RESEARCH ARTICLE

# Studies on the inactivation of medically important *Candida* species on agar surfaces using pulsed light

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## Abstract

Development of a pulsed-light (PL) approach to inanimate surface decontamination is timely, as the incidence of yeast-related infections in healthcare remains unacceptably high. Critical electrical and biological factors governing the efficacy of PL for the *in vitro* inactivation of medically important yeast were established in this study. Predetermined cell numbers of yeast were inoculated separately on agar plates and were flashed with $\leq$ 90 pulses of broad-spectrum light under varying operating conditions, and their inactivation was measured. Significant differences in inactivation among different yeasts occurred depending on the intensity of the applied lamp discharge energy and the amount of pulsing applied. Levels of yeast sensitivity also varied depending on the distance between the light source and the treatment surface used, and the population size, type and age of cultures treated. Yeast strains were shown to be significantly more resistant to PL irradiation compared with similarly treated bacterial control cultures. A clear relationship was observed between the concentration of eluted proteins from treated yeast and the severity of PL conditions, with scanning electron micrographs showing irreversible cellular damage. Therefore, the findings from this study will enable further development and optimization of PL as a method of decontaminating surfaces in healthcare setting.

## Introduction

Invasive fungal infections have become major causes of morbidity and mortality among immunosuppressed patients (Ellis *et al.*, 2001; Pfaller *et al.*, 2003). *Candida* species are now a frequent cause of nosocomial blood stream infections in critically ill patients in developed countries. At a teaching hospital in Taiwan, yeast infection was identified as the leading cause of nosocomial infection (Chen *et al.*, 1997). The increasing prevalence of yeast infections in healthcare highlights the need for improved decontamination approaches. Prevention of infection is a superior approach compared with the cost and consequences of treatment of infection (Solberg, 2000). Conventional surface decontamination approaches such as chemical biocides have recognized limitations that include unwanted microbial adaptation, subsequent cross-resistance to front-line antifungal agents and lingering chemical residues (Orozco *et al.*, 1998; Lambert *et al.*, 2001; Randall *et al.*, 2001; Gebel *et al.*, 2002). Numerous studies have also highlighted limitations of decontamination techniques such as conventional, continuous-wave UV mercury lamps of low and medium pressure that include microbial repair and the necessity for lengthy durations of exposure to obtain suitable levels of decontamination (Bintsis *et al.*, 2000).

While pulsed light (PL) is an approach that has received considerable attention as a strategy for decontaminating food, packaging, water and air (Dunn *et al.*, 1997; Gómez-López *et al.*, 2007), it is also a strong candidate for contact-surface decontamination in the healthcare setting. The emitted flash from the PL source has a high peak power and usually consists of wavelengths ranging from UVA to near infrared that is also rich with shorter UVC germicidal wavelengths (MacGregor *et al.*, 1998; Gómez-López *et al.*, 2007). PL is produced using techniques that multiply power manifold by storing electricity in a capacitor over relatively long times (fractions of a second) and releasing it in a short time (millionths or thousandths of a second) using sophisticated pulse compression techniques (Rowan *et al.*, 1999; Gómez-López *et al.*, 2007). PL technology has been the subject of recent review (Elmnasser *et al.*, 2007; Gómez-López *et al.*, 2007), with emphasis strongly placed on

decontamination efficacy for food and water applications that aptly reflects the focus of research in this field of study to date. A strong advantage of using pulsed xenon lamps over continuous low- to medium-pressure conventional UV lamps is that the former has a characteristic high-peak power dissipation, which allows for more rapid microbial inactivation.

While current findings from the literature suggest that development of a PL approach appears promising, most of the studies to date have focused on food or water applications using a limited range of electrophysical or biological parameters, such as the use of a single lamp discharge energy (J) or fluence (UV dose $cm^{-2}$) and/or using a single distance from the light source to the target treatment area. These PL studies have been reviewed recently for efficacy in terms of inactivating food-related spoilage organisms and potential microbial pathogens (Elmnasser *et al.*, 2007; Gómez-López *et al.*, 2007), and include studies carried out using lamp discharge energies of $3\,J\,cm^{-2}$ (MacGregor *et al.*, 1998; Rowan *et al.*, 1999), $7\,J\,cm^{-2}$ (Marquenie *et al.*, 2003; Gómez-López *et al.*, 2005a), $0.99\,J\,cm^{-2}$ (Krishnamurthy *et al.*, 2004) and $1\,J\,cm^{-2}$ (Wekhof *et al.*, 2001). To the best of our knowledge, no published study to date has investigated the *in vitro* decontamination efficacy of varying critical electrophysical parameters and biological conditions on PL-treated yeast of clinical significance that were artificially seeded on agar surfaces.

