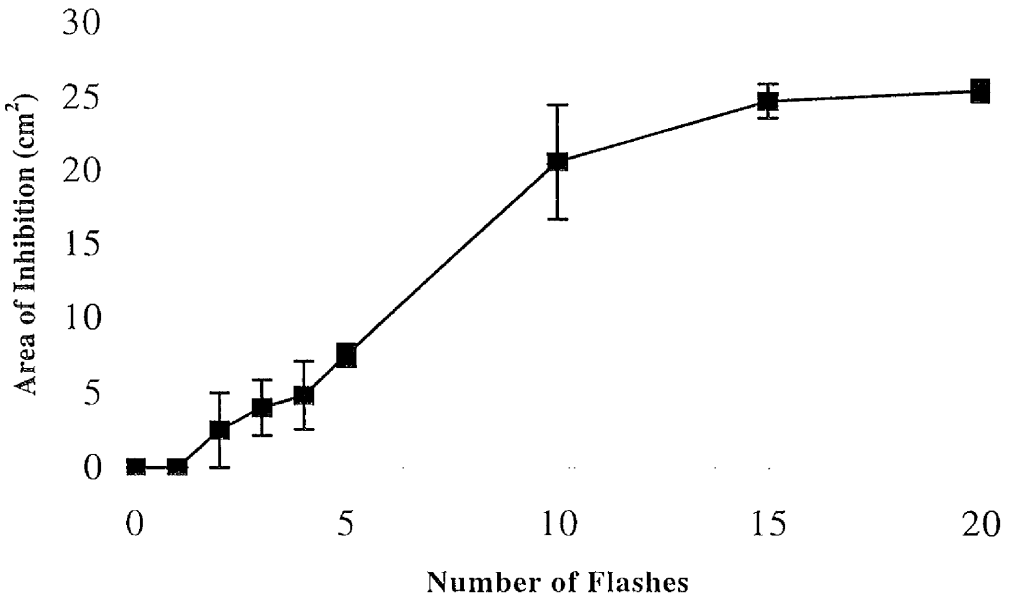


Figure 20 Diameters of inhibition caused by pulsed light. As the number of pulses was increased, the diameter of clearance increased. After 20 pulses, the maximum clearance was approached.

3.3.0 Increased bactericidal action of laser irradiation and chemicals

The combination of UV and laser irradiation gave increased killing, but this proved to be additive. In this section, laser heating was combined with the killing action of lactic acid and hydrogen peroxide, in an attempt to make the inactivation of spores more efficient.

3.3.1 Monitoring the absorbance spectra of chemicals at 1060nm wavelength

Lactic acid (1.34M), hydrogen peroxide (30% v/v) and distilled water were placed in a scanning specrophotometer, which measured the absorbency over a wavelength range of 800 to 1300nm. An absorbency value of between -1 and 1 was given for each wavelength. The absorbency for each chemical at 1060nm, the transition wavelength of the Nd:YAG laser, was investigated. The absorbency scan for **distilled water** is shown in **Figure 21**. At 1060nm an absorbency value of 0.1 was obtained. The absorbency of Nd:YAG laser irradiation by the test chemicals was compared to that of water. The absorbency spectrum of **lactic acid** (1.34 M) is shown in **Figure 22** with an air blank (red line) and a distilled water blank (purple line). The absorbency at 1060nm was only 0.05. This means that samples of lactic acid may take slightly longer to rise in temperature after laser exposure than samples of water that had received laser irradiation. The absorbency spectrum for **hydrogen peroxide** (30% v/v) is shown in **Figure 23**. The absorbency for the chemical compared to an air blank is shown by the red line. The absorbency for the chemical compared to distilled water blank is shown by the blue line. The absorbency of hydrogen peroxide at 1060nm with an air blank was again 0.1. This means that the absorbency of the hydrogen peroxide, was very similar to that of distilled water and there should be very little difference in the absorbency and therefore the rate of heating by the Nd:YAG laser. This is important as it means that in subsequent experiments, any rise in chemical activity will be primarily due to the temperature reached and not because the chemical is absorbing all the laser energy, this means controls of distilled water would be acceptable. The lines representing the water blank show that there is very little difference in the absorbency at 1060 nm between water and the chemicals. The water blank lines are very close to the 0 absorbency value. Note that these results were obtained at room temperature, the absorption values may vary as some function of temperature.

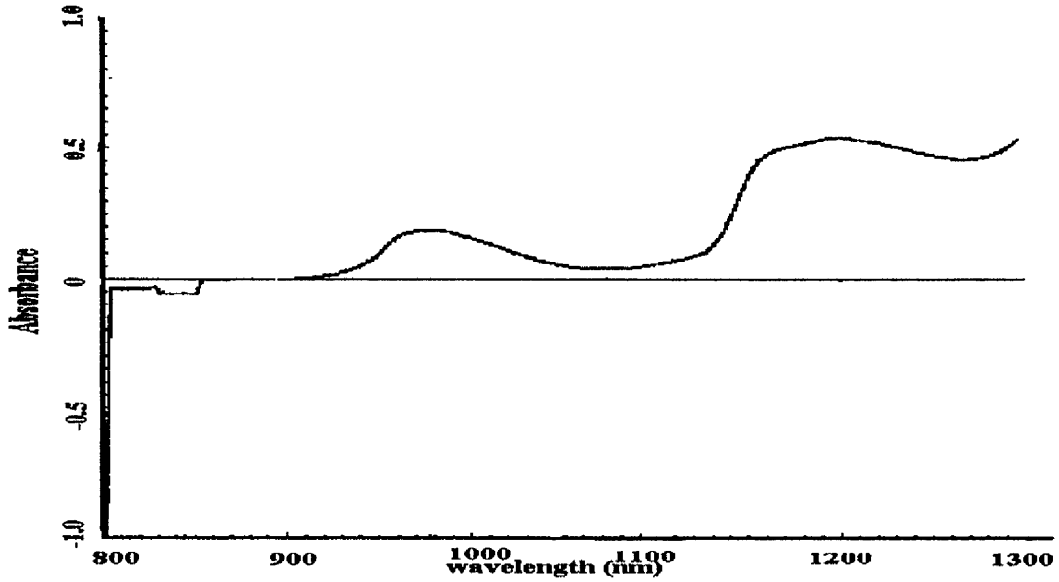


Figure 21 The absorbency spectra of distilled water over the wavelength range, 800 - 1300nm. The absorbency of 1060nm irradiation by water is 0.1. Notice that although water absorbs quite well at this wavelength there are higher peaks 1000 and above 1150nm.

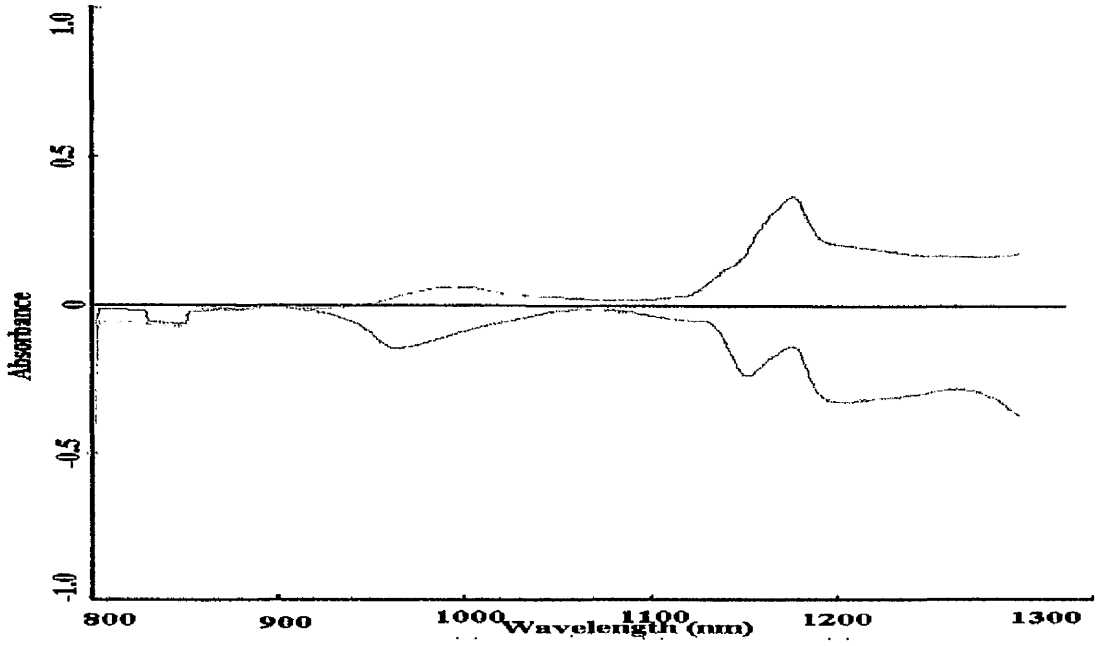


Figure 22 The absorbency spectra of 1.34M lactic acid over the wavelength range, 800 - 1300nm. The red line represents the absorbency of the chemical compared with an air blank. The purple line represents the absorbency of the chemical compared with distilled water blank. The absorbency of 1060nm irradiation by the lactic acid was 0.05. This value is just slightly lower than that obtained for distilled water.

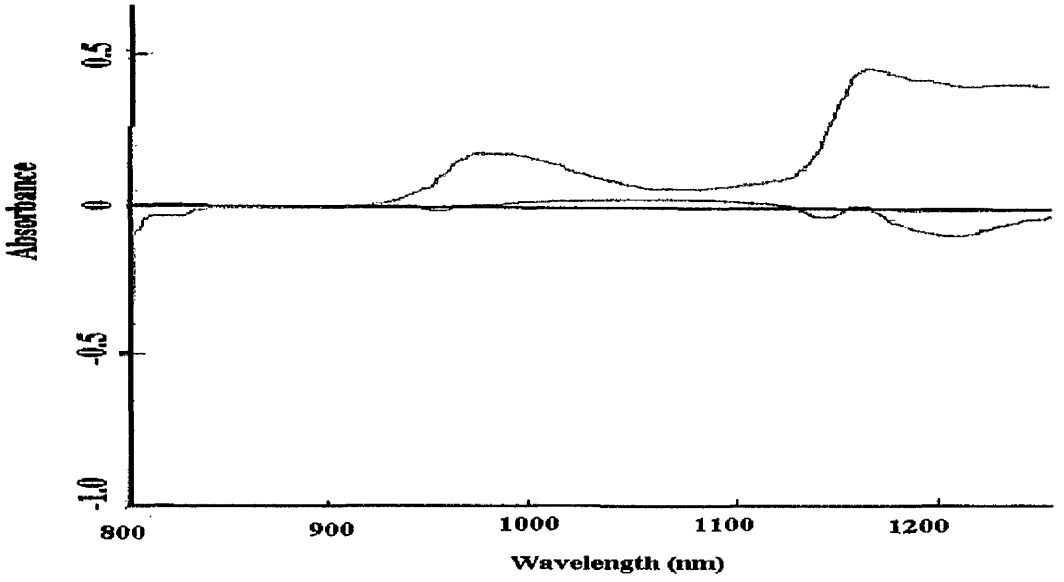


Figure 23 The absorbency spectra of 30% (v/v) hydrogen peroxide over the wavelength range, 800 to 1300nm. The red line represents the absorbency of the chemical compared with an air blank. The blue line represents the absorbency of the chemical compared with distilled water blank. The absorbency of 1060nm irradiation by the lactic acid was 0.1. This value was the same as that obtained for distilled water.

3.3.2 Lactic acid treatment of bacterial endospores over time

Aliquots (200µl) of *B. cereus* spore suspension (1 x 10^6 $cfuml^{-1}$) were pipetted into 1.8 ml of lactic acid of concentration, 1.34 or 2.68M, for varying time lengths, 0.5, 1.0, 1.5, 2.0, 5.0 or 10 min. As a control, spores were pipetted into the acid and a viable count immediately made. As a further control, spores were placed in 1.8 ml of distilled water for 10 min.

These results showed that by adding the spores to increasing concentrations of acid, an initial drop in viability was observed, i.e. 1.34M for 0.5 min gave a log viable count number of 5.85, this reduced to 5.28 when the spores were placed in 2.68M acid. For 0.5 min, the control, (distilled water) gave a log viable count of 5.98 after 0.5 min of exposure. From the results however, it was apparent that prolonged exposure, 10 min, did not cause a significant reduction in the viability of the spore suspension. There was approximately a 0.5 D-value decrease observed after 10 min. The results are shown in **Figure 24**. A 2 sample T test was used to compare the results. Only the 2.68 molar acid gave a significant effect ($p = 0.042$), the 1.34 molar acid gave a p value of 0.97 (95% confidence limits). The acid did give a significant effect if the concentration was high enough and exposure was long enough. These concentrations were acceptable for subsequent experiments involving heating. This was because the spore suspensions were only be exposed to the lactic acid for a few minutes while the heat was applied. Any increased killing effect could therefore be attributable to the heating as opposed to the length of time in the acid.

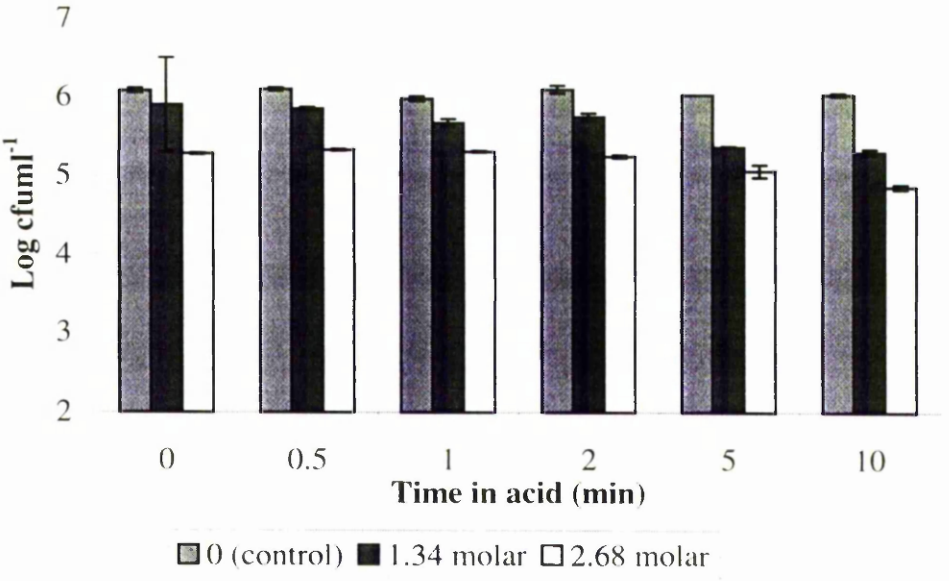


Figure 24 Death of spores exposed to varying times in acid. There was little decrease in the spore numbers over the 10 min period, but the initial concentration of acid had an effect on the initial viable count of the spores. The results were in triplicate and the error bars (where visible) were the standard error of the mean.

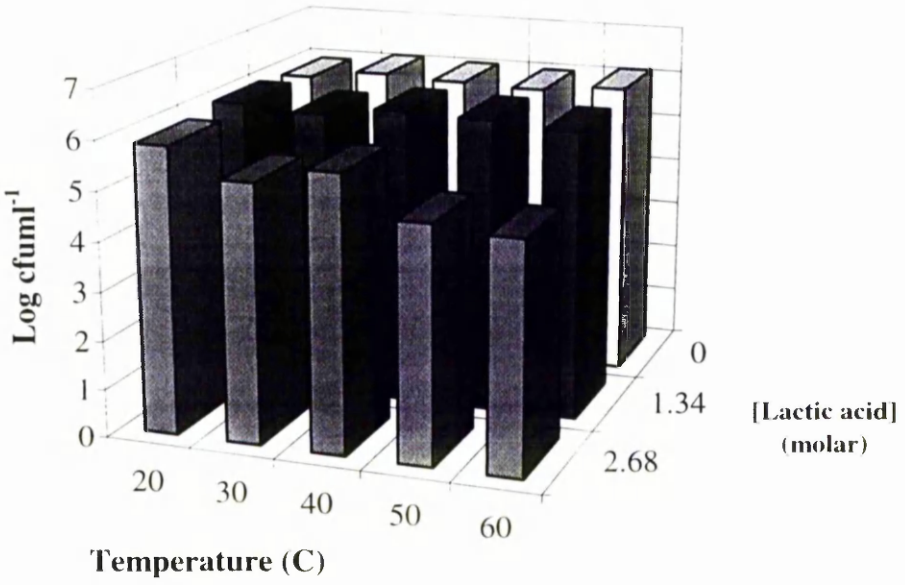
3.3.3 The effect of lactic acid, on bacterial endospores at 20 to 60°C

It was planned to heat lactic acid and observe for increased spore killing. It was decided that this would be done in two ways, either by laser irradiation or a water-bath. Aliquots (200µl) of *B. cereus* spore suspension (1 x 10^6 $cfuml^{-1}$) were pipetted into 1.8 ml of lactic acid of concentration, 1.34 or 2.68M. The mixture was subjected to Nd:YAG laser irradiation (10J, 10 Hz and 8 msec pulse width, the beam diameter measured 15.5mm), until the temperature reached 30, 40, 50 or 60°C. As a control, spores were placed in 1.8 ml of distilled water and exposed to laser irradiation until the required temperature was reached.

The results from these experiments were compared to those obtained by aliquots (200µl) of *B. cereus* spore suspension (1 x 10^6 $cfuml^{-1}$) that were pipetted into 1.8 ml of pre-heated lactic acid of concentration 1.34 or 2.68 M for 45 s and a viable count made. The pre-heated temperatures (water-bath) were 30, 40, 50 or 60°C. As a control, spores were placed in 1.8 ml of distilled water pre-heated to 30, 40, 50 or 60°C, and viable counts made. As a control for these experiments, spores were added to the lactic acid concentrations at room temperature, for 45s. A viable count was then made. The sporicidal action at elevated temperatures was compared to this control. The results are shown in **Figure 25.**

These results showed that for the distilled water control, there was no effect on spore viability as the temperature of the suspension was increased to 60°C with Nd:YAG laser irradiation. When the distilled water was replaced with 1.34M lactic acid, there was still no effect on the spore suspension viability, even after the temperature of the mixture was raised to 60°C. There was however, an increased sporicidal action observed when the concentration of the lactic acid was increased to 2.68M and the mixture was heated with Nd:YAG laser irradiation to 60°C (this acid concentration was not lethal at room temperature). There was now a 1.17D decrease observed in the viability of the suspension. There was now killing observed at 60°C, temperatures, which is normally sub-lethal to spores.

a)



b)

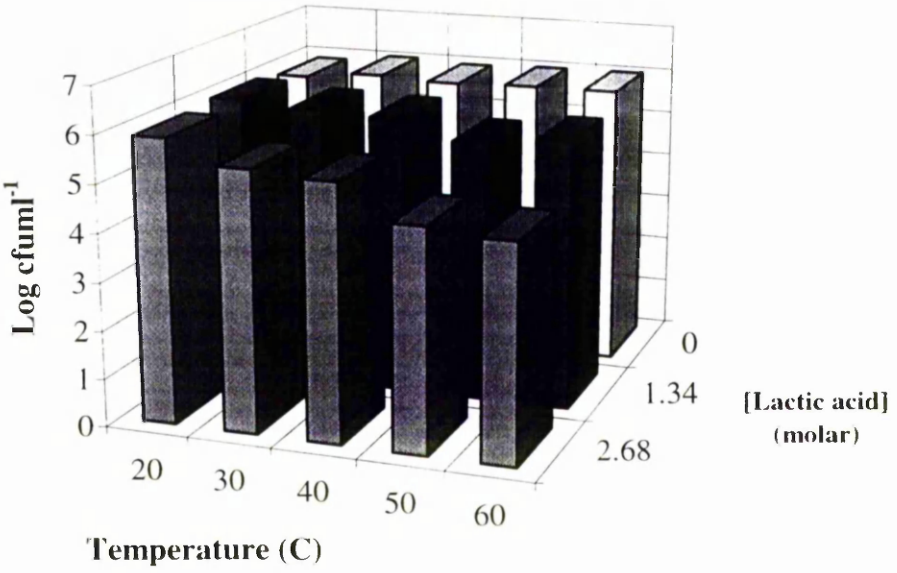


Figure 25 Effect of temperature on varying concentrations of acid. Both graphs show that as the concentration of acid is increased, the starting number of spores (at room temperature 20°C), decreases. Graph a, shows the effect of heating with laser light. The viable count of the spores decreased as the temperature of the acid tests is increased. There is a greater increase in killing in the 2.34 molar acid test. Graph b, shows the effect of exposing the spores to pre-heated acid of different temperatures. This heating was by water-bath. The pre-heated acid also showed an increased killing action. The experiment was carried out in triplicate.

When the spore suspension was exposed to pre-heated distilled water (up to 60°C), in a water-bath for 45s, there was again, no effect on the viability. This showed the heat resistance of the spore suspension and that 60°C was not lethal. When however, the spore suspension was added to lactic acid of concentration 1.34M, pre-heated to 60°C, the viability of the spore suspension reduced slightly by 0.36D. By adding the spore suspension to an increased concentration of pre-heated acid, 2.68M, there was an increased reduction in viability observed. When the acid had been pre-heated to 60°C there was a 1.42D reduction observed. What was also important, was that a 1.3D reduction was observed after 45s exposure at 50°C, this temperature is only sufficient for the killing of vegetative bacteria. Two sample t-tests were used to statistically compare the laser and water-bath results. The 60°C tests with the distilled water and 1.34 molar acid were not significantly different ($p = 0.28$ and 0.52). The results for the 2.68 molar acid however, did show a difference. The 50°C test gave a p value of 0.048 (≤ 0.05 is deemed significant) and the 60°C test gave a p value of 0.019. This meant there was a significant difference between the preheating and laser heating (95% confidence limits). These experiments have shown sporicidal action was caused by the Nd:YAG laser irradiation, at low acid concentrations and relatively low temperatures. The rapid heating of the mixture by laser irradiation was not quite as effective as pre-heating the acid using a water-bath to the required temperature and adding the spores to it, but an increased effect was observed.

3.3.4 Hydrogen peroxide treatment of bacterial endospores over time

After the results of the lactic acid, it was decided to try another sporicidal agent, hydrogen peroxide. As with the lactic acid, preliminary experiments were required to measure the effectiveness of the chemical.

Aliquots (200µl) of *B. cereus* spores (1×10^6 cfuml^{-1}) were pipetted into 1.8 ml of different concentrations of hydrogen peroxide (0.5, 1.0 or 1.5 % v/v). The spores were exposed to the chemical for different lengths of time, 1, 2, 5 or 10 min (for the zero time control, spores were placed in the H_2O_2 and an aliquot immediately removed on which a viable count was made).