## Materials and methods

A bench-top pulsed power source (PUV-1, Samtech Ltd, Glasgow) was used to power a low-pressure (60 kPa) xenon-filled flashlamp (Heraeus Noblelight XAP type NL4006 series constructed from a clear UV-transparent quartz tube), which produced a high-intensity diverging beam of polychromatic PL, and was used in this study as per Wang *et al.* (2005). The PL has a broadband emission spectrum extending from the UV to the infrared region with a rich UV content and its intensity also depends on the level of the voltage applied. The fabricant stated that the discharge tube represents a line source of limited length and, consequently, the light formed an elliptical, equi-intensity profile over the sample plane eliminating shading effects. This resulted in an *c.* 30% variation in luminous intensity between the centre and the edge of the sample. The light source has an automatic frequency-control function that allows it to operate at $1\,pulse\,s^{-1}$ that was used throughout this study. Light exposure was homogenous as the xenon lamp measuring $9\,cm \times 0.75\,cm$ was longer than the 8.5-cm-diameter polystyrene Petri dishes used in the tests, which were placed directly below the lamp. For standard treatments (unless otherwise noted), the light source was mounted 8 cm above the treatment area that was designed specifically to accommodate a standard Petri dish and was set as the minimum or lower threshold distance by the fabricant. This was to ensure that full coverage of the agar surface occurred and to eliminate possible shading effects.

In this study, standard treatments involved surface spread-inoculating 8.5-cm Petri dishes separately with predetermined numbers of test microorganisms that were subjected to lamp discharge energies of 3.2, 7.2, 12.8 and 20 J. The number of pulses of light used ranged from 0 (untreated control) to 90 pulses. The influence of varying distance from the light source from the inoculated Petri dishes (range 8–18 cm) on inactivation efficacy was also investigated by rotating the treatment shelf in the PL system to the desired distance. The lethality of the PL process under varying experimental conditions was determined by treating predetermined numbers of test organisms that were inoculated on agar surfaces, and by enumerating survivors post-treatments (expressed in terms of $\log_{10}$ CFUs or CFU $cm^{-2}$). Subtraction of the logarithm of the count after using different combinations of treatment from the logarithm of the count before processing provided a measure of process lethality. The time dependence of the log inactivation rate using a fixed lamp discharge energy (J) at different distances between the light source and the treatment surface was also measured for all test microorganisms using the model of Kühn *et al.* (2003). These death rate inactivation plots follow a first-order dependence [$\log_{10}(N_t/N_0) = -kt$, where $k$ is the rate constant]. $N_0$ is the initial concentration of bacteria and $N_t$ is the microbial concentration after pulsing for time $t$ at discharge energy (J). This rate constant $k$ defines the sensitivity of a microorganism to a defined PL treatment and is unique to each microbial species; the higher the $k$ value the more sensitive the test strain to the treatment process. All experiments were carried out in triplicate using the same culture to avoid sample variability.

The species of test microorganisms used in these experiments, their origin, clinical relevance and corresponding selective culture media are summarized in Table 1. The nonpathogenic distillery yeast *Saccharomyces cerevisiae* and potential pathogenic bacteria *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were also included in decontamination studies for comparative purposes. All test strains were maintained in Microbank storage vials (Cruinn Diagnostic, Ireland) at $-70\,^{\circ}C$. Identification of three randomly selected isolates of each yeast strain was confirmed before and after experimental studies using of the germ-tube assay with occasional use of the VITEK yeast biochemical card and API-32C systems (bioMérieux, France) as per the methods described by Hsu *et al.* (2003), while the identity of each bacterial pathogen was confirmed using appropriate biochemical and physiological tests (including use of the biochemical galleries API 20 E and API Staph) as per the methods described by Barrow & Feltham

**Table 1.** Origin, clinical significance and cultivation temperature of test microorganisms

| Test microorganisms | Code | Origin* | Clinical significance† | BSL‡ | Growth temperature (°C) |
|---|---|---|---|---|---|
| Yeast | | | | | |
| *Candida albicans* | 6250 | NUIG | Blood | 1 | 35 |
| | 2570 | NUIG | Sputum | 1 | 35 |
| | R810 | NUIG | Sputum | 1 | 35 |
| | R854 | NUIG | Sputum | 1 | 35 |
| | D7100 | NUIG | Wound | 1 | 35 |
| | 10231 | ATCC | Bronchomycosis | 1 | 35 |
| | 90028 | ATCC | Blood | 1 | 35 |
| *Candida krusei* | 6258 | ATCC | Sputum | 1 | 35 |
| | 32672 | ATCC | Blood | 1 | 35 |
| *Candida glabrata* | 2001 | ATCC | Faeces | 1 | 35 |
| | 15545 | ATCC | Fatal septicaemia | 1 | 35 |
| *Candida tropicalis* | 750 | ATCC | Bronchomycosis | 1 | 35 |
| | 18526 | ATCC | Abscess | | |
| *Candida parapsilosis* | 10233 | ATCC | Periarthritis | 1 | 30 |
| *Saccharomyces cerevisiae* | 9763 | ATCC | None | 1 | 30 |
| Bacteria | | | | | |
| *Escherichia coli* | 25922 | ATCC | Clinical isolate | 1 | 37 |
| *Staphylococcus aureus* | 25923 | ATCC | Clinical isolate | 2 | 37 |

*Code for origin of isolates: Department of Bacteriology, National University Hospital Galway (NUIG, Ireland), American Type Culture Collection (ATCC), Rockville, MD.

†Designates clinical sample and/or symptoms from which respective test strains were isolated or associated with humans.

‡Laboratory Biosafety Level (BSL) designation of test microorganisms.

(2003) and Rowan *et al.* (2008). Strains were stored at 4 °C on agar slopes of malt extract agar (MEA; Oxoid, Basingstoke, UK) and checked monthly for purity and renewed. To prepare the test samples, yeast test strains were streaked to purity from porous beads taken from Microbank vials, and an isolated colony was then transferred to 50-mL malt extract broth adjusted to pH $5.6 \pm 0.2$ °C and cultivated with shaking at 125 oscillations $min^{-1}$ for 24–30 h at the appropriate growth temperature for each test organism (as listed in Table 1) to reach the early stationary phase. The OD was then adjusted at 640 nm to 2.0 (*c.* $10^9$ CFU $mL^{-1}$) by spectrophotometric (model UV-120-02 instrument, Shimadzu Corp., Kyoto, Japan) determination using 0.1 M phosphate-buffered saline (PBS; pH 7.2) (confirmed via an aerobic plate count). Aliquots of 0.5 mL from the $10^{-1}$ dilution of this $OD_{640\,nm}$-adjusted culture were then inoculated onto triplicate Sabouraud dextrose agar (SDA; Oxoid) and MEA plates (both adjusted to pH $5.6 \pm 0.2$ °C) using the spread plate technique and after drying for 1 h on the laboratory bench to avoid light attenuation due to PBS, plates were flashed and then incubated without further treatment at temperatures described in Table 1 for 48 h before enumeration. Care was taken to ensure that $15 \pm 0.1$ mL of molten agar was aseptically transferred by a pipette to Petri dishes, thus maintaining a standardized depth of agar for all studies. After incubation, separate colonies were enumerated and as described earlier, the limit of detection was one colony. Typical colonies of each test strain were randomly selected from respective agar plates after 24 and 48 h at 37 °C with the highest dilution, and their identity was confirmed as described above.

To determine the influence of yeast population size on the decontamination efficacy of PL, several agar media in Petri dishes were inoculated with 0.5-mL aliquots of the 10-fold dilution of 24-h $OD_{640\,nm}$-adjusted culture on MEA plates to yield initial cell populations of *c.* 3, 5, 7 and 9 $log_{10}$ CFU $cm^{-2}$. Plates were removed from the PL treatment chamber after a predetermined number of flashes and survivors were enumerated by means of the above plating method. To determine the influence of population age on the sensitivity of test strains to PL treatments, cultures were grown for 14 h (late exponential) and were spectrophotometrically adjusted to *c.* 9 $log_{10}$ CFU $mL^{-1}$ (confirmed by a plate count). Plates were then inoculated, PL treated and survivors were enumerated as per the methods described above.