The results showed that after placing the spores in the varying concentrations of hydrogen peroxide, there was very little effect on the spore viability. After a 2min

exposure to the chemical, the log viable count of the three tests and the log viable count of the control only varied by 0.11. It must be noted that after 10 min in 1.5% (v/v) hydrogen peroxide, the log viable count had reduced to 5.15. This was approximately 0.8D lower than the time zero control. The results are graphed in **Table 13**. The results were analysed statistically, with a 2 sample t-test. Only the 1.5% (v/v) H_2O_2 test proved to be statistically significant ($p = 0.0092$) under 95% confidence limits.

The spore suspensions would be in the chemical for only a few minutes maximum during the following experiments so these concentrations were deemed usable.

Table 13 The effect upon spore suspensions exposed to varying concentrations of hydrogen peroxide, for varying lengths of time. There is very little difference in the viability of the spores in terms of the concentration of H_2O_2. There is also very little difference in the viability of the suspensions after extended exposure to the chemical. Note however there is a 0.8D decrease observable in the 1.5% (v/v) H_2O_2 test after 10 min of exposure. The experiment was carried out in triplicate.

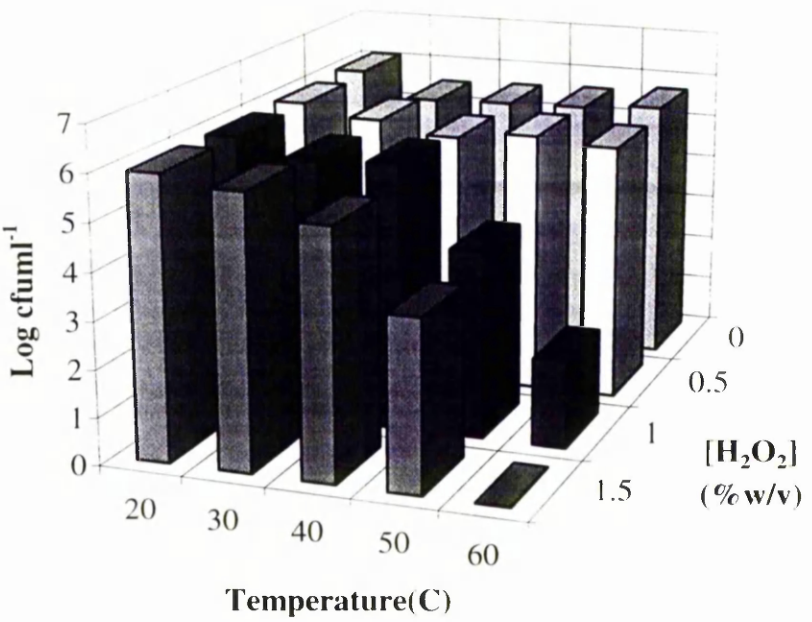
Concentration of hydrogen peroxide (% v/v)	Time of spores in H_2O_2 (min)	Log cfuml^{-1} (Standard Error of Mean)
0 (control)	0	5.93 (0.03)
	1	5.85 (0)
	2	5.87 (0.06)
	5	5.83 (0.01)
	10	5.83 (0.04)
0.5	0	5.57 (0.41)
	1	5.53 (0.39)
	2	5.81 (0.01)
	5	5.76 (0.05)
	10	5.81 (0)
1.0	0	5.83 (0.03)
	1	5.93 (0.02)
	2	5.76 (0.05)
	5	5.67 (0.03)
	10	5.66 (0.02)
1.5	0	5.93 (0)
	1	5.87 (0.02)
	2	5.83 (0.02)
	5	5.55 (0.01)
	10	5.15 (0.01)

3.3.5 The effect of hydrogen peroxide on bacterial endospores, raised from 20 to 60°C.

Aliquots (200µl) of *B. cereus* spores (1 x 10^6 $cfuml^{-1}$) were pipetted into 1.8 ml of different concentrations of hydrogen peroxide (0.5, 1.0 or 1.5% v/v). The spore/chemical mixture was raised in temperature by Nd:YAG laser irradiation, 10J and 10Hz, 15.5mm beam diameter and an 8 msec pulse-width. The temperature was raised to 30, 40, 50, 60 or 70°C. As controls, the hydrogen peroxide was replaced with distilled water, and aliquots of spores were placed in each test concentration for 45s at room temperature. These results were compared with those obtained after placing aliquots into H_2O_2 (for 45s) which had been pre-heated in a water-bath. The controls were the same for this part of the experiment. The results are shown in **Figure 26**.

The results showed that there was an increased sporicidal effect on the spores after heating with laser irradiation. With the distilled water control, there was no observable effect on the spore suspension viability after raising the temperature to 60°C. This showed that the spore suspension did not contain vegetative cells. The 0.5% (v/v) H_2O_2 test also did not show an increased sporicidal action after heating, again the spores survived the treatment. The 1.0% (v/v) H_2O_2 test, however, did show a decrease in spore viability after heating. A 1.99-D reduction in viability was observed after the spore/chemical mixture was raised in temperature to 50°C. This temperature is much lower than the temperature that would be required without the chemical, the spores receive an 80°C heating for ten minutes during preparation. When the spore/chemical mixtures were heated to 60°C, a 4.12-D reduction in viability was observed. When the H_2O_2 concentration was increased to 1.5% (v/v), there was an increased sporicidal action after heating. When the suspension was raised in temperature to 50°C, a decrease in viability of 2.36-D was observed (1.99 for 1.0% (v/v) H_2O_2 test). When the temperature was raised to 60°C, the viability at room temperature (5.97 log $cfuml^{-1}$) was lost completely, i.e. complete killing was observed. This means that sterility was achieved at only 60°C, with a chemical concentration that did not effect the cells at room temperature.

a)



b)

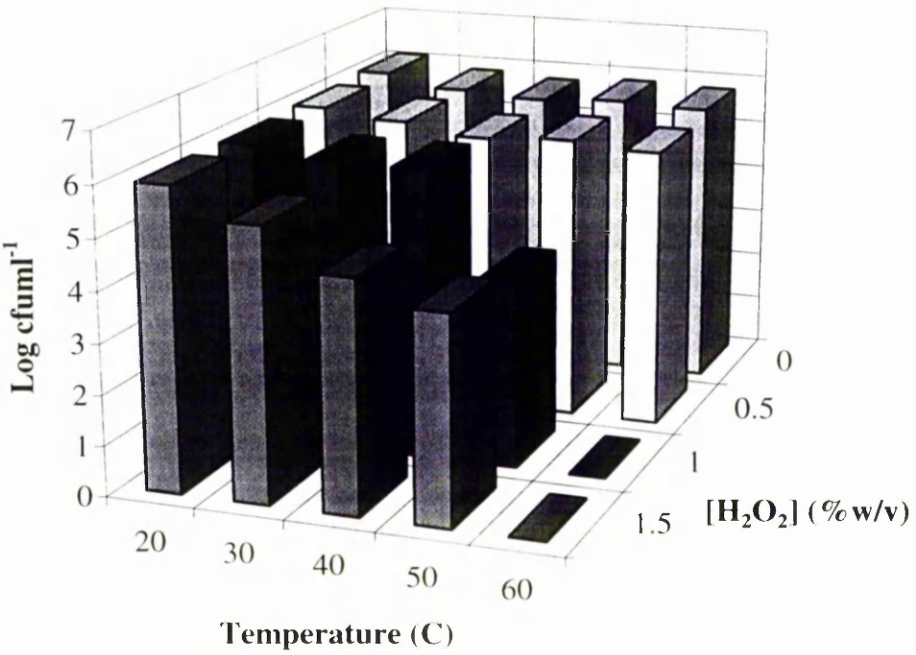


Figure 26 The effect on viability after heating in varying concentrations of hydrogen peroxide. Graph a) shows the effect of heating the spore/H_2O_2 mixture with Nd:YAG laser irradiation. There was no reduction in viability after heating the distilled water control or the 0.5% (v/v) H_2O_2 test. After heating the 1.0% (v/v) H_2O_2 test to 60°C, there was a 4.12-D decrease. This increased for the 1.5% (v/v) H_2O_2 test, to 5.96-D, complete killing. Graph b) shows the effect of placing the aliquot in pre-heated H_2O_2 for 45s. There was no reduction in viability after heating the distilled water control or the 0.5% (v/v) H_2O_2 test. The 1.0 and 1.5% (v/v) H_2O_2 tests gave complete killing after 45s exposure.

The spore aliquots were placed in H_2O_2 of each concentration, pre-heated to 30, 40, 50 and 60°C, for 45s. The pre-heated distilled water control had no effect on the spore suspension viability, this was also the case for the 0.5% (v/v) H_2O_2 test. This was comparable to the negative result with the laser irradiation heating. When the spore suspension aliquot was placed in pre-heated 1.0% (v/v) H_2O_2 for 45s, there was a decrease in viability observed. When the spore/H_2O_2 mixture was at 50°C for 45s, it caused a 2.2-D decrease in the spore viability. This was slightly higher than the reduction observed when laser irradiation heated the H_2O_2 test from room temperature to 50°C. Complete killing was observed when the spore suspension was placed in the chemical, pre-heated to 60°C. Again this was slightly better than the effect of heating with laser irradiation, where low numbers of viable organisms were still observable after heating to 60°C. When spores were placed in the 1.5% (v/v) H_2O_2 test, pre-heated to 40°C, it caused a 0.8-D reduction after the 45s exposure. This temperature is lower than the 50°C normally required for killing of vegetative cells. At 50°C, a reduction of 1.86-D was observed, this was lower than that observed with the laser heating. When the aliquot of spores was placed in the test at 60°C for 45s complete killing was observed, this was also shown with laser heating. The results were analysed statistically with 2 sample t-tests. The 60°C test results for distilled water and 0.5%v/v H_2O_2, did not differ significantly for the heating methods. With a 1% (v/v) solution though, there was a difference when the temperature reached 60°C, probability, p, was 0.023 (95% confidence limits). The 50°C tests did not vary significantly at this concentration. The results obtained for the 1.5% (v/v) H_2O_2 tests raised in temperature to 50°C by laser or by pre-heating in a water-bath did vary statistically. A probability value of 0.0091 was obtained (highly significant). The 60°C tests did not vary for the laser heated method and pre-heated method and both gave complete killing.

3.3.6 Killing of spores with H_2O_2 and glacial acetic acid, and the increased effect caused by heating - Sporicidal action of acetic acid

It was decided to continue with the hydrogen peroxide after such positive results were found. It was decided also to look at the sporicidal effect when combined with acetic acid; the antibacterial nature of this chemical has been known for hundreds of years (pickling) and is likely to be accepted for sterilisation by industry.

In most of these experiments, viable counts were not made, the tests aimed for complete killing, any surviving spores constituted a negative result.

The first part of this experiment investigated the killing effect of the acid. Aliquots (200µl) of *B. cereus* spores were added to 1.8 ml of 1M acetic acid for varying lengths of time; 1, 2, 5 or 10 min, then a viable count was made. Spores were also placed in 1.8 ml of sterile distilled water as a time zero control and the results are shown in **Figure 27.**

The results showed that the acid, at this concentration, had very little effect on the spores. It had been planned that in the subsequent experiments the spores would be exposed to less than 1 min of exposure, so this concentration, or lower would be used as it did not have an effect.

3.3.7 Complete killing of spores with pre-heated hydrogen peroxide and glacial acetic acid

Aliquots (10µl) of *B. cereus* 9373 spores were placed in a test-tube containing water, 1M acetic acid and 30% (v/v) hydrogen peroxide. The total volume was 5 ml. The working concentration of the acid was 0.25M and the peroxide, 1.5% (v/v). The spores were also added to tubes containing no acid or peroxide, water and peroxide, or water and acid. As a control, spores were added to just water, and one tube did not receive spores.

The spores were exposed to the agents for 1 min at 60 or 70°C. After 1 min, 5 ml of nutrient broth, 10µl of blood and 125µl of 1M NaOH were added to the tube. The alkali and blood were to stop further sporicidal action. The tubes were then incubated at 37°C and observed for turbidity, turbid tubes were deemed not sterilised.

The experiment was then repeated with the 5ml of test liquid pre-heated to 70°C in a water-bath. The results are shown in **Table 14.**

The results showed that by pre-heating hydrogen peroxide to 70°C, complete sterility could be attained, that was not achieved at room temperature (20°C) or 60°C for the same exposure length. This equated to 3-D values of a decrease.

The pre-heated acid did not give the increased effect sought after, in any of the tests.

3.3.8 Complete killing of spores with laser irradiation, hydrogen peroxide and glacial acetic acid

The above experiment was repeated but the samples were heated with Nd:YAG laser irradiation. The minimum volume used in these experiments was 2ml. This meant that all the volumes in the previous experiment were increased by a factor of four. The 2ml sample of spores, acid and/or hydrogen peroxide were exposed to Nd:YAG laser irradiation of 10 Joules and 10 Hertz. The beam diameter was 16mm. The samples were irradiated until the temperature reached 60 or 70°C. A control did not receive laser exposure (temperature 20°C). The results are shown in **Table 15.** The results were quite similar to the water-bath heated samples, but there were some differences. In one test there was complete killing at 60°C after laser treating spores in hydrogen peroxide, this effect was not observed in the pre-heated chemical tests. There was also a difference in that at 70°C, with both acid and peroxide, there was not always complete sterilisation observed. It is not known whether this was due to a protecting effect by the acid that was observed when laser irradiation was involved but not when the mixture was preheated. The absorbance spectrophotometry (**see Figure 28**) of the acid did not appear to show a great level of absorption at the Nd:YAG wavelength, so it is not immediately obvious if the result is due to a protective effect or an anomaly.

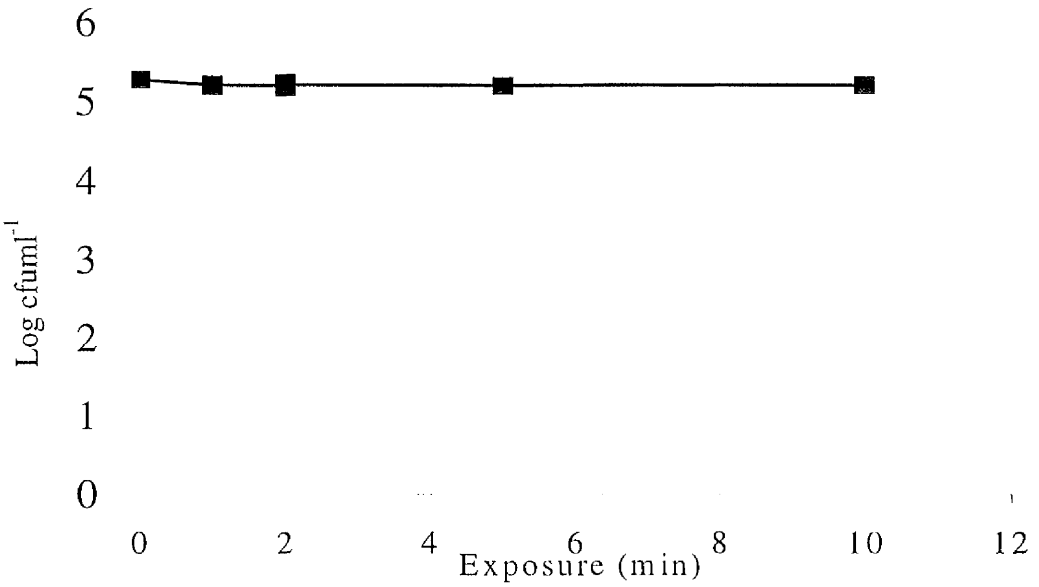


Figure 27 The effect of 1M acetic acid on spores over time. There was very little decrease in spore viability even after a 10 min exposure. The experiment was done in triplicate. The error bars are the standard error of the mean.

Figure 28 Absorption spectrum of acetic acid. There is a peak of absorption near 1169nm.

Table 14 Effect of exposing *B. cereus* spores to acid and/or H_2O_2 for 1 min at room temperature, 60 or 70°C. The experiment was carried out three times, in triplicate. The errors are the standard error of the mean.

Temperature (°C)	Spores	Water	Acid	H_2O_2	Average turbid tubes (out of three)	Standard error of mean (n=3)
20		✔			0	0
20	✔	✔			3	0
20	✔	✔	✔	✔	3	0
20	✔	✔	✔		3	0
20	✔	✔		✔	3	0
60		✔			0	0
60	✔	✔			3	0
60	✔	✔	✔	✔	3	0
60	✔	✔	✔		3	0
60	✔	✔		✔	3	0
70		✔			0	0
70	✔	✔			3	0
70	✔	✔	✔	✔	0	0
70	✔	✔	✔		3	0
70	✔	✔		✔	0	0

Table 15 Effect of exposing *B. cereus* spores to acid and/or H_2O_2 with Nd:YAG laser irradiation taking the temperature to 60 or 70°C. As a control, samples did not receive laser irradiation but exposure to the chemicals for1 min at room temperature.

Temperature (°C)	Spores	Water	Acid	H_2O_2	Average turbid tubes (out of three)	Standard error of mean (n=3)
20		✔			0	0
20	✔	✔			3	0
20	✔	✔	✔	✔	3	0
20	✔	✔	✔		3	0
20	✔	✔		✔	3	0
60		✔			0	0
60	✔	✔			3	0
60	✔	✔	✔	✔	3	0
60	✔	✔	✔		3	0
60	✔	✔		✔	2.7	0.34
70		✔			0	0
70	✔	✔			3	0
70	✔	✔	✔	✔	0.67	0.34
70	✔	✔	✔		3	0
70	✔	✔		✔	0	0

3.4.0 Spores of fungal species

In this section the ability to inactivate fungal spores with Nd:YAG and CO_2 laser irradiation was examined. In particular, the efficacy of CO_2 laser-scanning systems was assessed. The clearance of spores from the surface of food was attempted, and the success of this was compared with the clearing of lawns of spores on agar.

3.4.1 Exposure of fungal spores to laser irradiation

3.4.1.1 Nd:YAG laser treatment of liquid fungal suspensions

Aliquots, 2 ml, of a *Rhizopus* spore suspension and a *Penicillium* spore suspension were exposed to 100W Nd:YAG laser irradiation, with a 15.5 mm beam diameter. The rise in temperature was monitored and controlled via a computer controlling the laser paramaters. The suspensions were heated to 50, 60, 70 or 80°C. The energy densities applied and the resultant decreases in viability are shown below in **Tables 16 and 17**.
The fungal spores were not killed off at a temperature of 50°C, the temperature at which vegetative bacteria are killed. The spores of both fungal species were however, decreased, by approximately 2D-values as 60°C was reached. This reduction was increased as the temperature was increased to 70 and 80°C. The findings would imply that the *Rhizopus* spores were more resistant than the *Penicillium* spores. A statistical comparison of the 80°C values for each fungal species was made with a two sample t-test. The probability, P, was 0.5 (95% confidence limits), this means statistically there is not a significant difference between the values (which were complete killing and a log viable count value of 1.7). The graph showing energy density applied, however shows that more laser light was applied to the *Rhizopus* suspension than was applied to the *Penicillium*. It was likely that this was because of the difference in the spore concentrations, the suspension with the lower numbers taking longer to absorb the irradiation, the pigmentation of the suspension could also be important.

Table 16 Decrease in *Rhizopus* spore suspension viability after heating with Nd:YAG laser irradiation. The results are the mean of three trials.

Temperature (°C)	Laser exposure (s)	Energy Density (Jcm^{-2})	Log $cfuml^{-1}$ (SEM)
20 (control)	0	0	4.18 (0.06)
50	25.25	1335.98	4.24 (0.01)
60	34.25	1812.17	2.19 (0.01)
70	43.65	2309.52	2.02 (0.01)
80	48.2	2550.265	1.70 (1.0)

Table 17 Decrease in *Penicillium* spore suspension viability after heating with Nd:YAG laser irradiation. The results are the mean of three trials.

Temperature (°C)	Laser exposure (s)	Energy Density (Jcm^{-2})	Log $cfuml^{-1}$ (SEM)
20 (control)	0	0	5.37 (0.01)
50	22.5	1193.025	5.06 (0.02)
60	33.85	1794.84	3.43 (0.03)
70	46.1	2444.375	1.60 (0.96)
80	69.6	3690.42	0

3.4.2 Laser irradiation treatment of fungal spores on agar surfaces

3.4.2.1 Nd:YAG laser irradiation treatment on agar surfaces

Lawns of *Penicillium* spores were prepared by spreading 200µl of a 3.7 x 10^5 $cfuml^{-1}$ spore suspension over the surface of a czapeks dox agar plate (C,Agar plate). The prepared lawns were exposed with collimated 100W or 200W Nd:YAG laser irradiation, with a 12mm beam diameter. The lawns received varying exposure lengths: 0 (control), 1, 2, 3, 4 or 5 s exposures. This corresponded to an energy density range of a) 0 - 442.32 Jcm^{-2} and b) 0 - 884.64 Jcm^{-2}. There was no clearing of the lawns observed. It is apparent that the Nd:YAG laser was not effective against fungal spores on the surface of agar plates. The high and low power CO_2 lasers gave good clearing at relatively low energy densities (results shown below). The low energy densities of Nd:YAG laser irradiation were chosen as they were below the tolerance limits of cheese i.e. these laser parameters would not melt cheese.

3.4.2.2 CO_2 laser scan of fungal spores

Lawns of *Rhizopus* and *Penicillium* spores were prepared by spreading 200µl of spore suspension over the surface of a sabouraud dextrose agar plate. The plates were scanned with irradiation from a 1kW CO_2 laser. The scan was produced by aiming the laser irradiation at a rotating mirror. The laser beam diameter was 11.5mm. By increasing the distance the test plate was from the mirror, the scanning speed could be increased. The scan speed was varied from 197.7 to 341cms^{-1}. The diameter of the zone of inhibition obtained by laser treating the spore lawn was recorded for each scan speed, and the results are shown below in **Tables 18 and 19**.

Table 18 Diameter of clearance in *Rhizopus* spores caused by scanned CO_2 laser irradiation. The scan speeds and the distance of the plates from focal head are also shown. The experiment was in triplicate.

Distance from mirror (cm)	Scan speed (cms^{-1})	Width of clearance (mm) (SEM)	Rate of clearance (mms^{-1})
27.7	197.7	8.0 (0.25)	1.6×10^4
32.7	233.4	8.0 (0.1)	1.9×10^4
37.6	268.3	8.0 (0)	2.1×10^4
42.7	304.1	6.25 (0.25)	1.9×10^4
47.6	341.0	4.5 (0)	1.5×10^4

Table 19 Diameter of clearance in *Penicillium* spores caused by scanned CO_2 laser irradiation. The experiment was in triplicate.

Distance from mirror (cm)	Scan speed (cms^{-1})	Width of clearance (mm) (SEM)	Rate of clearance (mms^{-1})
27.7	197.7	7.25 (0.5)	1.4×10^4
32.7	233.4	7.1 (0.5)	1.7×10^4
37.6	268.3	7.0 (0)	1.9×10^4
42.7	304.1	6.75 (0.25)	2.1×10^4
47.6	341.0	6.0 (0)	2.0×10^4

The results of these experiments showed that the 1 kW CO_2 laser is effective in the clearing of fungal spores of these different species on agar surfaces. The fungal spore lawns are cleared effectively (6 – 8mm), up to a scan speed of 341.0 cms^{-1}. It should be noted that the observed inactivation caused by the laser irradiation remains fairly constant from scan speeds of 197.7 - 268.3 cms^{-1}. After this speed is increased further however, there is a noticeable decrease in the effectiveness of the system and the zone of inhibition is decreased. The results are interesting in that the *Rhizopus* spore lawns originally seemed more sensitive to the irradiation, 8mm diameter of inhibition where as the *Penicillium* spore lawns only showed a 7.25mm diameter of inhibition. When the scan speed was increased however, this trend was reversed, with the *Rhizopus* spore lawns showing only a 4.5 mm diameter of clearance, while the *Penicillium* spore lawns gave a 6mm diameter of inhibition. The results show conclusively that as the scan speed was increased, the clearing the laser irradiation caused decreased, this is because the laser irradiation spends a shorter time on each area of the test surface. A two sample t-test was carried out to compare statistically, the two fungal species. The probability value was 0.001 (95% confidence limits), so there was a statistical highly significant difference between the species.

3.4.2.3 13W CO_2 laser scanning

Lawns of *Penicillium* and *Fusarium* spores were prepared. The plates were scanned with irradiation from a 13 W CO_2 laser. The laser beam was 3.5mm. The scan speed and power could be altered by computer control. The scan speed chosen was 100 mms^{-1}. The power of the scanned irradiation could be altered, with computer control. Various powers of laser irradiation were tested for fungal spore killing. The powers of irradiation were 0 (control), and 2 - 13W. The diameter of the zone of inhibition obtained by laser treating the spore lawn was recorded for each scan speed, the results are in **Tables 20 and 21**. Examples of laser treated lawns of spores are shown in **Figures 29 and 30**.

Table 20 Width of clearance in *Penicillium* spores caused by scanned CO_2 laser irradiation at 100mms^{-1} at various power levels. The results are the mean of four trials.

Power (W)	Zone of inhibition (mm) (SEM)
0	0 (0)
2	0 (0)
3	0 (0)
4	0 (0)
5	0 (0)
6	0.2 (0.2)
7	0.5 (0.2)
8	0.5 (0)
9	0.6 (0.3)
10	1.1 (0.1)
11	1.1 (0.1)
12	1.3 (0.4)
13	1.3 (0.3)

Table 21 Width of clearance in *Fusarium* spores caused by scanned CO_2 laser irradiation at 100mms^{-1} at various power levels. The results are the mean of four trials.

Power (W)	Zone of inhibition (mm) (SEM)
0	0 (0)
2	0 (0)
3	0 (0)
4	0 (0)
5	0 (0)
6	0.2 (0.2)
7	0.7 (0.1)
8	1.1 (0.1)
9	1.4 (0.1)
10	1.9 (0.1)
11	1.9 (0.1)
12	1.9 (0.1)
13	1.9 (0.1)

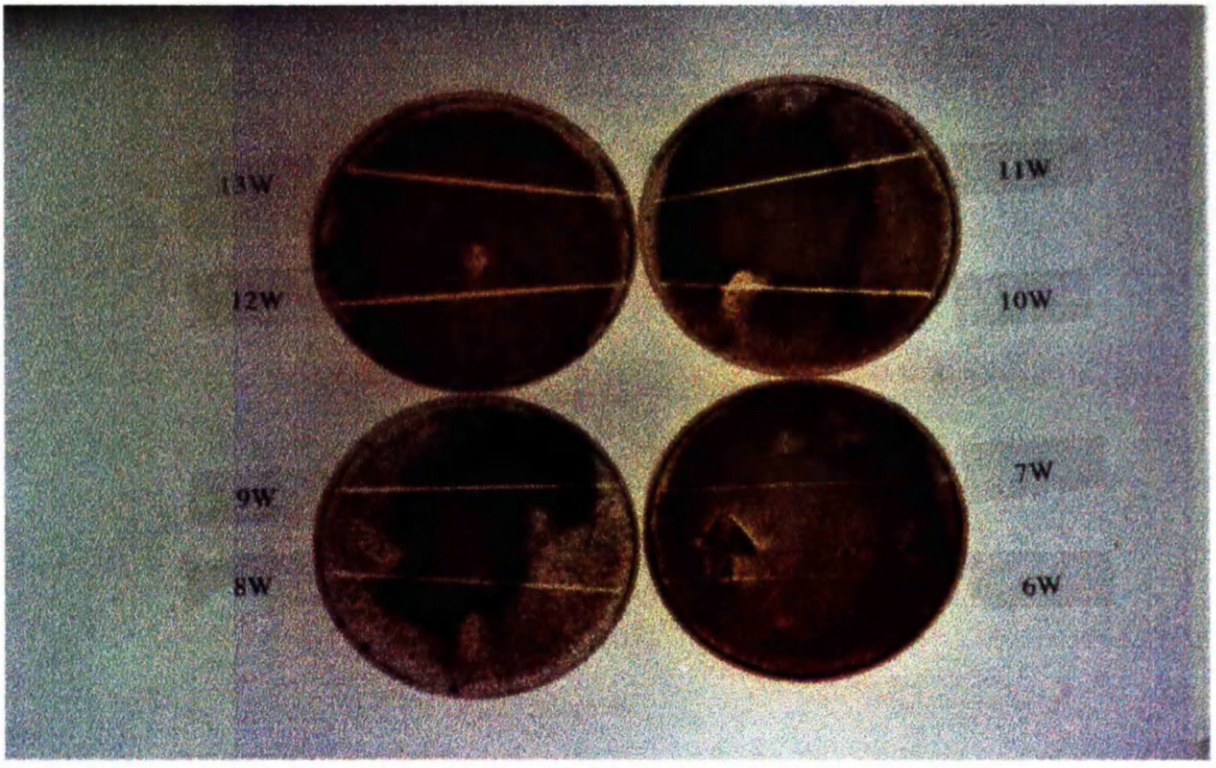


Figure 29 Examples of laser treated lawns of *Penicillium* spores after incubation. The spores have grown except where they have been inactivated by the laser irradiation. The lawns received laser irradiation scans of power 6-13 W, the scan speed was 100 mms^{-1}.

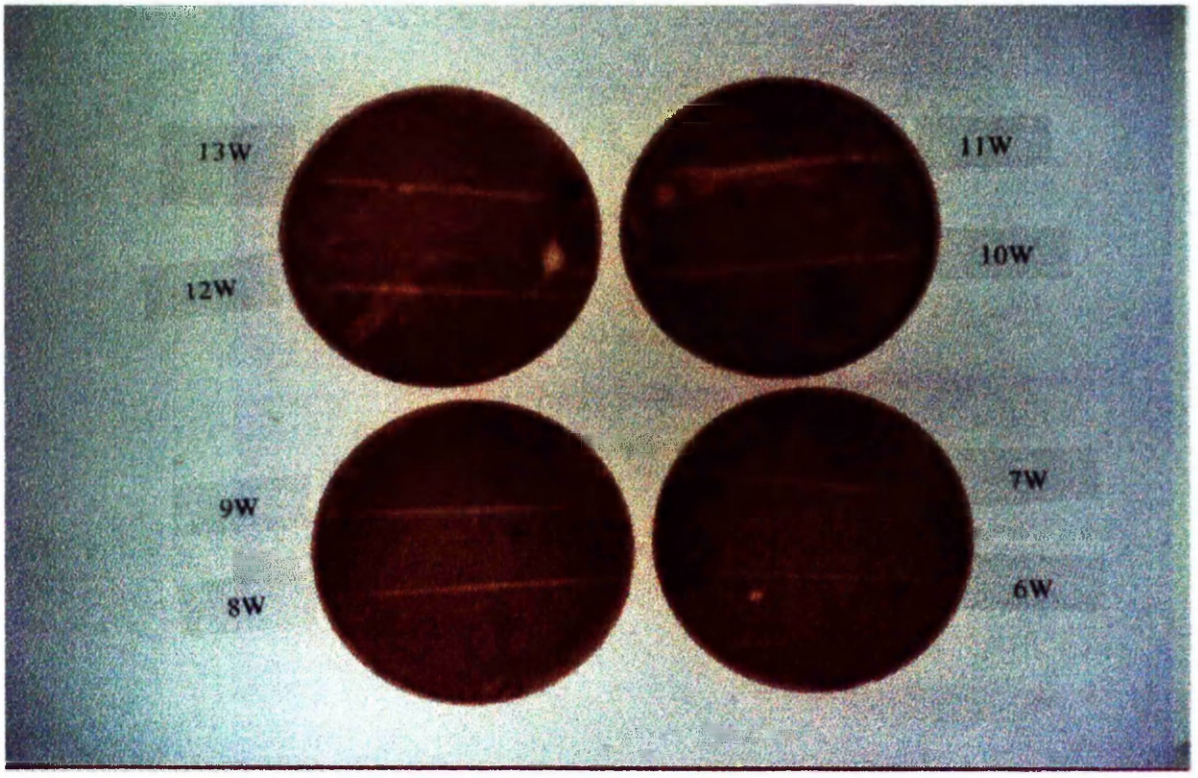


Figure 30 Examples of laser treated lawns of *Fusarium* spores after incubation. The spores have grown except where they have been inactivated by the laser irradiation. The lawns received laser irradiation scans of power 6-13 W, the scan speed was 100 mms^{-1}.

These experiments showed that the 13W CO_2 laser scanning system was effective against the spores of *Fusarium* and *Penicillium* species. The scanning speed chosen, 100 mms^{-1}, allowed clearance of the lawns with a power setting as low as 6W. There was however no clearing at levels below 6 W. Clearance of the *Penicillium* spore lawns reached a maximum at 9W with a mean clearance of 1.3mm observed. There was increased killing of the *Fusarium* spore lawn, 1.9 mm of clearance was observed after a scan of 10W power, but again the same trend was apparent. Increasing the power of the laser did not increase this level of inactivation further. The results are plotted as rate of clearance (speed x width of clearance observed) against power **Figure 31.** This shows the amount of clearance per second. The maximum value reached was 190mm^2s^{-1}, this was for the *Fusarium* tests. The *Penicillium* tests reached an average of 130mm^2s^{-1}.

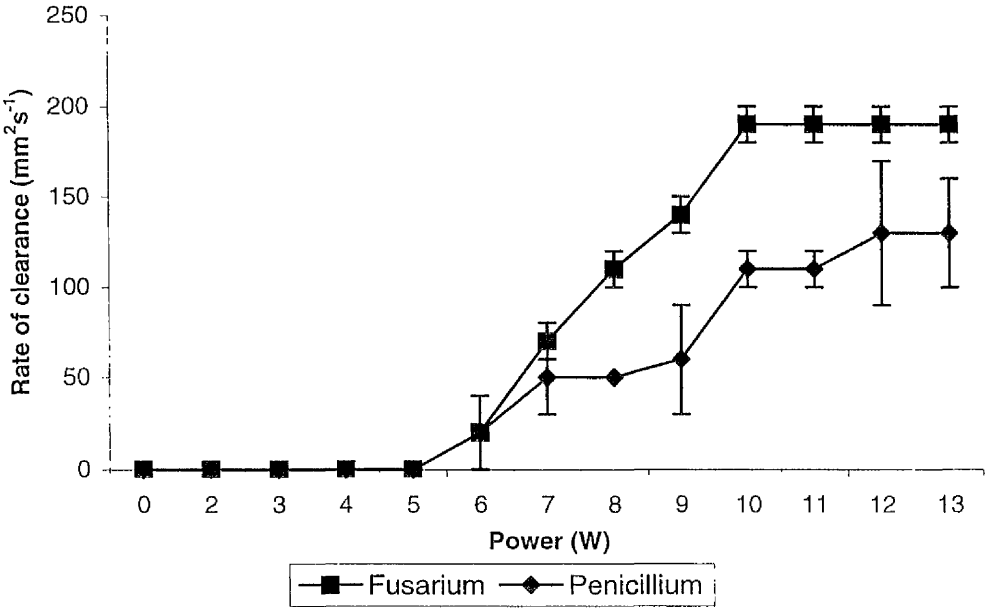


Figure 31 The graph shows the increase in clearance per second caused by increasing power of laser irradiation. The clearance rate (V.W) was calculated by multiplying the width of the clear zone by the speed of the scan. The maximum values reached were 130 and 190mm^2s^{-1} for *Penicillium* and *Fusarium* respectively.

3.4.3 Laser sterilisation of the surface of sliced fruit

A *Rhizopus* spore suspension was spread on the surface of an aseptically prepared slice of cherimoya fruit. (approximately 10 cm by 10cm section). The inoculated fruit was exposed to 1020W continuous wave CO_2 laser irradiation of various laser scan speeds. The beam diameter of the irradiation was 11.5 cm. The mirror for the scan had a rotational speed of 0.88cms^{-1}. The scan speeds were varied from 197.68 - 376.09 cms^{-1}. No clearing of the *Rhizopus* spores was observed. The CO_2 laser was unable to cause inactivation of the spores on the surface of the moist fruit. The fruit was allowed to dry somewhat before exposure, but was still damp. These same scanning speeds were shown to give effective clearing of fungal spores from the surface of agar plates, section (**3.4.2.2**). It may have been possible that the juice was running into the treated area of the fruit causing cross contamination after exposure.

A *Penicillium* sp. spore and a *Fusarium* sp. spore suspension were spread on the surface of aseptically prepared slices of star fruit (approximately 10 cm by 10 cm section). The inoculated fruit was exposed to 100W Nd:YAG laser irradiation, 12mm beam diameter. The fruit slices received various exposure lengths 0 (control), 1, 2, 5 and 10s. This corresponded to an energy density range of 0-885 Jcm^{-2}. There was no clearing observed.

The Nd:YAG laser irradiation applied to the surface of the star fruit was ineffective. The exposure time of the irradiation was long - 10s, so it is unlikely that exposure lengths greater than this would be an appropriate time-scale for a practical process. The fruit was not damaged by the laser treatment, although the treated area did look drier. Once again, the fungal species the fruit had been artificially contaminated with grew very well, with the fruit obviously providing an ample growth medium. There was no sign of clearance even at the onset of growth.

From all these experiments, laser irradiation was not effective at inactivation of fungal spores, artificially contaminating the surface of cherimoya or star fruit. The Nd:YAG laser irradiation method was unsuccessful, but was not very effective on the surface of agar plates. The CO_2 laser, however, proved to be very effective at clearing fungal spores from the surface of agar plates, but when the same conditions and parameters

were extended to the surface of fruit, then no clearing was observed. Higher levels of treatment would be required.

3.4.4 Laser sterilization of unwrapped cheese

3.4.4.1 CO_2 laser scanning treatments

A *Penicillium* spore suspension (1.15×10^6 $cfuml^{-1}$) was prepared and 100µl was spread on the surface of a piece of cheddar cheese (approximately 6.2 cm by 2.1 cm section), aseptically removed from its wrapping. The inoculated cheese was exposed to 1020W continuous wave CO_2 laser irradiation of various laser scan speeds. The beam diameter of the irradiation was 11.5 cm. The mirror for the scan had a rotational speed of $0.88cms^{-1}$. The scanning speeds varied from 196.3 - 553.1 cms^{-1}. The power of CO_2 laser irradiation, and the speed of the laser scan, were comparable to the parameters that were reported in **section 3.4.2.2**. In these experiments, the laser irradiation caused the inactivation of a zone up to 8 mm across. However, once these same parameters were applied to the surface of artificially contaminated cheese, the laser irradiation proved ineffective.

As with the fruit experiments, the fungal spores were not cleared from the cheese surface with the 1 kW CO_2 laser beam scanned at speeds that caused the killing of spores of the same fungal species on agar plates.

A *Penicillium sp* spore suspension was spread on the surface pieces of edam cheese, aseptically removed from the wrapping. (approximately 6 cm by 2 cm sections). The inoculated cheese was exposed to 13W maximum power CO_2 laser irradiation with a beam diameter of 3.5mm. The laser irradiation was scanned at $100mms^{-1}$. The laser power applied was 3, 5, 7, 9, 11, or 13W. The results are shown in **Table 22 and Figure 32.**

Table 22 Diameter of clearance in *Penicillium* spores on cheese caused by various powers of CO_2 laser irradiation, scanning at 100 mms^{-1}. The results are the mean of four trials.

Power (W)	Zone of inhibition (mm) (SEM)
0 (control)	0 (0)
3	0 (0)
5	0.9 (0.1)
7	1.9 (0.1)
9	1.6 (0.5)
11	2.0 (0)
13	2.3 (0.1)

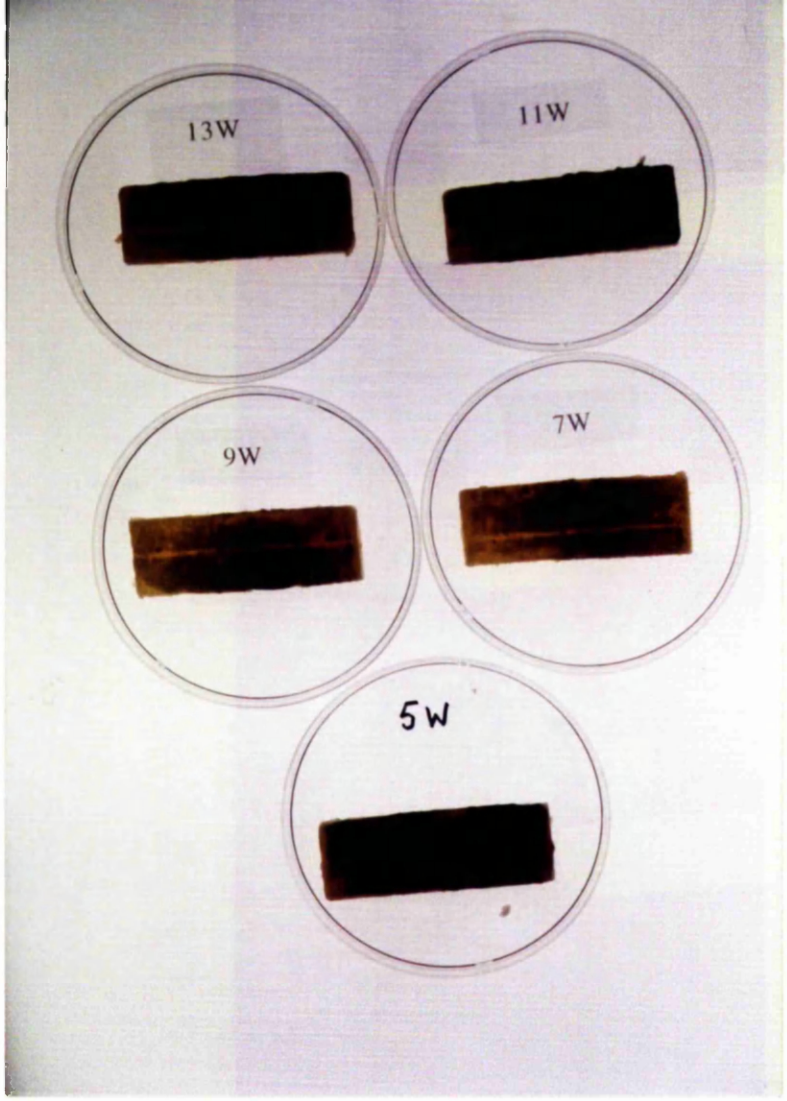


Figure 32 An example of artificially contaminated cheese after laser irradiation.

The results of this experiment were very clear and favourable compared with those of **sections 3.4.2.1 and 3.4.2.2** in which the 13 W of laser power was applied to the surface of inoculated agar plates. In this experiment it was apparent that again, 3W was too low a level to induce fungal spore inactivation. The levels of inactivation measured at higher laser irradiation levels were greater than those obtained from the agar experiments. A mean zone of clearance of 2.3 mm was obtained with a power level of 10W. This power level only gave a zone of inhibition of 1.3 and 1.9 mm (species dependent).

The results from the *Penicillium* sp. experiment showed that the clearance of fungal spores on the surface of cheese caused by the 13 W CO_2 laser scanning system was as effective as the clearing caused on the surface of inoculated agar plates. In this experiment however, an exposure of 5 W laser was able to cause on average, a diameter zone of inhibition of 0.9mm. This wattage was too low to cause inactivation on agar plates. It is also interesting to note that on agar, an exposure of 9W only caused a 0.6mm zone of inhibition, on the cheese, this value increased to 1.6 mm.

A maximum clearance was observed at 13 W. The clearance at this power setting was 2.3 mm.

Penicillium spore contaminated cheddar cheese, aseptically removed from its wrapping was exposed to 13W maximum power CO_2 laser irradiation with a beam diameter of 2.5mm. The laser irradiation was scanned at 125, 150 or 200mms^{-1}. The powers applied were 5, 6, or 10W. The results are shown in **Table 23**.

In this experiment the scan speed was increased from 100 to 125, 150 and 200mms^{-1}. This information is important as for an effective scanning system, in an industrial application, higher speeds may be desirable.

In all cases the 10 W test proved very effective, a 2mm diameter of clearance was observable from a 2.5 mm beam diameter. It was apparent that as the power was increased, the diameter of clearance increased.

Mucor sp. spore contaminated edam cheese, aseptically removed from it's wrapping was exposed to a 13W CO_2 laser beam of diameter of 3.5 mm. The laser irradiation was spirally scanned at 20 revmin^{-1}. The settings were varied with computer control so an almost complete spiral scan could be applied, i.e. where the lines of the scan touched each other, so the whole surface received laser irradiation. The power was varied via

the computer. The power applied was 0, 2, or 10W. The results are shown in **Table 24.** This experiment was very successful. It showed that the spiral scan was more than capable of completely scanning an object, in this case cheese, and sterilising the surface of the cheese inoculated with fungal spores. The scan was set up so the lines almost touched each other. The entire exposure lasted approximately 2.5 min, but this encompassed the whole cheese and sterilisation was achieved with power of only 2 W. Some growth was observed on the test cheese, this was due to incomplete scanning, i.e. space between the scan lines. This may have occurred due to tolerance of the system there may have been some fluctuation in the spiral velocity, the laser beam lateral scan velocity, the power may have reduced during the scanning or the energy density applied may have been on the border of sterilisation.

3.4.1.2 Non scanning laser treatment

Cheddar cheese, contaminated with either *Mucor sp* or *Penicillium sp* spore suspension, was exposed to 200W Nd:YAG laser irradiation, with a beam diameter of 10mm. The energy density range applied to the cheese was 0 - 510 Jcm^{-2}. No clearing was observed.