Heating of the surface of agar plates inoculated with test bacteria was investigated using thermal imaging (IRI 4010, InfraRed Integrated Systems Ltd, Northampton, UK) as per the modified method of Nugent & Higginbotham (2007). Plates were positioned 8 cm away from the light source and were flashed with $\leq 90$ pulses of increasing lamp intensity, namely 3.2, 7.2, 12.8 and 20 J per pulse. An image was taken of the treated plates immediately post-treatment and examined for thermal data using relevant software (IRISYS 4000 SERIES IMAGER, v. 1.0.0.14). Each data point represents a mean

value of 12 randomly selected points over three replicate treatments.

To test the possibility that exposure of treated yeast to sunlight may repair damaged cells through a photoreactivation process, agar plates were inoculated with *c.* 7 $\log_{10}$ CFU $cm^{-2}$ of test cells and were exposed to 75, 50, 20 and 15 pulses of light at 3.2, 7.2, 12.8 and 20 J, respectively (i.e. reducing numbers by *c.* 5 log orders at each discharge energy). After treatment, the first three plates were immediately wrapped in an aluminium foil post-treatment; the other three similarly treated plates were exposed to direct sunlight by placement on the laboratory bench illuminated by sunlight for 4 h. The plates were incubated for 48 h at appropriate temperatures for each test microorganism (Table 1) as described above. The experiment was conducted in triplicate.

Damage to the yeast membrane induced by PL treatment was investigated by determining the concentration of eluted proteins released from cells treated in a 10-mL PBS suspension as per the method of Takeshita *et al.* (2003) with modifications. Treated and untreated cell suspensions were kept on ice to prevent protease activity during studies, and samples were initially centrifuged at 10 000 ***g*** for 10 min at 4 °C in order to collect the supernatant. The concentrations of eluted protein (expressed in μg $mL^{-1}$) in the supernatants were determined spectrophotometrically at 562 nm using the commercially available bovine serum albumin (BSA) protein assay kit (Pierce Chemicals). A series of BSA standards was prepared using the dilution range of 0–200 μg $mL^{-1}$ and aliquots of 150 μL of each standard and sample were separately transferred into microtitre plate wells. Thereafter, 150 μL of working reagent was added to each well and then shaken for 30 s using an automated plate shaker. Microtitre plates were then incubated at 37 °C for 2 h before cooling to room temperature in order to determine $A_{562\,nm}$. A standard curve of blank, corrected for 562 nm readings for each BSA standard vs. concentration of sample (μg $mL^{-1}$), was used to determine eluted protein levels for each sample. Samples were run in triplicate.

Scanning electron micrograph (SEM) images of untreated and PL-treated yeast cells were obtained by adopting the method of Beveridge *et al.* (2004). Samples were viewed using a Philips 500 SEM. Student's *t*-tests and ANOVA balanced model (MINITAB software release 13; Minitab Inc., State College, PA) were used to compare the effects of the relationship of independent variables on light treatments.

## Results

### Sensitivity of test yeast to PL treatments

There were marked variations in the sensitivity to treatments among species of yeast treated with PL irradiation.

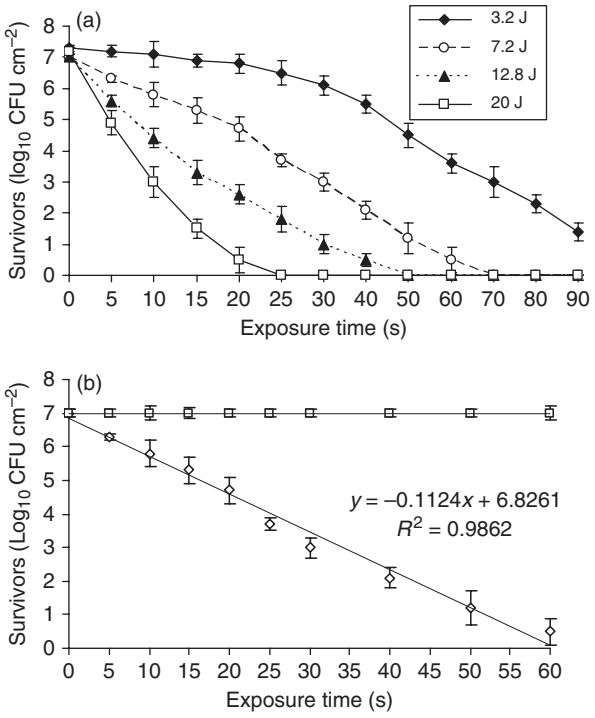


**Fig. 1.** (a) Inactivation kinetic data plot of *Candida albicans* 90028 after pulsing at an 8-cm distance from the light source using lamp discharge energies of 3.2, 7.2, 12.8 and 20 J. (b) Determination of the inactivation rate constant *k* value for *C. albicans 90028* after pulsing at 8 cm at 12.8 J. (□) untreated control. Bars indicate ± SD.

Greater susceptibility of yeast to PL treatment occurred with the use of increasing discharge energies, which is clearly illustrated for *Candida albicans* 90028 treated on SDA surfaces (Fig. 1a). All yeasts produced similar inactivation plots when exposed to increasing lamp discharge energies, and a clear pattern emerged where yeast treated at the lowest discharge energy of 3.2 J exhibited a sigmoid-shaped data plot with a pronounced initial shoulder effect. However, use of more intense discharge energies (≥7.2 J) produced linearized kinetic data for all yeasts tested with a marked absence of the initial shoulder or resistance phase. Interestingly, the Gram-negative bacterial control strain *E. coli* 25922 did not exhibit a shoulder effect irrespective of the intensity of discharge energy applied. Determination of the corresponding death rate constant *k* value of 0.11 for inactivation data generated at the lamp discharge energy of 12.8 J for *C. albicans* 90028 (shown in Fig. 1a) is illustrated in Fig. 1b, where the slope (or *k* value) of the linearized data plot provides a measure of the log reduction in cell population ($\log_{10}$ CFU $cm^{-2}$) per pulse or second of exposure. The lower the *k* value, the greater the resistance of the treated yeast to the applied PL treatment, and this means of evaluating *in vitro* inactivation performance was used to

**Table 2.** Comparison of inactivation rate constant *k* values for test microorganisms obtained at different lamp discharge energies where samples were treated at an 8-cm distance from the light source

| | | Reduction rate (*k*) at different discharge energies (J)* | | | |
|---|---|---|---|---|---|
| Test microorganism | Code | 3.2 | 7.2 | 12.8 | 20 |
| Yeast | | | | | |
| *Candida albicans* | 6250 | 0.09 ± 0.02 B | 0.16 ± 0.02 C | 0.32 ± 0.02 D | 0.37 ± 0.03 D |
| | 2574 | 0.05 ± 0.01 A | 0.18 ± 0.02 C | 0.34 ± 0.04 D | 0.38 ± 0.02 D |
| | R810 | 0.05 ± 0.01 A | 0.10 ± 0.02 B | 0.25 ± 0.01 C | 0.35 ± 0.03 D |
| | R854 | 0.05 ± 0.02 A | 0.11 ± 0.01 B | 0.24 ± 0.03 C | 0.43 ± 0.01 E |
| | D7100 | 0.09 ± 0.02 B | 0.15 ± 0.01 C | 0.29 ± 0.01 D | 0.45 ± 0.03 E |
| | 10231 | 0.09 ± 0.01 B | 0.15 ± 0.01 C | 0.30 ± 0.01 D | 0.45 ± 0.02 E |
| | 90028 | 0.05 ± 0.01 A | 0.11 ± 0.02 B | 0.25 ± 0.01 C | 0.39 ± 0.01 D |
| *Candida krusei* | 6258 | 0.03 ± 0.01 A | 0.09 ± 0.02 B | 0.21 ± 0.03 C | 0.30 ± 0.03 D |
| | 32672 | 0.03 ± 0.02 A | 0.08 ± 0.03 B | 0.22 ± 0.04 C | 0.31 ± 0.02 D |
| *Candida glabrata* | 2001 | 0.10 ± 0.03 B | 0.19 ± 0.04 C | 0.35 ± 0.03 D | 0.40 ± 0.05 E |
| | 15545 | 0.08 ± 0.01 B | 0.18 ± 0.03 C | 0.33 ± 0.02 D | 0.44 ± 0.03 E |
| *Candida tropicalis* | 750 | 0.09 ± 0.01 B | 0.20 ± 0.01 C | 0.33 ± 0.03 D | 0.47 ± 0.03 E |
| | 18526 | 0.10 ± 0.03 B | 0.22 ± 0.03 C | 0.38 ± 0.03 D | 0.50 ± 0.02 E |
| *Candida parapsilosis* | 10233 | 0.09 ± 0.01 B | 0.17 ± 0.03 C | 0.29 ± 0.01 D | 0.44 ± 0.01 E |
| *Saccharomyces cerevisiae* | 9763 | 0.07 ± 0.00 B | 0.20 ± 0.02 C | 0.35 ± 0.04 D | 0.48 ± 0.04 E |
| Gram-negative bacteria | | | | | |
| *Escherichia coli* | 25922 | 0.19 ± 0.02 C | 0.52 ± 0.04 F | 1.41 ± 0.03 I | 1.61 ± 0.05 J |
| *Staphylococcus aureus* | 25923 | 0.12 ± 0.01 B | 0.38 ± 0.03 D | 0.90 ± 0.02 G | 1.29 ± 0.03 H |

*Inactivation kinetic (*k*) data.
Values with different capitalized letters are significantly different at $P \leq 0.05$.

compare the sensitivities of all clinical yeasts in similar studies using increasing lamp discharge energies (Table 2).