Penicillium sp spore contaminated cheddar cheese, aseptically removed from it's wrapping was exposed to 200W Nd:YAG laser irradiation, with a beam diameter of 12mm. The energy density range applied to the cheese was 0 – 1769 Jcm^{-2}. The results are shown in **Figure 32.** In the first series of experiments, the Nd:YAG laser irradiation was ineffective against the spores of both *Mucor* and *Penicillium sp* spores. Energy densities of 510 Jcm^{-2} were applied to the spores. These experiments were tried at a later date with Nd:YAG irradiation of a higher energy density on the spores of *Penicillium sp*. Clearance of the *Penicillium* spores on the surface of the cheese was observed. The higher energy density levels applied, reaching 1769 Jcm^{-2} caused a zone of inhibition 11.9 mm in diameter. It should be noted that a 4 mm zone of inhibition was now observable after an energy density of 354 Jcm^{-2} had been applied. This is a lower level of laser irradiation than had been applied previously, yet was now causing clearance. All tests caused damage to the cheese, and the energy density of 1769 caused the cheese to bubble/boil. The cheese and spore suspensions were prepared in exactly the same way as previously, it is not known whether the laser started to work more efficiently, between the times

Table 23 Diameter of clearance in *Penicillium* spores on cheese caused by various powers of CO_2 laser irradiation, scanning at various speeds. The results are the mean of three trials, the standard error of the mean is also shown.

Scan speed (mms^{-1})	Power (W)	Width of clearance (mm) (SEM)	Rate of clearance (mms^{-1})
200	5	0.5 (0.29)	100
200	6	0.83 (0.17)	166
200	10	1.92 (0.08)	384
150	5	0 (0)	0
150	6	1.17 (0.17)	176
150	10	2.0 (0)	300
125	5	0.75 (0.14)	94
125	6	1.17 (0.6)	146
125	10	2.0 (0)	250

Table 24 Clearance caused by spirally scanning CO_2 laser irradiation.

Power (W)	Growth
0	copious
2	very little
10	very little

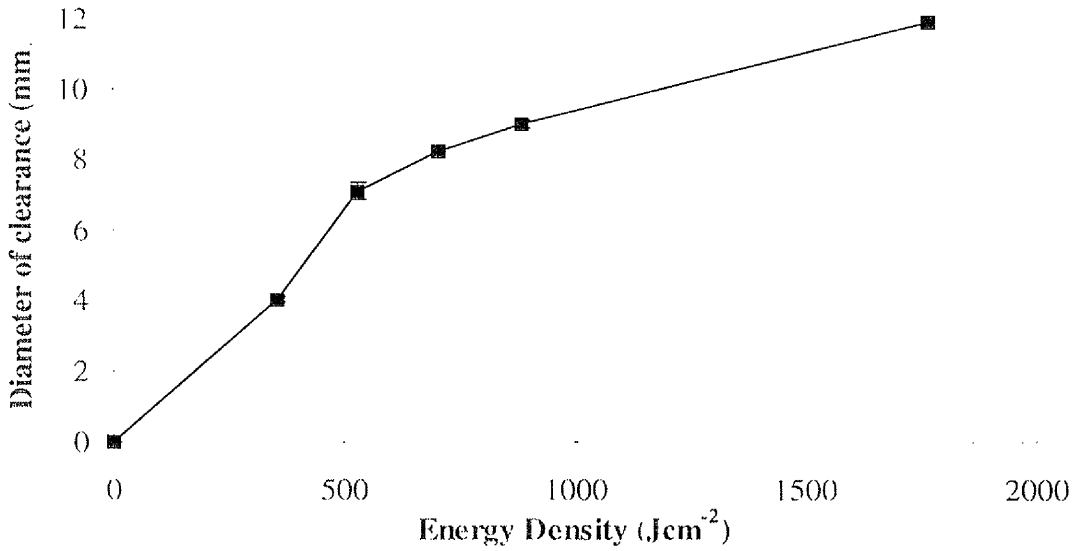


Figure 33 The increased clearing of Penicillium spores by Nd:YAG laser irradiation on the surface of cheese caused by an increase in applied energy density. The experiment was in triplicate and the error bars (where visible) are the standard error of the mean.

when these experiments were done, or whether the properties of the cheese were slightly different.

Mucor sp. spore suspension contaminated edam cheese was exposed to a single exposure of 500W maximum power CO_2 laser irradiation with a beam diameter of 11 mm. Various exposures were applied, 0 (control), 1, 2, 3, 4, 5 or 6 ms exposure. There was no clearance observed.

Mucor sp. spore contaminated edam cheese, were then exposed to a single exposure of 1 kW maximum power CO_2 laser irradiation with a beam diameter of 11 mm. Various exposures were applied, 0 (control), 2, 4, 8, or 10 ms exposure. The results are shown in **Table 25**. In these experiments, the 500W power CO_2 laser irradiation proved to be ineffective at the exposure lengths chosen. The 1kW CO_2 laser system however, caused clearing of the fungal spores after a 4 msec exposure, and an energy density of only 60 Jcm^{-2}. As the exposure was increased to 10 msec the zone of inhibition increased. It should be noted that the zone of inhibition was larger than the actual beam diameter. The cheese was undamaged at the exposure lengths chosen, so this effect was not caused by the cheese melting, but more likely because of lateral diffusion of heat across the surface. A zone of inhibition, 12 x 16mm was achieved from an 11 mm beam diameter after 10ms.

3.4.5 UV treatment of fungally contaminated unwrapped cheese

Penicillium sp. spore contaminated edam cheese, was exposed to UV irradiation, from 2 x 8W lamps, 24.2 cm from the cheese surface (output 200-400μWcm^{-2}). The results are **Table 26**.

After 1 min of UV irradiation, there was still not an observable effect shown on the fungal lawn. This time period was very large when compared to the 10s of Nd:YAG laser and the 10 msec exposures of CO_2 laser which had an effect on the fungal spores. Of course the UV irradiation levels may be increased if more rapid clearing was desired.

Table 25 The diameter of inhibition of *Mucor* spores on cheeses caused by a pulse of 1kW CO_2 laser of various exposure.

Exposure (msec)	Energy Density (Jcm^{-2})	Average zone of clearance (mm)
0	0	0
2	30.25	0
4	60.49	8 x 12
8	120.98	8 x 11
10	151.23	12 x 16

Table 26 Observation of decreased growth of *Penicillium* spores on cheese caused by UV irradiation.

UV exposure (s)	Growth
0	no
20	no
30	no
40	no
60	no
120	Growth only on centre of cheese surface

3.5.0 Mechanisms of laser inactivation

In this section fungal and bacterial spores were laser heated and heated in a water-bath, then examined under electron microscopy. The resulting effects caused by the treatments were compared in an attempt to identify the method of laser action.

3.5.1Electron Microscopy of heat treated spores

3.5.1.1 Transmission Electron Microscopy *Bacillus* spore suspensions heat-treated with laser irradiation and water-bath.

Aliquots (2ml) of *B. cereus* spore suspension (1 x 10^6) were raised in temperature to 80, 90 or 97°C with 10J and 10Hz with a 12mm beam diameter Nd:YAG laser irradiation. Further aliquots were heated in a water-bath at 80, 90 or 100°C for 10 min. A control did not receive heating. The samples were prepared for T.E.M. and analysed.

Figure 34 shows the expected structures of the typical *B. cereus* spore (x 60000), the central core is clearly observable, this is surrounded by the cortex region (clear area), this is in turn surrounded by the spore coat layer, and the irregularly shaped lipid exosporium layer. Notice that the spore core has an area of lighter coloured nuclear material along the periphery. **Figure 35** shows a spore that has been heated to 80°C with laser irradiation. The spore core and cortex are still clearly visible, but now the spore coat and exosporium are no longer clearly defined, the spore has the general appearance of a spore that has been affected. **Figure 36** shows the effect of heating spores at 80°C for 10 min in a water-bath. This field (x 6ooo) shows that not all spores are transected perfectly allowing the central region to be observed. This is due to the spacial arrangement of the spores, some are cut at the end, others through the edge or side. The cells marked show that there has been a similar effect as that caused by laser irradiation heating, again there is less definition in the outer layers of the spore. The effect of heating spores to 97°C with laser irradiation is shown in **Figure 37**. In this section the outline of the spore coat is still visible but the thin layer of nuclear material has become thicker, notice the cortex region is still clearly visible. The exosporium is not defined. **Figure 38** shows spores that have been exposed to a boiling water bath for 10min. Again the outer layers are barely discernible, and the ring of nuclear material is larger than that of the control. This area is less well defined and seems to be infringing

on the cortex region that appears smaller and less distinct. Both methods of heating appear to confer similar physiological effects upon the spores, the water-bath heating lasted for ten minutes, the laser for only 40s. The water-bath did appear to have a more pronounced affect on the spores, but a thermal effect was also administered by the laser irradiation, if to a lesser degree.

3.5.1.2 Scanning Electron Microscopy of *Bacillus* spore suspensions heat-treated with laser irradiation and water-bath

Aliquots (2ml) of *B. cereus* spore suspension (1 x 10^6) were raised in temperature to 80, 90 or 97°C with 10J 10Hz 12mm beam diameter Nd:YAG laser irradiation. Further aliquots were heated in a water-bath at 80, 90 or 100°C for 10 min. A control did not receive heating. The samples were prepared for S.E.M. and observed.

There was no change in the outside structure of the spores. There was no difference between the treated and control spores.

3.5.1.3 Scanning Electron Microscopy of *Bacillus* spores on stainless steel.

Bacillus cereus spores were air-dried on the surface of 0.2mm thick stainless steel discs. The prepared coupons of metal were exposed to 100W Nd:YAG laser irradiation (beam diameter 16mm) for 2.5s. This exposure ensured the dried suspensions were not burned. As controls, unexposed tests were prepared. Scanning electron microscopy was used to detect any changes caused by irradiation.

Figure 39 shows the surface of a prepared coupon, with the relatively clean and smooth steel on the left, and the spores on the right. **Figure 40** shows the control coupon (no laser treatment) in more detail. The surface was very uneven. After laser exposure, the surface was very different (**Figure 41**). The surface now seemed "pitted"

3.5.1.4 Scanning Electron Microscopy of fungal spore suspensions heat treated with laser irradiation and water-bath

Aliquots (2ml) of *Penicillium* spore suspension (1 x 10^6) were raised in temperature to 50, 60, 70 or 80°C with 10J, 10Hz with a 12mm beam diameter Nd:YAG laser irradiation. Further aliquots were heated in a water-bath at 50, 60, 70 or 80°C for 10 min. A control did not receive heating. The samples were prepared for S.E.M. and analysed. A control spore is shown in **Figure 42**, note the crenated surface. The results

showed that even after heating to 50°C, some spores showed signs of damage. The damage between the treatments was slightly different, the laser treated spores, showed signs of violent disruption, with the outside integrity being compromised, see **Figure 43**, the cells almost seemed to show ablation. The water-bath damaged cells, did not seem to be damaged in such a dramatic fashion, but the damage was still severe. The cells seemed to split apart, i.e. half spores were observable, as were smaller fragments, see **Figure 44**. It must be noted that even at 80°C, intact, apparently, non-damaged spores were observed. It is not known if these cells remained viable, or had been inactivated, without damage to the structure.

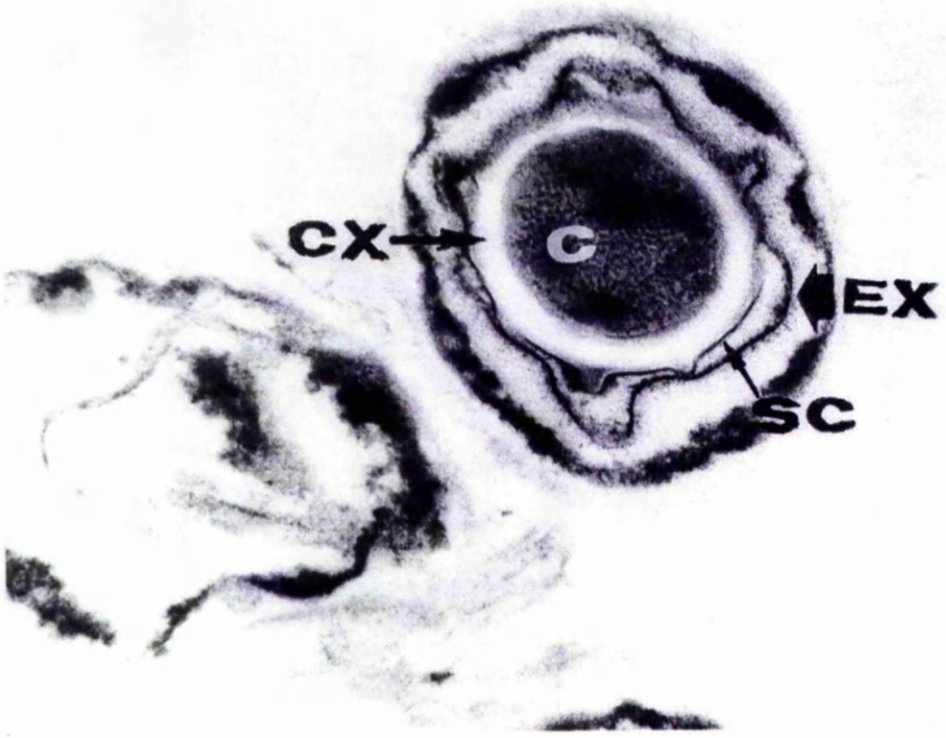


Figure 34 Electron micrograph of a *B. cereus* spore (x 60000). The spore has not been heat treated. The core, cortex region, spore coat and exosporium layers are all visible. Key CX = cortex, C = core, EX exosporium, SC = spore coat.

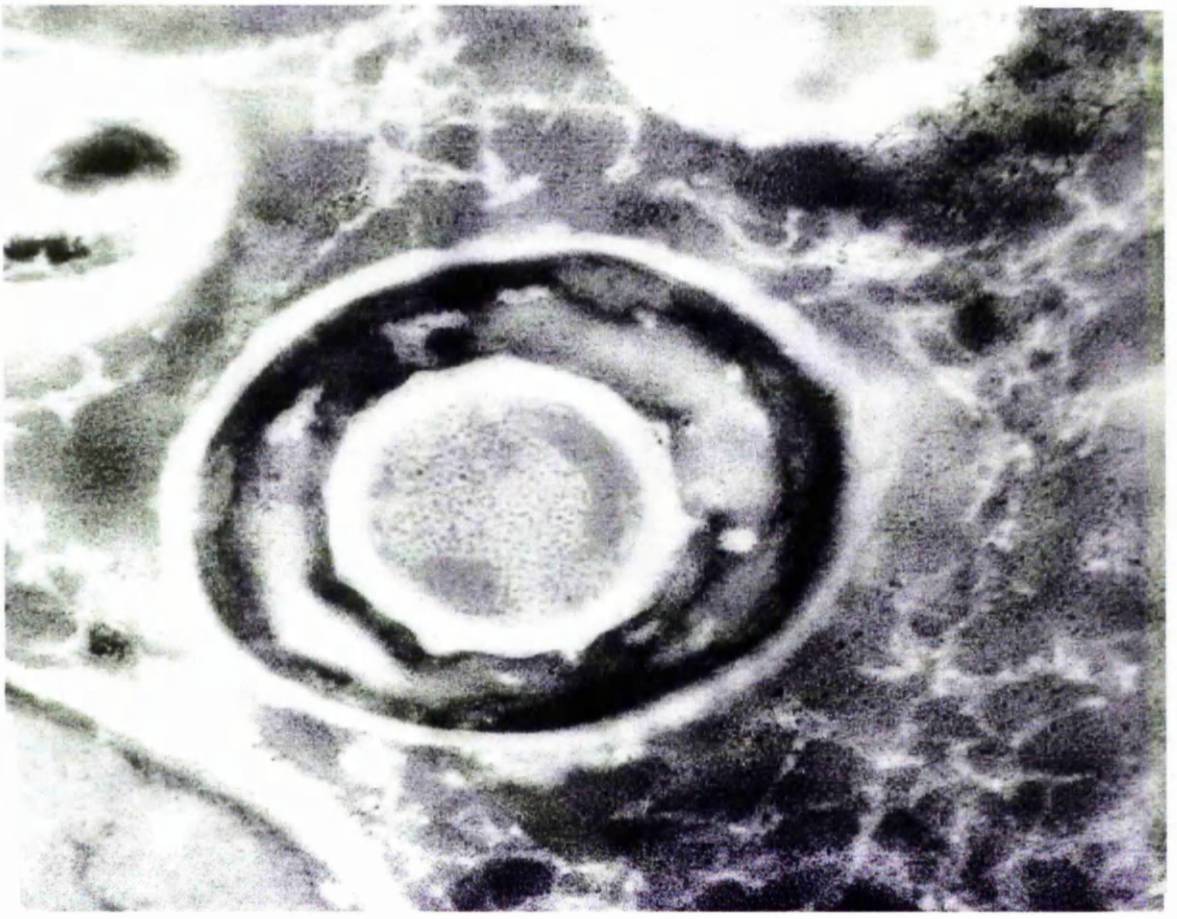


Figure 35 Electron micrograph of a *B. cereus* spore (x 60000) heated to 80°C with Nd:YAG laser irradiation. The core and cortex are still visible, but the coat and exosporium is less well defined.

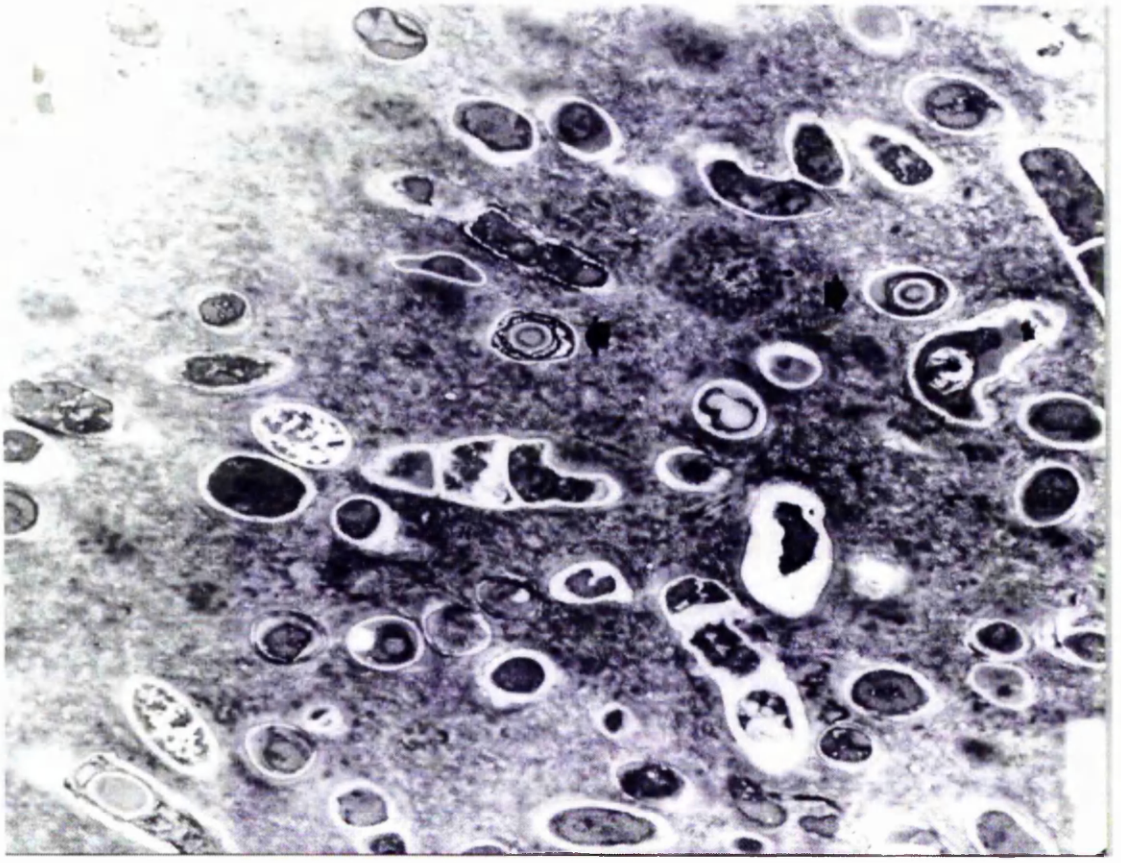


Figure 36 Electron micrograph of a *B. cereus* spore suspension (x 6000) that has been heated at 80°C for 10 min in a water bath. The clearest spores have been marked. Notice that many spores do not have an observable centre, due to their spatial arrangement and the angle of the slice when the cut was made.

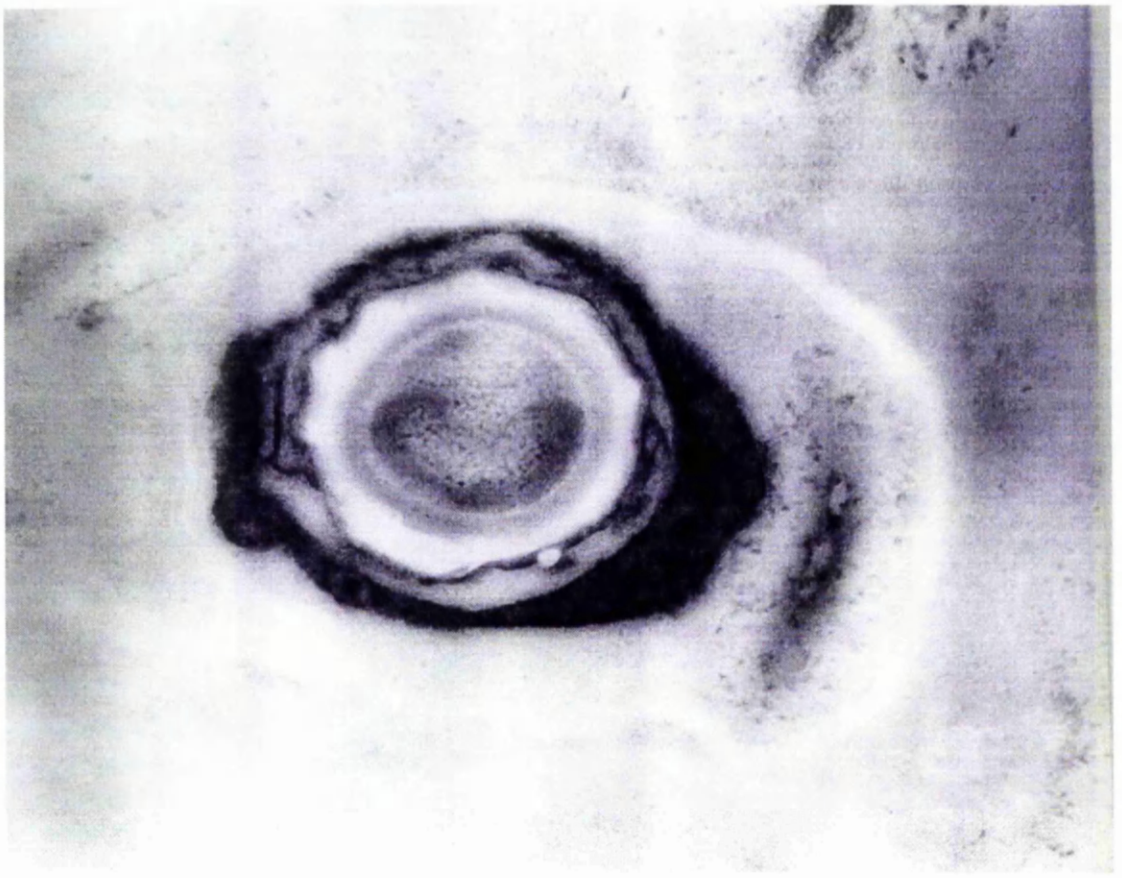


Figure 37 Electron micrograph showing a *B. cereus* spore (x 60000) that has been heated to 97°C with laser irradiation. The outer layers are not well defined and the area of nuclear material at the edge of the core has become enlarged.

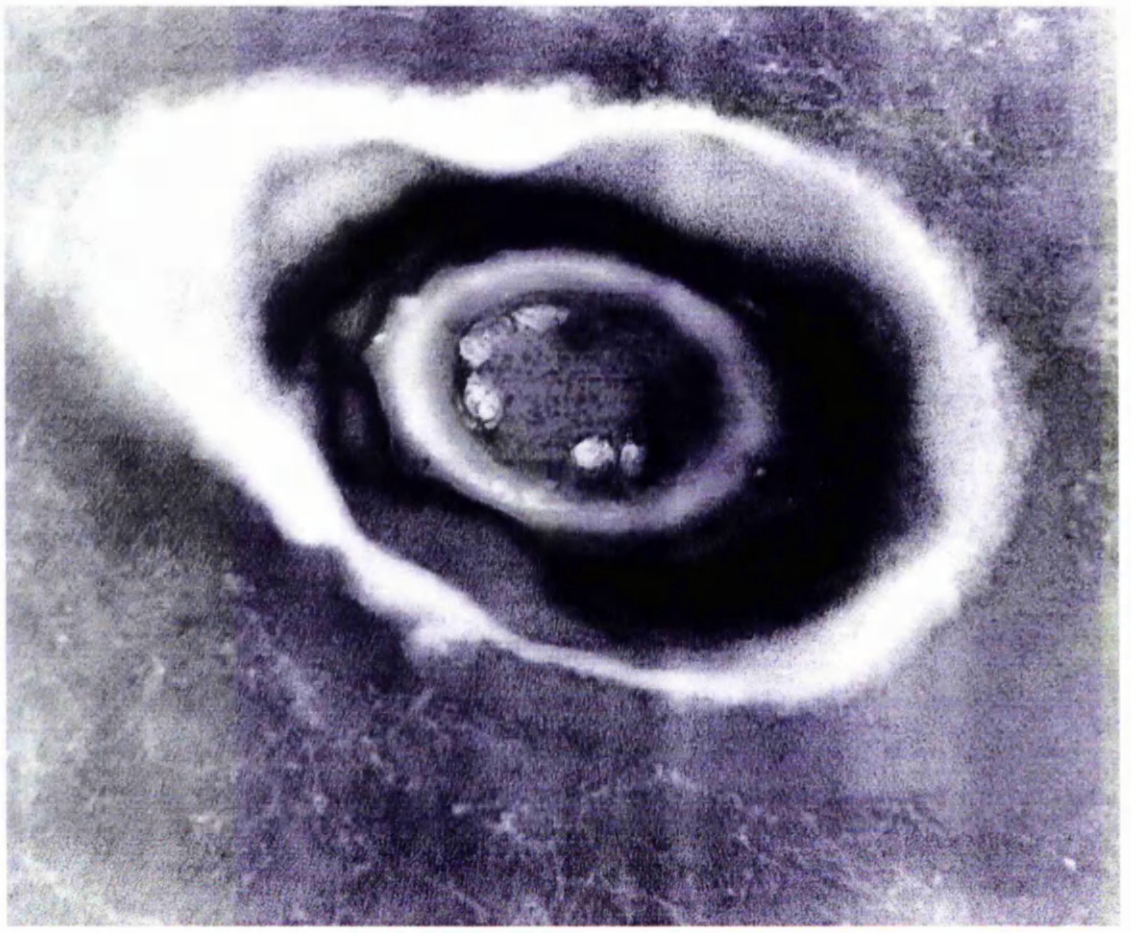


Figure 38 Electron micrograph showing the effect of heating a *B. cereus* spore for 10 min in a boiling water-bath, the outer layers are again less distinct but also the core nuclear material is encroaching the cortex region.

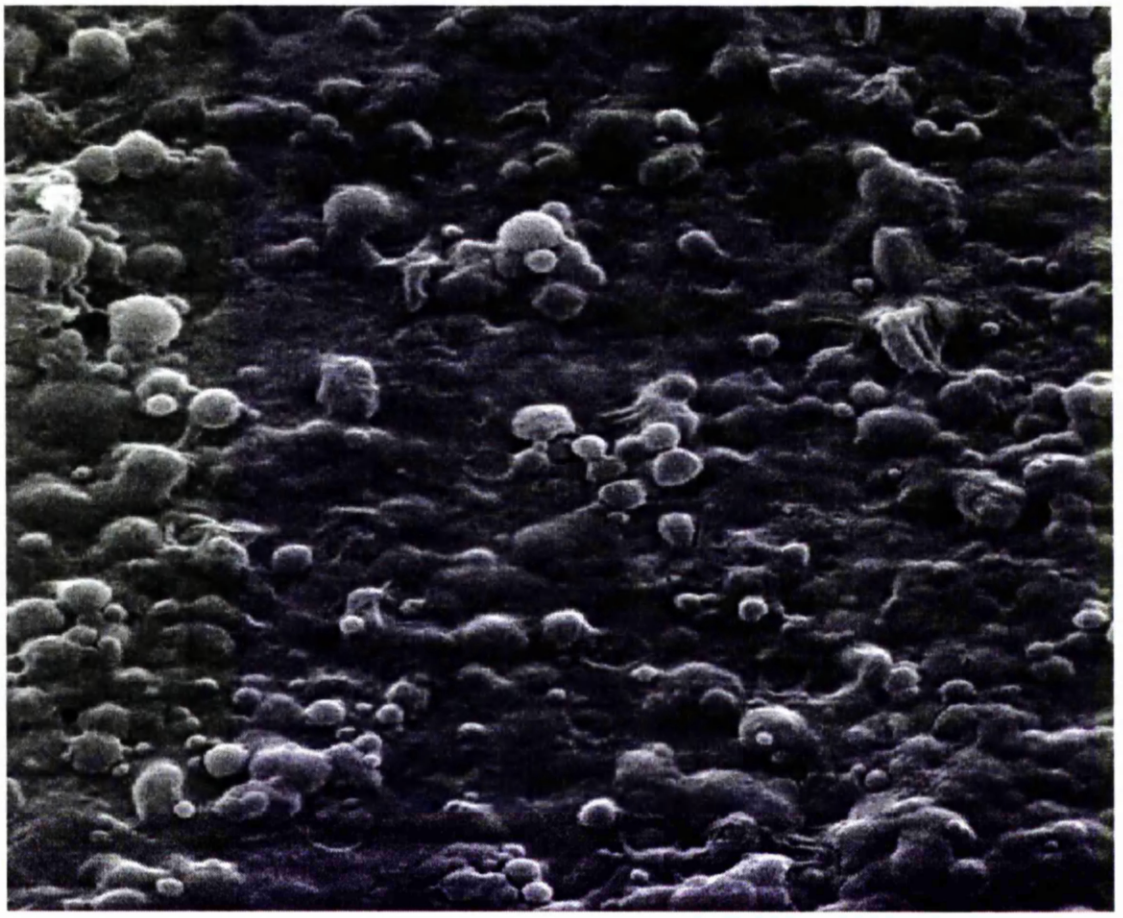


Figure 39 S.E.M micrograph of an untreated *Bacillus cereus* spores (Magnification x 15000) on the surface of stainless steel.

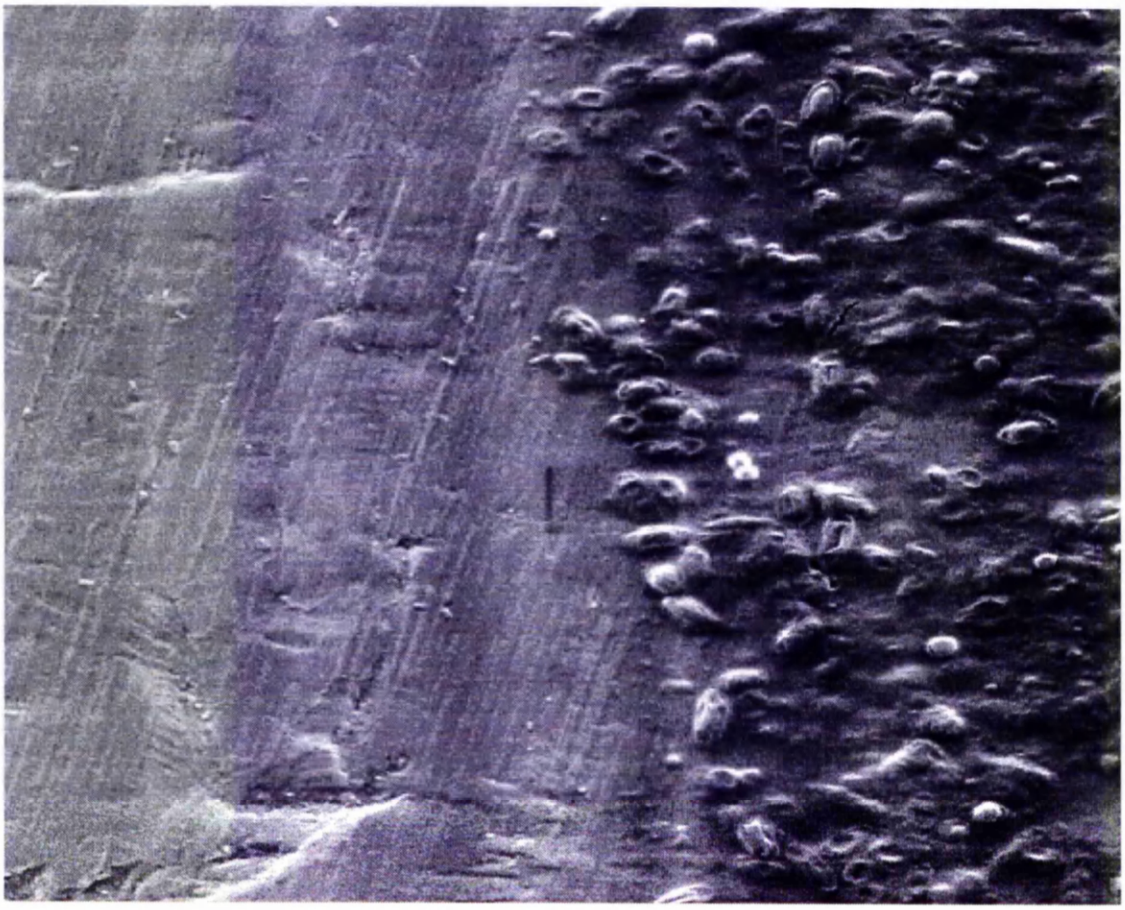


Figure 40 *B. cereus* spores (x5000) air dried on steel.

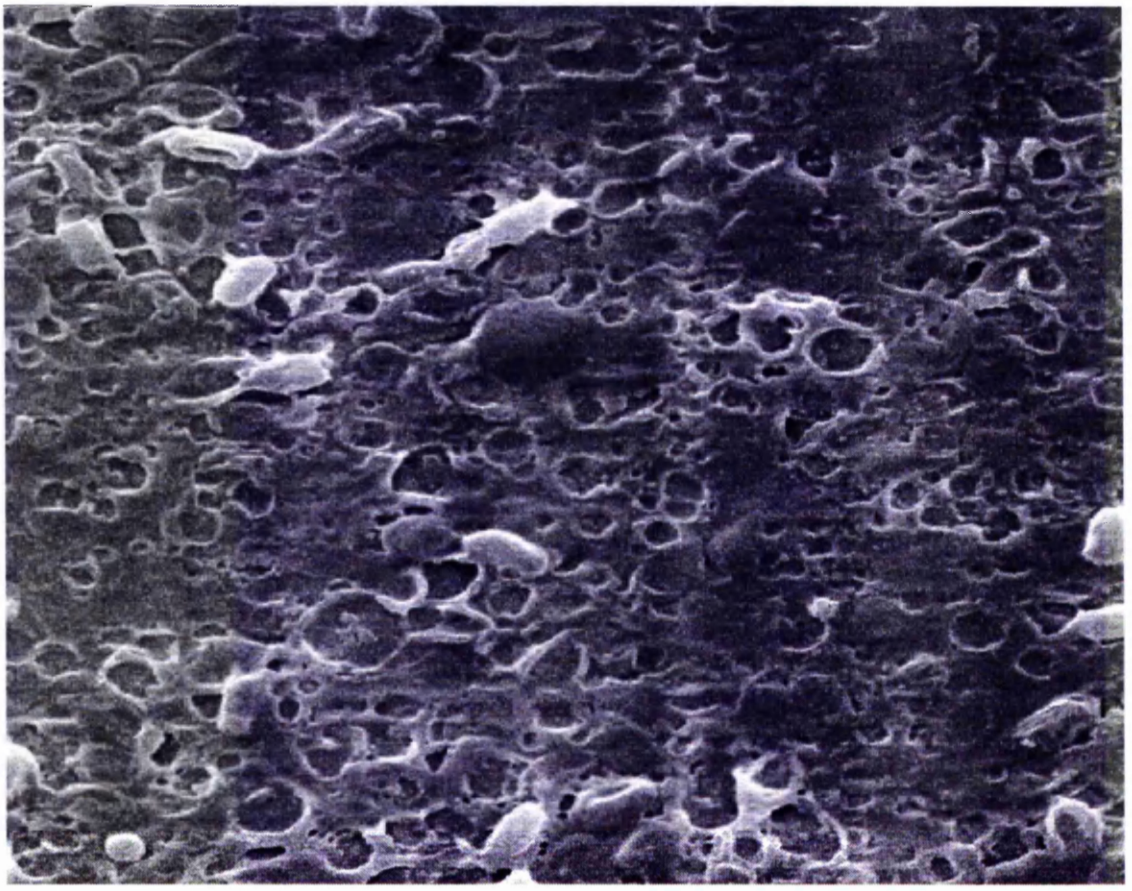


Figure 41 *B. cereus* spores (x15000) after 100W Nd:YAG exposure. The surface now was "pitted". The irradiation had caused a marked change in the appearance of the tests.

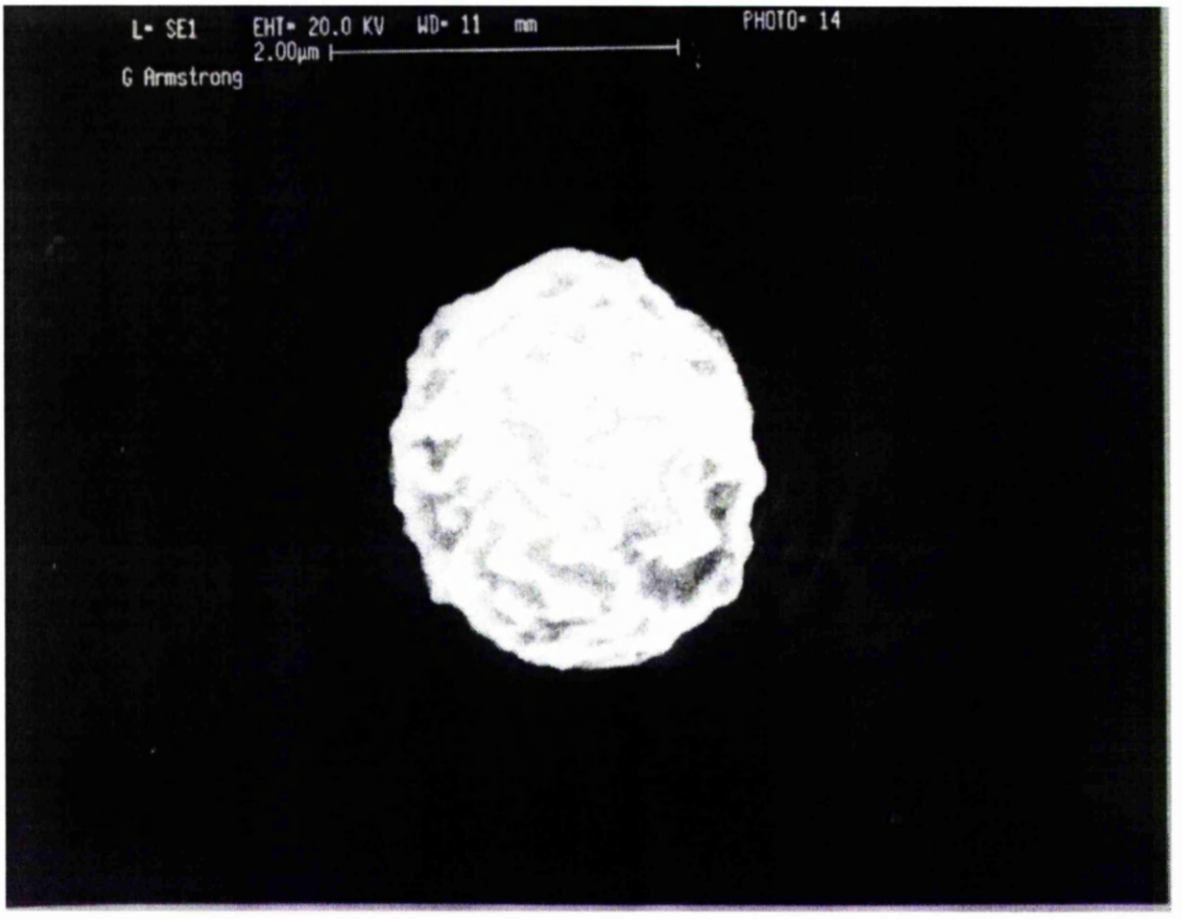


Figure 42 S.E.M micrograph of an untreated *Penicillium* spore.

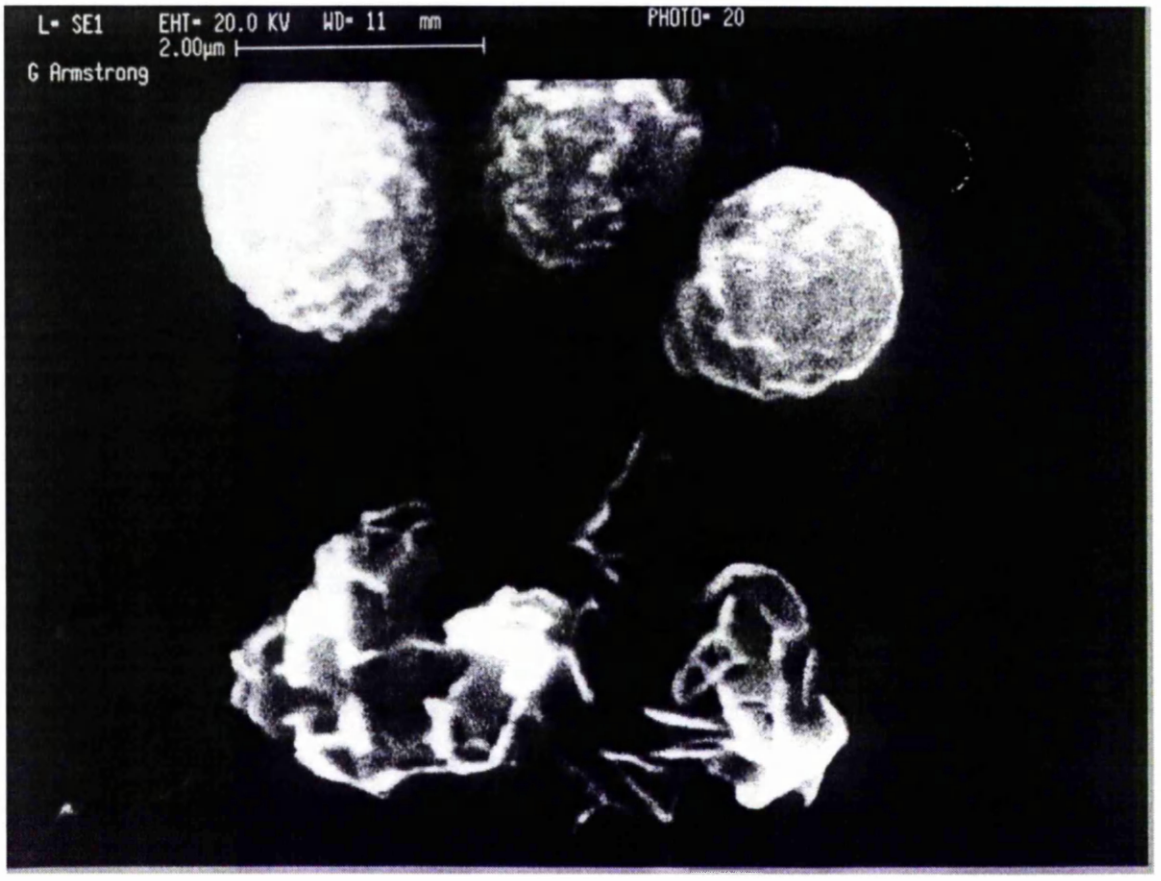


Figure 43 S.E.M micrograph of a *Penicillium* spore heated to 50°C with Nd:YAG laser irradiation. Note the damaged cell while others seem unscathed.

Figure 44 S.E.M micrograph of a *Penicillium* spore heated to 60°C for ten minutes in a water-bath. Note the segment of damaged cell, again, others seem unscathed.

4.0 Discussion

Chapter 4 Discussion

4.1.0 Spores of *Bacillus* species

4.1.1 Laser irradiation treatment of spores on surfaces

The work described has demonstrated that using laser irradiation method to sterilise solid surfaces is a viable prospect. Laser irradiation was especially efficient when applied to the surfaces of metals, in these investigations stainless steel was used. The laser irradiation showed itself to be effective, even though the surface itself was reflective. In these experiments, the surface of the steel was left undamaged whilst still giving excellent sterilisation of the bacterial load. The vegetative bacterial species that were investigated were sterilised in under 1.5 seconds with the Nd:YAG laser, and in milliseconds with the CO_2 laser. The rate of killing was very rapid. A more conventional method, for instance the autoclave, may take over an hour to come to pressure, reach the required temperature and cool down enough for the materials to be used.

It may be argued that if concentrated chemicals are used then bactericidal action can also be achieved relatively quickly. This may be true, but legislation and public opinion is reducing the use of chemicals (Hurst 1995). There is a movement towards a more "environmentally friendly" attitude. The use of lasers would be a far cleaner method of sterilisation. It is also worth bearing in mind that the bacterial load used in these experiments was artificially high. The concentration of bacterial numbers used in these studies, would be rare in an actual industrial environment, where the laser sterilisation system may be put to use. The laser is not totally without limits when applied to stainless steel surfaces and a sterilisation system can only be evaluated when applied to the most stringent tests available. *Bacillus cereus* endospores can be a source and causal agent of food poisoning, and the sterilisation of the heat resistant spores of the organism is more important than the ability to clear the vegetative cells. Therefore it is against spores that the laser must prove its worth.

The CO_2 laser was used on spores with plastic (1 kW and 10 msec exposure) and glass discs (640 W for 20 msec). These materials were chosen because they absorb the CO_2 wavelength more easily than steel. In both cases 1D value (90% killing) was obtained in 20ms or less (**Figure 4**). This exposure is more conducive to a scanning system,

which would be necessary if lasers were to be used in an industrial sterilisation set-up. An important factor in the glass experiment was the fact that one of the trials for the 20ms gave a high count. It is postulated that this was due to the irregular beam profile of the laser beam. The laser was operating in the TEM $_{01}$ mode. It is possible therefore that the higher viable count obtained was due to incomplete exposure in the test area. The higher count could also be due to a miss-fire or incomplete exposure or due to a slight misalignment. If lower average reduction was due to the spores not receiving the complete exposure, then a 3D-decrease was noted on the glass surface, which was an excellent result after only a 15 msec exposure. A scan of spores on stainless steel strips, with the 13W CO_2 laser and a speed of 100 mms^{-1} was attempted **Figure 5**, but there was no significant effect on spore viability. It should be noted that only dry samples were used with the CO_2 laser, as this wavelength of light is highly absorbed by water, and wet samples tended to splatter as they rapidly absorbed the laser energy. This was not wanted from a safety point of view i.e. aerosols containing bacterial species. Sadoudi *et al* (1997) describes the phenomenom of laser induced aerosols, citing it as a reason for re-contamination.

Nd:YAG laser irradiation was used to kill spores on the surface of steel.
A 6 D-value decrease was routinely achieved with the vegetative cells of *B. cereus,* with an energy density of 250Jcm^{-2} and exposure of 1.5s, **Figure 8,** this was not achieved when the test subject was bacterial endospores. No inactivation of spores was observed with the Nd:YAG laser, energy density of 778 Jcm^{-2} (exposure of 10s) **Figure 9**. This showed the importance of bacterial endospores as a test subject. A different method of laser treatment was investigated, in order to reduce the exposure time required for Nd:YAG laser light treatment of spores. It was hoped to achieve rapid, laser decontamination. It was hypothesised that if a small beam diameter was used in accordance with a high energy density pulse, then a single high energy, short duration pulse could possibly give killing, i.e. the smaller beam diameter would increase the energy density of the pulse. If successful, this may make the implementation of a scanning system more realistic. The high energy, short duration pulses, however, gave very little killing after a single pulse of light (exposure equivalent to the pulsewidth = 8 msec) **Figure 6**. The Nd:YAG laser was only capable of firing a 35 J pulse (with a chosen beam diameter of 8mm). This gave the sample on the disc a burnt appearance but was not enough to cause killing.

Wet and dry samples were tested. There was no statistically significant effect caused by the treatments and there was no significant difference between the treatments.

The experiment was repeated with *S. aureus*. This organism is slightly more resistant than the vegetative cells of *B. cereus*, but does not produce highly resistant spores. The laser parameters chosen were 30J energy, a pulse width of 4 msec and a beam diameter of 7mm. It was felt that any killing effect would be more prominently expressed in this organism than in the spore tests. Once again very little killing was observed **Figure 7**, it was therefore felt that this method was not the best method of sterilising solid surfaces.

As small beam areas were used in this branch of the experiment, discs were made with small indentations. This allowed the sample to remain in a confined spot that could be successfully targeted with the laser beam. It was however, felt that a problem of "shadowing" may occur, as a focussed beam was being used. This would mean that there may be some protection for the organisms at the edges of the depression. To nullify this a small spot of the protein, bovine serum albumin (BSA) was placed in the centre of a flat disc and this was used to confine the organism on the desired spot. This method proved successful. This method, however, brought to light a new disadvantage of using the laser irradiation on solid surfaces, in that the protein used on the discs, was sometimes charred by the laser irradiation. This produced a black residue that was often difficult to remove. This may not be tolerated in an actual operating system, as it may be detrimental to subsequent processing. Protein was not used for subsequent experiments for this reason and also that the protein may begin the onset of germination. Another disadvantage with this method was that the smaller beam diameter and increased energy density would be closer to the damage threshold of the metal.

As mentioned, when 778 Jcm^{-2} of Nd:YAG laser irradiation was applied to spores on the surface of stainless steel discs, there was a 3 D-value decrease in the viability of the spore suspension (exposure 10s). It was decided to increase the exposure in the hope of achieving greater spore reduction.

This was achieved with Nd:YAG laser irradiation of 10J and 10Hz, beam diameter 16mm. The exposure required was 36s, an energy density of $1800Jcm^{-2}$ (giving over a 4 D-value decrease in viability), **Figure 10.** The exposure time was too long for a practical system. In order to reduce the exposure, the spore samples on the steel were exposed to the same parameters of Nd:YAG irradiation but the beam diameter was reduced from 16mm to 8mm. The energy density remained the same, the exposure was

reduced to 9s. The killing levels were found to be the same as those for irradiation of 16mm. This was excellent as it meant that the killing process had been speeded up, **Figure 11**. The next logical step would be to reduce the beam diameter further. This was not done, as the sample size was 5mm and the risk of misalignment would be increased, giving the chance of false positive results. The 8mm beam diameter caused wet samples of spores to "splatter" upon treatment. For this reason only dry samples were used.

The results did raise an important question. The energy density used in both sets of experiments was the same, 1800Jcm^{-2}, but for the 8mm beam diameter tests, a larger percentage of the beam came into contact with the, yet the killing remained the same.

Using data from (Yeo, 1999) the percentage of the beam in contact with the sample was 72% and 43% respectively (taking beam profile into consideration). This information was found by plotting the beam profile of the laser using a pyroelectric array.

If the killing was due to the photonic interaction of the laser irradiation with the spores, then it would be reasonable to expect that the exposure with the 8mm beam diameter would cause more efficient killing. This is because more of the laser beam was in contact with the dried suspension, but the inactivation was the same for the 16mm and the 8mm tests, 1800Jcm^{-2} being required for complete inactivation.

It was hypothesised that the killing may in fact be due to an indirect heating of the spores by the laser heated metal. If this could be proven, then it would indicate the laser killing mechanism for spores death.

Discs of stainless steel were exposed to various levels of Nd:YAG laser irradiation of beam diameter 8 or 16mm and by using calorimetry, a cooling curve was obtained, the values are tabulated in **Tables 8 and 9.** The bulk temperatures of the steel were compared in **Figure 12**. The found bulk temperature of the disc (average mass 5.6g) after exposure to a laser energy density of 2000Jcm^{-2} was 130°C for the 8mm beam diameter test and 140°C for the 16mm test. There was not a significant difference between the results.

The discs received exposure of equivalent energy densities, reached approximately the same temperature, and the level of killing was the same even though different amounts of laser irradiation actually came into contact with the spores (as described above). It is likely that the main mechanism of spore killing on the steel surface was due to an

indirect effect. The laser irradiation heated the steel and the heat transferred to the spores.

To show that indirect heating was an important killing factor, 0.2mm thick stainless steel coupons were used instead of the 5.6g discs, **Figure 13**. This was done because the thinner, lower mass metal would heat up rapidly after a lower laser irradiation energy density. This would support further the indirect heating theory because if the laser irradiation killed the spores directly, then the lower energy density used, would not give killing, but if killing was due to transfer of heat from the metal, then the low mass metal (0.8g) could still facilitate killing.

Sterilisation of the metal was achieved with 10J and 10Hz Nd:YAG laser irradiation, beam diameter 16mm after 2.5s, an energy density of $125Jcm^{-2}$, compared to $1800Jcm^{-2}$ in the previous experiment. This supported the hypothesis that killing was at least by heat transfer from the substrate to the spores.

The important factor was the laser energy density required was reduced, only 125 Jcm^{-2} was required for the 0.83g coupons and $1800Jcm^{-2}$ for the 5.6g coupons. So it was apparent that in quantifying laser sterilisation of surfaces, it is not sufficient to give a bactericidal energy density, but the mass and conductive nature of the exposed material must also be considered. This conclusion would also have important ramifications for the development of a scanning or industrial system. If the target material was a large metal table for instance, it would require a greater energy density to raise the bulk temperature to that required than the levels found for the test coupons used in this experiment. The lateral/radial heat loss in a table would be much greater in relation to those in the 20mm x 20mm coupon (thickness 0.2mm, mass 0.83g) of laser irradiated metal. From the first series of experiments it was apparent that the eradication of bacterial endospores from solid surfaces required high energy density levels and/or exposure lengths than that required for vegetative cells. A 4 to 5D decrease **was** obtained at low energy density levels with thin metal (0.2mm thick).

This work has shown that more information must be given when commenting on the sterilisation of organisms on metal with laser irradiation. Many existing papers lack such information, i.e. Hooks *et al* (1980) claimed sterilisation of stainless steel reamers. The mass of the steel was not mentioned.

This important implication of test surface interaction was not fully considered before this study here.

The results described here also have furthered the knowledge in the method of spore sterilisation on metal. The experimented showing that even when a smaller percentage of the beam was directly affecting the spores, if the bulk temperature of the metal was the same, the same killing was observed. The thinner metal experiments showed that less energy density was required for sterilisation on the thin metal (with lower mass). Taking these experiments together it seems obvious that the direct interaction between the photon and spore is not as important as the bulk temperature of the metal. The transfer of the heat from the metal to the spores must be therefore the main killing mechanism.

4.1.2 Exposure of bacterial endospores to laser irradiation before and after ultra violet irradiation

It was felt that if a synergistic system involving laser irradiation and another bactericidal agent could be found, then not only would the system be more practical than that currently available for the agents themselves, but also further insight could be gained towards the mechanisms involved in laser action.

The first of these investigated was laser and Ultra Violet (UV) irradiation obtained from a conventional UV lamp.

Both the Nd:YAG and the CO_2 laser seemed to be ineffective against the spores on agar surfaces, the agar and occasionally the plastic was damaged before the spores were killed **Section 3.2.1.** Agar is normally considered a good surface to show the inactivation of vegetative organisms as it gives a clear zone where killing has been achieved and this allows the effect of the beam site to be easily deduced. The agar does however melt at approximately 80°C and is therefore not necessarily a good surface on which to attempt the killing of spores that can survive greater than 100°C. The Nd:YAG laser was applied to lawns of both *B. cereus* and *B. subtilis*, at energy density levels of 3000Jcm^{-2}. There was no clearance of the lawns after exposure. As previously mentioned, there was a reduction when stainless steel discs were used after an energy density range reaching 800 Jcm^{-2}. This means that over three times as high an energy density did not cause clearing on the agar surface. In an attempt to increase the amount of laser irradiation absorbed, a reflective piece of aluminium was placed below the petri-dish, to reflect any unabsorbed irradiation back up through the agar. This caused the bottom of the petri-dish to melt, so could not be considered a useful innovation for this application. The CO_2 lasers did not prove any more successful. Irradiation from a 1 kW laser was applied to lawns of *B. cereus* spores, for exposures of 500 ms. There was no clearing of the lawn, but large indentations were made where the water in the agar absorbed the irradiation and was boiled off. The exposures were increased to 1500 and 2000 msec, but this only succeeded in damaging the dish and the agar. A scan was attempted with 500 W power CO_2 laser at 10m min^{-1}. There was no clearing observed.

This experiment shows that for certain surfaces, laser light alone may not be sufficient, this section examined the effect of adding UV irradiation to laser light in the hope of increased bactericidal properties. UV and laser were first examined on agar plates **Figures 14 and 15**.

The previous experiments showed that with no UV exposure, an Nd:YAG laser exposure of 3000Jcm^{-2} gave no clearing of the spore lawn. In this experiment, the same 3000 Jcm^{-2} (10J and 10Hz or 20J and 10Hz) was followed by an exposure of UV irradiation (2 x 8W) of varying lengths. The control test of laser exposure alone gave an average clearance diameter of 1.5 mm (although some of the trials gave no clearing). The results were described as a percentage of the possible clearing **Figure 16**, this refers to the 14 mm beam diameter, i.e. 14 mm diameter clearing = 100%. The clearance of 1.5 mm equates to a percentage diameter of clearance of 10.71%. A two minutes exposure of UV irradiation increased this value to 42.86%, by increasing the exposure to 2-10 minutes the percentage diameter of clearance was raised further to 75-82%. After 6 min however, a distinct detrimental effect was observed on the spore lawn, i.e. clear zones were beginning to appear in places not exposed to laser irradiation.

One interesting effect was that even though a constant energy density of 3000Jcm^{-2} was used, when the power used was 100 W (10J and 10Hz) i.e. longer exposure lower power, the clearing effect was reduced. i.e. only 71% after 4-10 min, and virtually no clearing after 2 min of UV. The results were compared with a 2 sample t-test, and they did not prove statistically significant. High power, shorter exposure has been shown to be more effective in relation to *S. aureus* (Yeo 1998).

This preliminary experiment did not ascertain whether the laser clearance was due to a synergistic effect or merely because there was decreased spore numbers after the UV treatment (additive effect).

The effect of heating spores (liquid aliquots) with laser irradiation was investigated in an attempt to quantify the effect further, **Figure 17**. *B. cereus* spore suspensions were raised in temperature to 90°C. This experiment was interesting in that it showed that reduction in the viability of a liquid spore suspension (0.5-D) could be achieved in 30s and that the suspension itself only needed to be raised to a temperature of 90°C. Heating of the suspension was almost sub-lethal.

The next group of experiments, combined the heating effect of the laser irradiation with UV irradiation **Figure 18**. A laser treatment alone was compared with UV then laser,

and laser then UV. The UV exposure was 10s from 2 x 8W lamps. The spores received the secondary treatment immediately after the first. The first notable result from this was that the *B. cereus* spores were reduced in viability by 2.4-D, after heating to 90°C. This may have been due to the prepared spore suspensions being less heat tolerant than those previously. There was an observable increase in sporicidal action when the additional treatment was given (i.e. UV). This was to be expected. When the laser exposure was followed by the UV, there was a 2.67D decrease observed. It was interesting to observe that when the UV was administered first, this value increased to 3.37-D. This would imply that the UV is sensitising the spores to the laser treatment, and that when laser is administered first, most spore damage is caused by the heating caused by laser irradiation, and the UV subsequent treatment may merely add to the killing effect. It may be however, that the UV penetrability of the suspension was altered by laser treatment, i.e. by heating and thus altering the absorption co-efficient. This would mean the UV could not cause as much damage as previously possible due to a change in penetrability. When the results were analysed statistically, it was found that there was no significant difference between the laser heated to 90°C sample, and either of the UV and laser to 90°C tests. Statistically there was no significant difference in the two UV laser tests.

It is widely recognised that laser light and UV irradiation can provide bactericidal action, UV sporicidal action has been attributed to damage occuring between thymine residues on the same strand of DNA, this produces the spore photo-product (SP), and can cause a potentially lethal effect on the spore. Spores have a SP-specific repair system which rapidly and efficiently repair the lesion in the first minute of germination, (Munakata 1972, 1974, Nicholson 1990). A multifactorial experiment was designed which aimed to investigate the lethal effect of the laser and UV irradiation, as a positive interaction may enable the laser target to be more easily recognised. An example could be laser induced mutants that lack the *uvr* gene (encoding nucleotide excision repair apparatus), or the *spl* gene (which encodes SP lyase) would be more sensitive to UV irradiation. DNA damage due to the laser pre-treatment, if causing alterations to these areas will cause a significant increase in the killing efficacy of the following UV irradiation, causing a synergistic rather than additive effect.

Figure 20 showed that the laser irradiation had a significant effect on the spores, as did the UV irradiation, this was borne out by the statistical analysis (ANOVA). The laser

light was always very significant as a single test factor. The UV irradiation was not as significant (at the exposure chosen) but still proved to have a significant effect as a single test agent. There was however no significant interaction between the laser light and the UV irradiation. This would imply that any effects with the laser and UV are merely additive, and not of a synergistic nature.

Levinson (1960) reported that "radiation may enduce spore permeability changes, simulating the responses characterizing germination, even in the absence of glucose". The induced germination of spores has also been shown to be caused by heat. This means that the laser light could cause germination indirectly by the heating of the stainless steel discs. Should the laser irradiation cause induced heat activation or mechanical germination by the nature of the radiation, then the spores would be more susceptible to the UV irradiation. It was for this reason that a high and low delay factor was incorporated into the multifactorial experiment, in order to examine the importance of the time lag between the treatments, i.e. can the laser light induce changes in the time period between treatments?

The delay was only shown to be of significance when a high (6s) laser exposure was applied. This could mean that the spores, after high enough laser exposure are beginning to lose their resistance properties, and given a longer delay, the UV secondary treatment could prove more effective. This is an area that deserves further research, but in practical terms a delay of over 15 min would be unacceptable in many fields of industry.

4.1.3 Increased bactericidal action of laser irradiation and chemicals

The increased bactericidal action of chemicals upon heating is well documented, as are the benefits that can arise from chemical sterilisation. Opthalmological instruments have been shown to be excellent subjects for chemical sterilisation methods as moist heat treatments can blunt, and damage the instruments. (Wilkins, 1994)

This study aimed to compare the increase in killing efficiency in chemical that had been heated with a water-bath and from laser heating. Two chemicals were chosen, hydrogen peroxide, (H_2O_2), a clear, colourless liquid, and lactic acid (2-hydroxypropionic acid, $C_3H_6O_3$), this is a colourless or slightly yellow, syrupy liquid when concentrated, and is miscible with water. These particular chemicals were chosen, primarily for their known increased action on heating, but also because the chemicals did not contain chlorine based compounds. It was felt that this was very important as chlorine compounds are less environmentally friendly and also have problems associated with them in terms of degradation of metal surfaces, and are unpleasant for employees. Hydrogen peroxide breaks down to release oxygen, leaving no residue. Lactic acid is present in foods i.e. yoghurt, and has been used in practice on meat (New Scientist, 20/9/97). Lactic acid is also a constituent of the human body.

The study planned to show Nd:YAG laser irradiation treatment could give a synergistic effect when combined with a chemical. The ideal scenario for a laser system would be to flood a surface with low concentrations of a chemical, and then scan with relatively low levels laser irradiation, this would overcome any problems associated with shadowing so imperfections in the surface would not necessarily allow bacterial survival.

The standard industrial, concentration of hydrogen peroxide, is 30% (w/v) at 85°C (Soon Yung Shin, 1994). It was aimed to reduce both concentration and temperature while still causing a decrease in remaining viable spores. An assay (K_2MnO_4) was also used to ensure that the concentration before heating with water-bath or laser was the same as the concentration afterwards, this proved to be the case. There was also no visible production of oxygen gas bubbles after irradiation treatment. This was important as it was a visible indication of the chemical integrity and activity (hydrogen peroxide breaks down to form water and oxygen), but gas bubble formation would hinder the laser irradiation heating process. In the synergism experiments it was planned to expose the spores to the chemical for a maximum of 2 minutes. This included the time for the sample preparation, exposure and viable counting. It was therefore important to be able

to say with certainty that the increased killing effect (if any) was due to the heating of the chemical, not because longer heating lengths meant longer exposures to the chemical. *B. cereus* spores were exposed to H_2O_2 for up to 10 minutes, **Table 13.** Various concentrations of chemical were used: 0.5, 1.0 and 1.5% (v/v), these corresponded to the concentrations that were planned for subsequent experiments. The distilled water control, the 0.5% and the 1.0% (v/v) tests did not prove to have a statistically significant effect after the 10 minute exposure. The 1.5% (v/v) test did prove to have a significant effect after 10 min, $p = 0.0092$, under 95% confidence limits. The 2 sample t-test also proved that the effect at time 2 min was not statistically different from the distilled water control ($p = 0.2$).

Spore suspensions were also exposed to lactic acid of concentrations, 1.34 and 2.68 molar, **Figure 24**. The distilled water control, and the 1.34M acid did not have a significant statistical effect even after 10 min. The 2.68 test did have a significant effect on the spores (2 value t test $p = 0.042$, 95% confidence limits). Again the 2 min test did not prove significantly different from the time zero control, $p = 0.3$. The results meant that the concentration could confidently be chosen for subsequent experiments. The chemicals at these concentrations would be sub-lethal to the spores and any killing after heating (compared with a water control) can be attributed to an increased action of the chemical after heating.

The spore suspensions were then placed in the hydrogen peroxide concentrations and raised in temperature with Nd:YAG laser irradiation 10J and 10Hz, beam diameter 15.5mm until the temperature was raised to 30, 40, 50 or 60°C, **Figure 26**. These temperatures are far lower than the temperatures normally associated with spore killing, i.e. 90°C or above. The distilled water control, and the 0.5% (v/v) H_2O_2 test, after heating to 60°C did not show a significant effect on the spores. When the 1.0% (v/v) H_2O_2 test was heated to 50°C, the effect (2 sample t-test) began to approach significance, $p = 0.046$. When the temperature was raised further to 60°C, this effect became highly significant, $p = 0.017$, 95 % confidence limits. The 1.5% v/v H_2O_2 test was also significant after heating to 50°C, $p = 0.047$, this significance rose as 60°C was reached where complete killing was observed. This quantifies to almost a 6D decrease in less than 30s. This level of killing would be acceptable in an industrial set-up. Spore aliquots were also exposed to pre-heated chemical for 45s. The pre-heating was done with a water-bath. This can be seen as the ideal, as the temperature is constant and no

rate of rapid heating is faster than chemical that is already at the required temperature. The actual method of attaining the temperature is therefore irrelevant. It merely provides a basis of comparison for the rapid heating of the laser against constant exposure at the required temperature. With the water-bath tests, again the distilled water control and the 0.5% (v/v) H_2O_2 test did not cause a significant effect on the spores after heating to 60°C. The comparison between the laser and water-bath tests at these concentrations and temperatures showed there was no significant difference, p = 0.19 and 0.69 respectively. The 1.0% (v/v) H_2O_2 test did however prove to be highly significant at both 50 and 60°C. (p = 0.003 and 0.008). Complete killing was observed at 60°C. When the laser and water-bath were compared however, there was not a significant difference at 50°C between the methods of heating. There did prove to be a significant difference at 60°C though, p = 0.017 95% confidence limits. This means that the laser is significantly less effective than exposing the spores to pre-heated chemical. The 1.5% (v/v0 H_2O_2 tests again proved to be highly significant at 50 and 60°C (as in laser tests) the p values were 0.016 and 0.038, again complete killing was observed at 60°C. The laser heated and pre-heated chemical did not show a significant difference this time at 50 or 60°C. This means that putting spores in 1.5% (v/v) H_2O_2 then laser heating the chemical with laser irradiation is comparable to pre-heating the chemical and then placing the spores in it, the laser is approaching the ideal.

The results were not quite as encouraging, when spores were placed in lactic acid of concentrations 1.34 and 2.68molar. These were also raised in temperature to 30, 40 50 or 60°C with Nd:YAG laser irradiation (10J, 10Hz and 15.5 mm beam diameter), **Figure 25**. The distilled water and the 1.34M acid test, did not show a significant effect on the spores after heating to 60°C. The 2.68 molar test did have a significant effect as the temperature reached 50°C, p = 0.009, and the effect remained significant when the temperature was increased to 60°C, p = 0.012. In actual values, this only equated to 1.17-D of a reduction. The laser and acid combination did, therefore, show an increased killing effect. It was compared with the results of spores being placed in pre-heated acid for 45s. The distilled water control and the 1.34M acid did not show a significant effect after pre-heating, and were not significantly different from the results from the laser heating. The pre-heated 2.68M acid also proved to have a statistically significant effect after being pre-heated to 50 and 60°C, p = 0.009 and 0.01 respectively. The laser and pre-heated 50°C tests were compared and there was a slightly significant

difference, p = 0.048 (95% confidence limits). The 60°C tests were highly significantly different (p = 0.01). This means that unlike the hydrogen peroxide tests, where both pre-heated and laser heated tests at 60°C gave sterility, the laser heated lactic acid tests were significantly less effective than the pre-heated acid. The laser irradiation heated tests did show an increased action, but it was not as effective as the "ideal", i.e. pre-heated so the heating up time was very short.

These results show that an increased effect is shown when these chemicals are heated with laser irradiation, there are many ways in which this may be developed, see **Future Work**.

An attempt was made to cause "complete inactivation". Acetic acid was used with H_2O_2. The chemicals were used alone and in combination. Sub-lethal concentrations of acid were used (1M), **Figure 27**. The test mixtures were preheated to 60 or 70°C by water-bath heating, or Nd:YAG laser irradiated, **Tables 14 and 15**.

The results for the 60°C tests were very similar, the acid alone and acid/H_2O_2 mixture, did not cause complete inactivation. The hydrogen peroxide alone, laser irradiation heated tests, in the main, could not cause complete inactivation, but some did show no turbidity. This raised an interesting point, if hydrogen peroxide, laser heated to 60°C sometimes caused complete killing, why did the addition of acid not help, but hinder the process.

When the tests were heated to 70°C, the same trend appeared, the hydrogen peroxide alone tests gave no turbidity, the average number of turbid tubes (out of three) for the acid/H_2O_2 tests was 0.67 (S.E.M. was 0.34).

It is possible that the addition of acid could be affecting the balance between the amounts of H_2O_2 molecules and dissociated hydrogen and oxygen, but this was not apparent from the results from the pre-heated tests. It may be a laser irradiation specific problem, with the acetic acid stopping the hydrogen peroxide inactivation efficiency after laser irradiation heating. The results would suggest that if chemicals are to be used in conjunction with Nd:YAG laser, than hydrogen peroxide should be used, rather than lactic acid, acetic acid or a mixture of acid/H_2O_2.

H_2O_2 at 5% (v/v), has been used on mushrooms (Sapers and Simmons 1998), work in this field has become more topical as an alternative for chlorine based chemicals has become more desirable. The combination of laser irradiation and hydrogen peroxide could mean lower concentrations of the chemical would be needed.

The absorbency spectrum of H_2O_2, acetic acid and lactic acid were obtained in order to ascertain whether the chemical would absorb the chosen Nd:YAG laser light wavelength to a greater extent than a water control. The distilled water absorbed 1060 nm irradiation better than the test chemicals. This means that the distilled water should be slightly more effective than the acid at absorbing the Nd:YAG laser irradiation. This shows that with these chemicals chosen any synergistic effect may be due to a thermal effect, rather than an increased activity due to excitation of the molecule by the photons of irradiation. If equipment was available that would allow a more in-depth spectrum i.e. broader range, then it may have been found that a prominent peak existed that corresponded to a known laser wavelength. It would be interesting to combine laser excited chemicals, rather than laser heated, excited chemicals.

4.2 Spores of fungal species

4.2.1 Exposure of fungal spores to laser irradiation

Many fungal species, rely on aerial dispersion as the primary method of spread. This is facilitated by the mass production and release of fungal spores. These sporing bodies can come into contact with humans, often causing serious problems, i.e. skin infections. Another serious connotation of such contact is through the problem of food contamination, as the spores may land on and germinate in our food. It was this problem which was addressed in the present study.

Cheese is a particular problem, as it can act as rich nutrient source, and many fungal species can exhibit growth at 4°C, the conventional temperature for refrigeration. Two of the fungal species in this study were isolated from cheese samples at 4°C, i.e. *Penicillium* sp. and *Mucor* sp. These were examined and identified microscopically.

Research in this field is justified, as the sterilisation of fungal contaminants found on the cheese before wrapping, may increase the shelf life of wrapped cheese.

The first aspect of fungal spore killing investigated was the effect of laser irradiation treatment of liquid suspensions, **Tables 16 and 17**. This experiment was important because contamination of fruit juices is an important problem in the industry. Another aspect of this work is the decontamination of wash water or ingredient water, which may help ensure that pre- or post- processing is less likely to be a source of fungal contamination.

The Nd:YAG laser was again chosen for the liquid work, ahead of the CO_2 laser (as this wavelength is absorbed too much by the water).

Aliquots of *Penicillium* fungal spores were raised in temperature to 50, 60, 70 and 80°C, with 10J and 10Hz 15.5 mm beam diameter Nd:YAG laser irradiation and the decrease in viability recorded against and untreated control. There was a slight decrease in viability observed after the spores were raised in temperature to 50°C, (approximately a 22.5s exposure). After the aliquot was raised in temperature to 60°C however, a 1.95D decrease was observed. At 70°C, a 3.77D decrease was observed. Upon reaching 80°C, there was complete killing, log $cfuml^{-1}$ of 5.37 to 1 (log value of zero). This shows that the fungal spores were able to survive at 50°C then started to lose viability at 60°C. This is the expected trend for fungal spores. The experiment

was also carried out with the spores of *Rhizopus*, in this case there was not complete killing at 80°C, but the numbers were reduced from 1.51 x 10^4 cfuml^{-1} to 50 cfuml^{-1}. The experiment can be looked at in two ways. In terms of temperature reached, and in terms of energy density applied. If the experiment is looked at in terms of temperature, the *Penicillium* spores were killed off more readily than those of the *Rhizopus* species. An approximate 4.5D decrease was observed at 80°C as opposed to 2.5D at the same temperature. The results are less dramatic when the laser energy density applied is taken into consideration. When an average energy density of 2550 Jcm^{-2} was applied in the *Rhizopus* tests, the temperature reached 80°C. In the Penicillium tests however, a temperature of only 70°C was reached after an average energy density of 2444 Jcm^{-2}. This means that in terms of energy density applied, the 80°C *Rhizopus* test can be compared with the 70°C *Penicillium* test. A 2 sample t test however showed that there was no significant difference between the spore species at either 70 or 80°C, p = 0.44 and 0.5 respectively, 95% confidence limits.

The main aim of this section was to investigate the viability of a laser irradiation sterilisation system, capable of inactivating fungal spores from surfaces and the surface of food in particular. The next step of this study looked at the ability of laser irradiation scanning systems to clear fungal lawns. The first surface to be used as a test was sabouraud dextrose agar (SDA) plates. These provided a flat surface that also allowed good growth of the test organism. By starting with the agar surface, it was possible to determine whether the fungal spores would be affected by the laser light before moving onto the surfaces of food, and also allowed the approximate energy densities required for inactivation. It also allowed a comparison between the actual food surface and a more " *in vitro*" laboratory test that was easily repeatable.

Lawns of fungal spores were prepared and exposed to a 1kW CO_2 laser irradiation system at various scanning speeds. This was achieved by adjusting the distance between the test and a rotating mirror, the scan speed was increased by increasing the distance between the test and mirror.

The spores of *Penicillium* and *Rhizopus* were tested, **Tables 18 and 19**. There was good clearing of the spore lawn at the lower scan speeds, for both fungal species, which stayed constant as the speed increased from 197.7 to 268.3 cm^{-2}. The *Rhizopus* spores, gave a 8mm zone of clearing at the laser level, the *Penicillium* gave a clear zone of 7 mm (beam diameter = 11.5mm). The scanning ability decreased as the speed was

increased to 304 and 341 cms^{-1}. This effect was most pronounced on the *Rhizopus* test, with the clear zone decreasing to 4.5mm, this was statistically significant (p = 0.09, 95% confidence limits) the *Penicillium* test only decreased to 6mm this was not significant (p = 0.13). There was still a large diameter of clearance, at the faster scan speed for both samples, but there was a significant difference between the species, p = 0.00, this means that the scanning laser system would have to take into account, the species of fungal spores, depending on the speeds applied.

The 13W CO_2 laser irradiation scanning system was also used to show clearance of the fungal spores from agar surfaces, **Tables 20 and 21, Figures 29 and 30**. This method also proved successful in that the laser light produced a clear zone of inhibition, where the exposed fungal spores did not grow. When a scan speed of $100mms^{-1}$ was chosen, a power of only 6W was required to give a distinct stripe of inhibited growth. As the power was increased, the diameter of inhibition was also increased. Spores of *Penicillium* and *Fusarium* were scanned with laser irradiation of beam diameter of 3.5mm. At a power level 10W, there was not a significant difference between the species. The *Penicillium* spores were only cleared over a 1.3mm area, whereas the *Fusarium* spores were cleared over a 1.9mm diameter. When the spores are produced, they have a distinctive coloration, i.e. the *Fusarium* spores are red, the *Penicillium* spores are blue/green, it was thought a possibility that this may have an effect on the irradiation absorbency but this was not found.

Both of the CO_2 scanning systems had shown the ability to clear fungal spore lawns on agar plates. This was not the case with the Nd:YAG laser. Exposures were used which gave energy densities known to exceed the tolerance of the cheese. Collimated Nd:YAG laser light energy levels of 10 and 20J were used, both with a pulse repetition frequency of 10Hz, with exposures reaching a maximum of 5s. This corresponded to an energy density range of 0-442 and 0-884 Jcm^{-2}. Neither of these energy densities was able to show a clear zone of inhibition on the surface of the agar plate lawned with *Penicillium* spores.

The next step was to see whether it was possible to repeat the clearing ability of the lasers, shown on agar, onto the surface of food, **Sections 3.4.3 and 3.4.4**.

The first food surface tested was the surface of sliced fruit. The cherimoya was chosen because these fruit, when sliced, are known to be particularly susceptible to contamination by the spores of *Rhizopus* species. Slices of the fruit, artificially contaminated with spores of the fungi, were exposed to laser light from the 1 kW CO_2

laser scanning system. The same parameters were chosen which had shown to be very effective upon the spores on the surface of the agar. This time there was no clearing of the fungal lawn, not even at the slowest speed of 197.7 cms^{-1}. This effect must be due to a combination of effects including, how the CO_2 laser irradiation wavelength is absorbed by the fruit, and also the heat distribution properties of the fruit. This means that the laser scanning system is not a useful one in relation to this fruit. The Nd:YAG laser was also used in an attempt to decontaminate slices of star-fruit. Energy density levels as high as 884 Jcm^{-2} were applied to the contaminated fruit but no clearing of the *Penicillium* or *Fusarium* sp. spores was observed. This is unfortunate as this was an application with a lot of possibilities, as a lot of fruit is lost to fungal contamination, whilst being transported to its destination.

After the initial lack of success in the decontamination of the surface of artificially infected fruit, the decontamination of cheese was examined.

Two main methods were investigated, the scanning systems of the CO_2 lasers, and by exposing the cheese to Nd:YAG laser light. The Nd:YAG laser system was only capable of a very low scanning speed (used for welding), so only static exposures were used i.e. exposure to a single area.

The 1 kW CO_2 laser scanning system, did not produce any clearance of *Penicillium* spores, at speeds which gave good clearing of spores on agar. This could mean that the present system must be altered to be of a practical use in the future. The 1kW CO_2 laser did prove effective however, when a rapid, single shot was applied to the contaminated cheese, **Table 25 and Figure 23**. A 4ms exposure gave a clearance of 8 x 12mm of *Mucor* spores, with a laser beam diameter of only 11.5mm. This is only an energy density of 30Jcm^{-2}. It is interesting to note that a zone of inhibition, 12 x 16mm was obtained when an exposure of 10ms was applied. This clearance is actually greater than the diameter of the laser beam. This effect must be caused by lateral diffusion of heat, which is significant enough to cause spore inactivation.

The small 13W CO_2 laser scanning system proved to be very useful indeed, **Table 22 and Figure 32**, as it caused fungal spore inactivation at various speed and power settings. The larger 1 kW laser did give 7.4 times as high power per unit but was scanned at 19.77 times faster, so the smaller laser compared very favourably indeed.

Faster linear scans were also investigated, **Table 23**. Linear scan speeds of 125, 150 and 200mms^{-1} all gave approximately 2mm diameter of inhibition in *Penicillium* spores,

when 10W of power was used. Again this would imply that a scanning laser irradiation system would practical.

A complete scan of the surface of cheese was attempted, **Table 24**. This involved a rotating table, across which a linear scan was fired. This created a spiral scan that completely covered the test sample with successive scans, the rotational and linear speeds were co-ordinated. The rotating table was set at 20 $revmin^{-1}$, and artificially contaminated (*Mucor*) cheese was scanned. The unscanned control cheeses gave complete overgrowth of fungus. The scanned cheese however gave almost zero growth. This was achieved with light of only 2W power. The only growth observed on these cheeses was where (it is assumed) the spiral scan lines did not quite meet, i.e. growth between the lines. It is interesting to note that this growth was sparse, if the area had not been scanned, vigorous growth would have been expected in these areas, this may be due to lateral diffusion of heat from the surrounding scanned areas, which, while not sufficient to cause complete inactivation, has still had an effect on growth, by damaging the fungal spores.

The effect of UV was also examined **Table 26**, a UV source, 2 x 8W bulbs of 254nm wavelength (output 200-400μWcm^{-2}) was applied to cheese, artificially contaminated with *Penicillium* sp. spores. The fungal growth was not apparently affected until 2min of exposure. In this case, the small CO_2 laser proved more than an equal to the UV (at the levels chosen). The 13W CO_2 laser scanning system has shown itself to be adequate for the clearance of fungal spores from the surface of cheese, and research in this area is extremely valid and could prove useful to the food industry.

4.3.0 Mechanisms of laser action

4.3.1 Electron microscopy of heat-treated spores

Scanning Electron Microscopy of heat treated fungal spores gave further insight into the laser action. The cells seemed to respond to both treatments, water-bath and laser heating, with a disrupting effect (the cells were broken). This was particularly done by the laser-heated samples, where the cell destruction was quite extreme. From the laser irradiation killing of fungi experiments, it is obvious that this ablation effect is not the determining killing effect. There must be an effect shown on the cell constituents, as many undamaged spores were observed. There may be a temperature distribution effect, some areas may be at lower temperatures which can cause killing, but when the heating is more severe then the integrity of the cell is also damaged, causing further cell death and destruction. The water-bath heating (10 min), may cause a general weakening of the spore, so it basically falls apart, whereas the laser heating causes the inside of the spore to rapidly heat up, and disruption is caused by the spore bursting.

The S.E.M. analysis of heat treated *B. cereus* spores, did not show anything of note, it is known that Gram -ve bacteria show ablation after heating, but Gram +ve bacteria showed no change in structure (Ward 1997). The strong spore structure was less likely than either to show damage and this was the case; no damage was observed in any of the tests. It was felt that T.E.M. analysis would show more.

The transmission electron micrographs shown in **Section 3.5.1** are very interesting in that they give more of an insight into the method of laser killing action. The micrographs showed that both methods of heating, laser and water-bath, produced a similar cytological effect on the individual spores. There seems to be a decrease in the cortex volume caused by both heating methods. It has been previously reported (Hunnel, 1978) that heating of spores (4 min 121°C) caused the cortex to disappear followed by the core filling the cell as it swells. The micrographs therefore seem to point to a rapid thermal effect on the spores caused by laser action. The prepared samples also showed that where some spores were severely affected by the heating, others were relatively unchanged from the control. This phenomenon has also been reported after heat treatment (Hunnel, 1978). The electron micrographs therefore seem to point, primarily, to a thermal effect on the liquid suspensions.

The SEM micrographs of the laser treated spores on the surface of stainless steel may also have pointed toward a thermal killing effect. After exposure to 100W of Nd:YAG

laser irradiation, at a level that was lethal, but did not produce a charring effect, it was apparent that there was a great deal of spore destruction. The surface of the dried suspension had turned from a lumpy mass to a flatter surface full of pits, with only a few spores being visible. It was not known though how this was achieved. The spore destruction may have been due to complete breakdown of the spores, leaving only a residue of lipid behind. The micrograph showing the area of "clean steel" next to the dried suspension, does show a few spores that appeared to be surrounded by material, that would leave the circular "imprint" if the spore was removed. It could just be that the laser treated samples left "spore-prints" after the spore was destroyed. What also must be taken into consideration is that Sadoudi and Cerf (1997) found that pulsing of laser irradiation caused aerosols that could be removed by a vacuum. It is possible in this instance that the spores were ablated (10Hz for 2.5s) again leaving the circular arrangement where the spores had previously been.

The treated tests did show up a lava-like morphology. Cobb *et al* (1992) laser treated tooth enamel. They found that the surface of the tooth took on a lava-like morphology too, but on a smaller size scale. This was attributed to rapid heating (making the surface molten) and then a rapid cooling. It is possible that the spore suspension was superheated causing the molten effect.

4.4.0 Applications for the work

The results discussed have important implications towards an actual, practical system. It can be envisioned that the technology could be aimed in two directions; the surfaces of materials, or of food.

The main surfaces of materials that could be targeted are ones (perhaps obviously) that require sterility either because the object itself needs to be sterile, i.e. reamers scalpel blades, or because the surface would come into contact with sterile objects, i.e. conveyors, food wrap. A target for a new sterilisation system had been found. The new system must confer an advantage over the methods currently available. The main methods available namely UV lamps and autoclaves, do have weaknesses. UV lamps can be effective, but suffer from the problem of shadowing and also as with lasers, have the problem of spore resistance. The irradiation must come into contact with the micro-organisms to be effective. Autoclaves also have drawbacks, as discussed previously, the time to reach (and decrease from) pressure can be extensive, sharp cutting edges can be dulled by the autoclaving, and some materials must be kept dry.

Having established a target and a need (or perhaps an open niche) for a new system, how could this be achieved? The primary requirement would be to direct the laser beam with a scanning mirror, or to place the item to be sterilised into the path of the beam. Both techniques have advantages (application dependent). By scanning the irradiation, a large area can be treated, whereas the other end of the market could be targeted if small objects are placed in the beam, i.e. reamers. This would allow small bench top systems to be built. This for example could be used in dental surgeries. By incorporating a turning element into the system, the whole of the object could be exposed to the beam. By scanning the irradiation with a mirror, larger objects could be sterilised. The laser sterilisation system would again benefit from some sort of turning or rolling action placed on the test, but would be ideally suited to applications where only one side (at a time) was sterilised, i.e. food wrap, conveyor belt or table. An X-Y translational table may be useful. The results discussed showed the problem of laser treating larger bodies of metal. An energy density of 1800 Jcm^{-2} was required for a piece of stainless steel of mass 5g. It may be necessary to pre-design tables that would be required to be sterilised, i.e. the work-top could be made with a thin layer of metal on top of an insulting material, that way lateral heat loss could be minimised.

Minimal processing would involve two or more of the methods discussed in this work. UV would be an easy addition to the laser system. A lamp or lamps could be mounted before or after the laser treatment point. This would be advantageous in many ways, one reason would be to minimise the laser and UV exposures, thus increasing the sterilisation process, that would increase the productivity of the system being sterilised. The laser could also help to overcome the UV problem of shadowing, by heating the surface of the material. This could be achieved with a level of precision not found with IR lamps. The UV could also help the killing of the laser alone, as the temperature labile nature of the material to be sterilised would become less important because by decreasing the laser exposure required, the subsequent temperature increase associated with the irradiation would also be decreased. Thus temperature sensitive targets could then be targeted.

Chemical sterilisation is common in industry, but by combining chemical sterilisation with a laser sterilisation system, many problems could be overcome. By heating some chemicals, the activity can be increased. This makes the process faster, and more efficient, and the concentration of the chemicals required can be reduced, enabling further savings. It can be envisioned that by dipping or spraying smaller objects, that

the laser could be easily integrated into the chemical system. Many companies use an automated fog disinfecting system. These are commonly used in the food industry to sterilise air and surfaces that come into contact with food. This could be automated so as to allow a spray of chemical, then a lag period to allow the mist droplets to settle, then laser treatment of the surface could follow. Scattering could become a problem though if both treatments were used concurrently.

The rapid heating of the chemical provided by the laser irradiation, would have the added benefit of allowing the chemical to be stored refrigerated or at room temperature. This would save in heating costs and protect chemicals that become labile after prolonged heating.

The integration of chemicals into the laser system, would have the added advantage of nullifying problems of shadowing by flooding "nooks and crannies" with chemical.

Another possible technology currently available that laser irradiation could be combined with is pulsed light. This was briefly evaluated during this work. Xenon and deuterium lamps are currently available, incorporating Broad-Spectrum Pulsed Light (BPSL) for the sterilisation of pharmaceuticals and packaging (also water). The broad spectrum includes 254nm (UV) so it is bactericidal. One company providing these systems is Pure-Pulse Technologies Inc. They claim that in 1s, a 7-Log decrease in viability of *Pseudomonas aeruginosa* is possible, as is a 6-Log decrease in viability of *B. stearothermophilus* (although spores were not mentioned). The companies light systems are used by Johnson and Johnson vision products, for contact lenses. The laser could be incorporated as some heat may overcome shadowing, while the flash lamps with the high energy low exposure pulses could be more rapid than conventional UV lamps.

Sterilisation of food is also a viable proposition for laser sterilisation. The work presented here has shown that laser irradiation is effective against fungal spores on the surface of cheese. As a final pre-wrap treatment, this could be effective. A more obvious food application, would be to combat the presence of enteric organisms on vegetables. This problem exists because manure used as fertiliser (and from current practices can be from human sources too) can allow a considerable bio-burden of possibly dangerous organisms to contaminate the vegetables. Vegetables found in shops and labelled "ready to eat" can often contain many micro-organisms, while the consumer may not know that washing of the vegetables is still required. The

sterilisation methods described above would also apply to vegetables. A combination system situated at the end of a sorting line (before packing) could eliminate a possible hazard. Again a secondary treatment could decrease the unwanted heating effect of the laser irradiation and chemicals could aid the substantial problem of shadowing.

5.0 Conclusions and Future Work

Chapter 5 Conclusions and Future work

The original work in this thesis can be summarised as follows. Spore killing was observed with the Nd:YAG and the CO_2 lasers. The parameters for this killing were described in a manner that can be easily followed by interested parties (a criticism of previous work in this field). Not only was this achieved but the importance of the mass of the substrate was realised, original papers on laser sterilisation rarely divulge the mass of the test substance and other important parameters. An insight into the mechanism of Nd:YAG laser killing was found – indirect heating being the primary effect thus the mass of the test substance was important. Transmission Electron Microscopy was also used and this work showed that the cytological effects of Nd:YAG heating of spores was similar to those seen after more conventional treatments (waterbath heating). The effects were observed after a much shorter exposure. This again implied that thermal death was caused by the irradiation.

Another original discovery found during this work was that an additive effect was found with Nd:YAG laser and UV irradiation. This had not been shown before, and also aided the study of the Nd:YAG killing mechanism.

By combining Nd:YAG laser with chemicals, rapid killing was observed that may have important implications for development of an industrial system.

Another important industrial application could be the laser scanning of cheese with CO_2 laser irradiation. This work could branch into many other areas.

The conclusions for each section are described in more detail below.

Lasers, UV light and chemical have been used to decontaminate a range of substrates representative of a number of industrial applications. The substrates include stainless steel, glass and plastic (PTFE) agar and liquid. The applications associated with these substrate media and decontamination requirements are diverse. They include for example, reducing mould formation on cheese and fruits, to medical or dental applications with decontamination of medical tools to prothesis. **Table 27 and 28** show a brief summary of the results from this work.

Table 27 Summary of results obtained with bacterial spores

Treatment	**Conditions**				
	Stainless steel	**PTFE**	**Glass**	**Agar**	**Liquid suspension**
Laser	100W Nd:YAG ($1800Jcm^{-2}$) – 5D reduction	1000W CO_2 ($8.3\ Jcm^{-2}$) – 1D reduction	640W CO_2 ($10.6\ Jcm^{-2}$) – 1D reduction	1000W CO_2 ($800Jcm^{-2}$) – No killing Nd:YAG ($3000Jcm^{-2}$) – No killing	Nd:YAG heating to 90°C – No killing
Laser + UV	Increased kill due to UV			Increased kill due to UV	Increased kill due to UV
Laser + chemicals					1.5% H_2O_2 + Nd:YAG – 6D reduction at 60°C

Table 28 Summary of results obtained with fungal spores

Laser	Conditions			
	Agar	**Fruit**	**Cheese**	**Suspension**
Nd:YAG				$1800Jcm^{-2}$ – 2D reduction at 60°C
Scanned CO_2 (1kW)	2×10^{-4} mm^2s^{-1} (1kW, $34cms^{-1}$ scan speed)	No clearance	No clearance	
Scanned CO_2 (13W)	$190mm^2s^{-1}$ clearance (6W, $100mms^{-1}$ scan speed)	No clearance	$90mm^2s^{-1}$ clearance (5W, $100mms^{-1}$ scan speed)	

The laser irradiation did give killing on its own. From the results it was apparent that although the CO_2 laser was relatively more efficient on plastic and glass than the Nd:YAG was on stainless steel (in terms of energy density applied) the material reached its damage threshold before any more than a 1D value reduction was obtained. This obviously would limit the future applications available for this laser type although future work could involve varying the materials used. There may be different types of plastic and glass that have a higher tolerance to the irradiation. It may also be advantageous to use the CO_2 laser irradiation with stainless steel. This was not done because of the high reflectivity that this radiation has on steel, but the increased thermo-tolerance this material has would allow the efficiency of the CO_2 laser to be quantified with higher killing levels. This in turn would be useful as it would show how good the CO_2 laser would or would not be if used on metal test subjects – a direct application for this would be dental reamers.

The Nd:YAG laser was effective at sterilising spores on the surface of stainless steel, and in a relatively short exposure time (9s) compared with conventional techniques. It was noted that the exposure time required was reliant on the beam diameter used and the thickness of the steel. Future work would involve further investigations into killing

efficiencies with different beam diameters, and also investigation into the relationships between the beam diameter used, the energy density applied and the temperature reached by the test substrate. It would be useful if the correlation (if any) between the mass of the substrate and the energy density required for killing could be found.

The results in this thesis showed that UV could be used with laser irradiation, but the effect was an additive one. With this in mind, it could be envisioned that UV could be used in association with laser irradiation under a number of different working scenarios.

One aspect of the multi-factorial work that could be expanded upon was that the delay may become more important if it was changed. Any increase in the delay may have to fall within the limits acceptable for a practical system, but in terms of investigating the mechanisms of laser action on spores it could prove useful.

Another interesting aspect of future work would be to see if a combination of laser and UV could improve on UV alone for the sterilisation of a flowing water system. In this way the laser could be used to augment an existing system, rather than looking to use UV to augment a future laser system.

In this work, chemicals were also used to increase the killing with laser irradiation. Future work would involve developing this to quantify the effect on solid surfaces and possibly some foodstuffs. The incorporation of chemical into a working system is also discussed further below. In this work only a few chemical species were used as test subjects, this could be expanded upon further, hopefully finding an even more effective chemical than hydrogen peroxide.

The results obtained with food (using fungal spores) were variable, it would not be imagined that the sterilisation of sliced fruit would merit much further research, but sterilisation of the outside of fruit and vegetables may prove very productive.

The results with cheese were very encouraging. A simple set-up allowed the whole surface of the cheese to be treated, and it would useful to try and scale up such a system to treat more samples (more like a production line).

It would also be advantageous to investigate further the importance of fungal species, this could involve the treatment of wood or other surfaces that are prone to fungal infection. It may prove that a more expensive target subject could offset the higher cost of the laser. The future work for this branch of the research would also involve an investigation into the shelf life and also any possible detrimental effect on the nutritional (and taste) value of the cheese.

The future work would obviously aim towards the completion of a working, practical system. This would primarily involve laser and UV irradiation in combination. This could be designed in two ways. The first would be to direct the chosen killing methods to a single target area i.e. a combined system. This would have the advantage of making the set-up more compact and therefore more desirable. The second set-up design would be for a sequential system. If a conveyor of some sort were used, the test subject could be moved below each killing device for the required exposure. The company installing the system may require a certain throughput of their product in a day, which would dictate the system specifications that may include chemical treatments either by a fog system discussed previously or by passing the test subject through a wash bath before laser or UV exposure. The rolling effect produced by some tables/belts would have the advantage of turning the product. This would mean that the whole surface could be exposed.

Another addition to this system would be a flashlamp component. This could augment or replace the UV system. There are different types available - xenon or deuterium lamps. Both types produce a broad range of wavelengths including UV. The deuterium lamps produce more UV, so would be the preferred choice. Flashlamps can be pulsed at a high repetition rate but there is a key problem with flashlamps in that the lifetime of the flashlamp is low, which leads to high consumable costs which are unacceptable to many end users. Due to variations in the flash, "the spectral content can vary from pulse to pulse" (The book of photon tools - Oriel instruments). This could mean that the amount of UV administered per pulse could vary. If this were the case, then perhaps a certain overexposure would be needed to ensure any shortfall due to the reduced UV emission would be compensated for, reducing lamp life further.

Once the system design was complete the efficiency of the single units and the total sterilisation system could be ascertained at varying belt speeds. Investigations into the best combinations of killing components could also be made, perhaps a two component system would be better than a three component system. The future work could also find that laser and flash lamp is better for inactivating organisms than laser and UV. Once the best combination was found the system would be built and tested in a working environment. One interesting piece of work that could help to further investigate the mechanisms of laser action would be to look for laser irradiation induced stress proteins.

Some organisms such as *E. coli* produce specific sized proteins in response to the cells being exposed to unfavourable conditions such as hot, cold or UV light. These proteins convey protection against further exposure. This work would involve whole cell or protein preparations. These preparations would be boiled (denaturing the proteins) and run through SDS-PAGE gels. By using the Nd:YAG laser to heat cells, any stress proteins produced could be compared to those produced in response to conventional heating methods such as water bath heating. It would also be possible to compare the laser irradiation induced proteins with the stress proteins produced in response to exposure from UV irradiation. What this experiment would show, is whether or not the cells do respond to laser heating in a "conventional manner" by making the same heat induced stress proteins caused by water bath heating. It may be found that the laser irradiation causes the production of unique stress proteins not seen with other sterilisation methods.

Lasers that emit in UV irradiation include the Nitrogen laser that produces short duration high peak power pulses. If the Nd:YAG produced laser specific stress proteins, the UV laser may also produce specific proteins, but these may also vary due to the change in wavelength. It may be found that the UV laser may produce stress proteins similar to those found after exposure from conventional UV lamps.

It would also be interesting to investigate the killing efficiency of the UV laser. It would rely on direct UV damage as opposed to heat damage (as proposed for the Nd:YAG laser in this study). Sadoudi *et al* (1997) showed that the UV was effective for killing (using a frequency trebled Nd:YAG) but proposed ablation by the pulses as the main killing mechanism.

Electron microscopy could also be used to analyse UV laser treated spores. As discussed, the Nd:YAG laser caused typical heat-like effects within the spore. In theory, the UV laser irradiation exposed spores should not show these consequences.

6.0 References

Chapter 6 References

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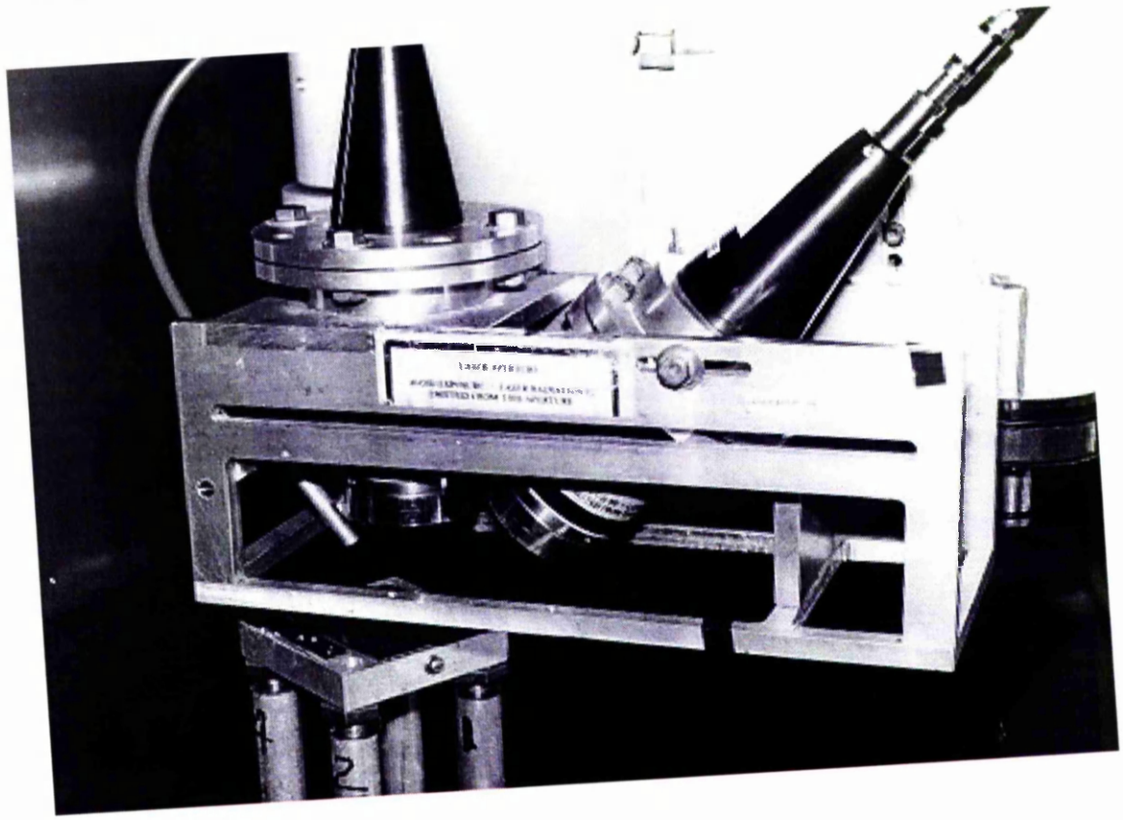
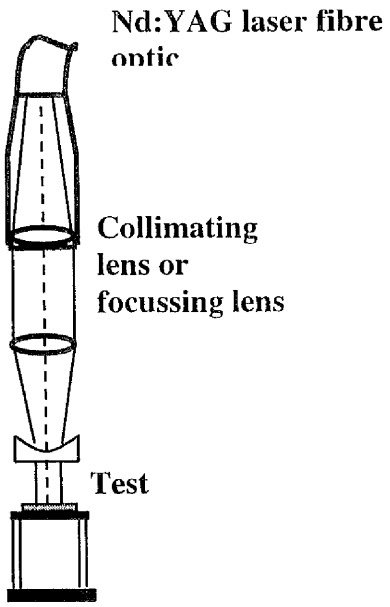
7.0 Appendices

Chapter 7 Appendices

7.1 Appendix 1 Nd:YAG laser



7.2 Appendix 2 Collimating beam assembly



Appendix 3 Introduction to lasers

Laser is an acronym for **l**ight **a**mplification by **s**timulated **e**mission of **r**adiation.

Laser irradiation varies from "normal" light sources in that it is very monochromatic, i.e. almost a single wavelength, this varies for each laser medium. Each wavelength has differing properties, i.e. absorption. The laser irradiation also shows far more unidirectionality than other light sources. This means that a laser can be aimed at a point a large distance away, with little loss of intensity and less divergence than with conventional sources. The intensity (brightness) of the beam is also far higher than that of other sources, it is this intensity which may cause the banning of laser pointers, as they may cause serious damage to the eye. The first visible light laser was made in 1960, when Maiman demonstrated his pink-ruby laser (wavelength 694.3 nm).

For the actual laser process to be possible, three basic criteria must be fulfilled - there must be an **active medium,** a state known as a **population inversion** must exist, a **feedback system** is required.

The active medium is a collection of atoms, molecules or ions that emit radiation in the optical range of electromagnetic radiation.

To explain how the active medium actually works, the process can be compared with the absorption and emission of visible light, i.e. it has been observed that ions and molecules often absorb visible light only at discrete wavelengths, while other atomic elements, when excited by atomic discharge, emit light at discrete wavelengths and not the continuous spectra.

Energy state transitions (due to simultaneous absorption or emission) can be described in probabilistic terms, i.e. some transitions and the subsequent emission or absorption are highly probable this is called an **allowed transition.** Some other transitions occur a thousand times less than these allowed transitions (sometimes not at all). These transitions are known as **forbidden transitions.**

This is the theory behind **simultaneous emission and absorption**. The lasing process relies on **stimulated emission.**

If a gas is treated with a beam of white light, some of the beam's energy can raise atoms from a **ground state** to a higher energy level. This is called **stimulated absorption** as incident radiation is required for the transition to occur. In nature, atoms will occupy the lowest possible energy level available to them. The atoms therefore release a

quantum of energy in order to "drop" down to a lower energy level again. This is known as **stimulated emission.** This quantum of energy can interact with already excited atoms. These atoms also release their excess energy but they release it, **in phase** with the stimulating radiation.. The liberated energy, stimulated by the "propagating wave" adds constructively, thus increasing the amplitude of the wave. The means the lasers beam is amplified and not attenuated. The Light Amplification by Stimulated Emission of Radiation is an integral part of the laser process.

Normally there are more atoms in the lower energy state than in the higher ones. By creating a **population inversion** this relationship can be reversed. This is fundamental to laser operation as it allows more stimulated emission to occur. The attainment of this population inversion is achieved by a process known as **laser pumping**. This involves pumping atoms indirectly, to higher energy levels. Pumping allows a build up of atoms in a higher energy state, it can also be achieved by optical discharge, electrical discharge, fuel combustion or pressure temperature cycling.

It is not enough for just a population inversion to exist, for lasing to occur. There are many energy losses that must be overcome first. This is called the threshold gain. This threshold must be overcome before laser oscillation can be great enough to provide sufficient energy for lasing. These power losses can occur, i.e. by non lasing transitions. For this reason, **a feedback system** is employed. Some lasers use a device known as a single pass light amplifier. This takes laser irradiation and amplifies it to increase the power output. This method can be used for ruby lasers and neodymium doped lasers, but most active media have too low a starting output for this single pass device. These media rely upon an **optical resonator**. This involves the use of mirrors that direct the irradiation beam back and forth through the active medium many times. This has the effect of increasing the pass length up to fifty times. This means that even if the single pass amplification is small, the total amplification after many passes can be very substantial. The increase in amplification increases until a steady level of oscillation is reached. After this level is achieved growth of the waves ceases and all additional energy supplied by the stimulated emission process then serves to maintain the laser output. The radiation inside the resonator diffracts somewhat as it passes through the active medium, this causes it to spill out past the mirrors, and be lost from the system. This is overcome by designing the resonator to contain the beam as much as possible. This is achieved by bending the mirrors inward towards the beam (making them slightly spherical and concave inward). The mirrors are aligned so that their

centres are each on a common line. **This is called the optic axis**. The pair of mirrors is positioned so that a stability condition is achieved, i.e. low losses. This means the collimated radiation is contained near the optic axis of the resonator. The most common laser resonator, employs one long radius of curvature mirror and one planar mirror. This is efficient and easy to align.

So by putting these three conditions together, the **active medium** has it's atoms pumped creating a **population inversion**, and the **resonator cavity** helps amplify the radiation up to it's optimum amount.

By replacing the planar mirror with a planar, partially reflective mirror, some of the planar, monochromatic light, passes through the mirror and can be passed along a fibre optic and this gives the actual laser beam.

Some lasers have a high gain active medium and can therefore tolerate a lot of power loss and still lase effectively. This doesn't mean that the laser is efficient, just because it has a high, steady state gain. The efficacy of the laser is determined by the ratio between the input and output energy. The CO_2 laser is one of the most efficient (5 to 35% efficient). The argon laser has a high activity but is very inefficient, (0.001-0.01%). Some other lasers are inefficient and also have a low gain.

Laser types

The CO_2 laser, is the most commonly found member of the group known as molecular gas lasers. This laser, operating in the infra red region, has a high power, high efficiency and the wavelength (10.6μm) is readily absorbed by almost all materials.

This laser does not undergo atomic transitions, it is slightly more complicated. Carbon dioxide gas molecules have three possible types of vibrational oscillations (mode). The molecule also has rotational energy for each vibrational mode. The energy levels for transitions correspond to these vibrational states.

In the CO_2 laser, molecules are pumped from a ground state to a higher level, and slow and fast transitions produce a population inversion. It should be noted that there are two metastable pathways, one causing a line at 9.6μm but the stronger line is at 10.6μm.

Nitrogen and helium are added to the CO_2 gas to improve the output (10% CO_2, 40% N_2, 50% He). The nitrogen molecules transfer energy to the CO_2 molecules by resonant collisions. The helium aids the population inversion process by speeding up the transition from the energy level that is the receiving end for the 10.6μm transition.

The high efficiency of the transitions from the 10.6 ⎵m level to the ground level state helps the CO_2 laser produce tremendous amounts of power in short pulses. Nanosecond pulses are possible.

The design of the CO_2 laser is complicated by the fact that almost everything absorbs at that wavelength, so the laser must be made of or coated with material that does not absorb. Germanium and sodium chloride are transparent at this wavelength. The high absorption means that even 20W beams can cut through metal, wood and asbestos when focussed properly.

The optical resonator has two long radius of curvature mirrors, the output mirror can made of gallium or germanium arsenide. The highly reflective mirror can be replaced by a diffraction grating to tune the wavelength around 9.6 or 10.6μm.

The CO_2 laser is often used in industry because of its all round high efficiency and effectiveness.

The **Nd:YAG** laser is a member of the doped insulator lasers. Doped insulator is a term used to describe a laser whose active medium is a regular array of atoms, such as a crystal, which has deliberately had impurity ions introduced into the crystal at the time of growth. This is the case with ruby, chromium 3+ ions give the crystal its red colour.

These lasers are often used in industry as they are rugged, simple to maintain and capable of achieving high peak required for many tasks.

In most cases the active medium of a doped insulator laser consists of an impurity or dopant in a crystalline insulator. The crystal atoms do not participate directly in the lasing action, but serve as a host lattice where the dopant resides.

The reason the crystal is made impure, is because in a pure crystal all of the energy levels are degenerate, the impurity provides multiple low lying energy levels.

The neodymium: yttrium aluminium garnet ($Y_3Al_{15}O_{12}$) or Nd:YAG laser has an active medium of neodymium 3+ ions doped into yttrium aluminium garnet. One percent of the Y^{3+} atoms are replaced by Nd^{3+}. Neodymium has an output in the infra red portion of the spectrum primarily with a wavelength of 1.06μm.

An advantage with this type of laser is that it uses a 4-level pumping scheme, so the process has a low threshold. The Nd:YAG laser uses flash-lamps to excite the ions to higher electronic states. Xenon lamps are used as they are easily cooled.

It should be noted that doped lasers do not operate with a continuous output, but as a large series of bursts of power (spikes). They are excellent for producing large bursts of power from a relatively small laser.

Appendix 4 The electromagnetic spectrum

Radiation type	Wavelength (μm)
Gamma rays	$10^{-5} - 10^{-6}$
X-rays	$5 \times 10^{-2} - 5 \times 10^{-4}$
Ultraviolet	$5 \times 10^{-2} - 0.38$
Visible light	0.38 – 0.78
Near infrared	~1
Thermal infrared	$1 - 10^{2}$
Microwave	$10^{2} - 10^{4}$
TV/Radio	$10^{4} - 10^{7}$

The visible spectrum

Colour	Wavelength (nm)
Violet/Indigo	380 - 440
Blue	440 – 490
Green	490 – 540
Yellow	540 – 600
Orange	600 – 630
Red	630 - 780

Appendix 5 Laser types

Type	Wavelength (nm)
Argon-fluoride excimer	193
Krypton-flouride excimer	249
Xenon-chloride excimer	308
Xenon-fluoride excimer	358
Organic dye (in solution)	300
Xenon-chloride excimer	308
Krypton ion	335 – 800
Nitrogen	337
Argon ion	450 – 530 (488 and 515 mainly)
Copper vapour	510, 578
Helium-neon	543, 633, 1150
Titanium sapphire	660 – 1060 (tunable)
Ruby	694
GaAlAs	750 - 900
Neodymium:YAG	1064 (frequency doubled = 532)
Hydrogen flouride	2600 - 3000
Carbon monoxide	5000 – 6000
Carbon dioxide	9000 – 11000 (10600 mainly)

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