A clear pattern was observed where more rapid reductions in predetermined populations of all test yeasts occurred with increased levels of pulsing with the use of higher discharge energies (Table 2). For instance, rapid reductions in all test microorganisms (*c.* 6 log orders) occurred in $\leq$ 15 pulses at 20 J on inoculated agar surfaces compared with a requirement for use of *c.* 90 pulses to attain the same level of *in vitro* inactivation for these test pathogens at the lower 3.2 J (Fig. 1a). The levels of susceptibility of test microorganisms to PL treatments differed significantly ($P < 0.05$) and are listed in order of decreasing resistance: *Candida krusei* > *C. albicans*, *Candida glabrata*, *Candida tropicalis*, *Candida parapsilosis*, *S. cerevisiae* > *S. aureus* > *E. coli* (Table 2). While there were marked variations in the susceptibility of individual strains of *C. albicans* to PL treatments (Table 2), no significant difference in sensitivity was observed between *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. parapsilosis* and *S. cerevisiae*. The marked difference in the sensitivity of test yeast and bacteria to PL exposure at 7.2 J is highlighted in Fig. 2.

The influence of the relative position between the test sample and the PL source on decontamination efficacy was also investigated. A clear pattern was observed, where yeasts treated at a shorter distance from the treatment surface to the PL light source were more sensitive to this decontamination approach. Figure 3 shows that while *C. albicans* 90028 is less sensitive to PL exposure at 7.2 J over a continuum of

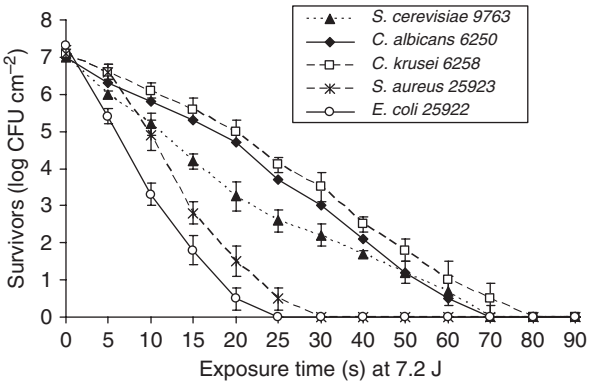


**Fig. 2.** Comparison of inactivation plots (expressed in $\log_{10}$ CFU cm$^{-2}$) of test yeast and control bacteria flashed with 12.8 J at an 8 cm distance from the light source. Bars indicate ± SD.

distances from the light source compared with similarly treated *E. coli* cells, both test cultures appear less sensitive when treated at greater distances from the PL system. In general, it took approximately four times as many pulses to achieve the same level of yeast inactivation at 18 cm as it did for similar studies carried out at 8 cm from the light source.

## Influence of microbial population size and age on decontamination efficacy

Test yeast may react differently to PL exposure depending on the population size and stage in the growth cycle. Figure 4

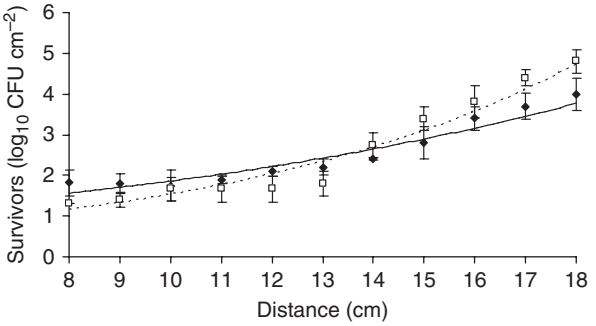


**Fig. 3.** Effect of the distance between the lamp source and the surface of spread-inoculated Petri dishes on the inactivation of *Candida albicans* 90028 (♦) and *Escherichia coli* 411 (□) at a lamp discharge energy of 12.8 J. Bars indicate ± SD. The solid line denotes the trend line.

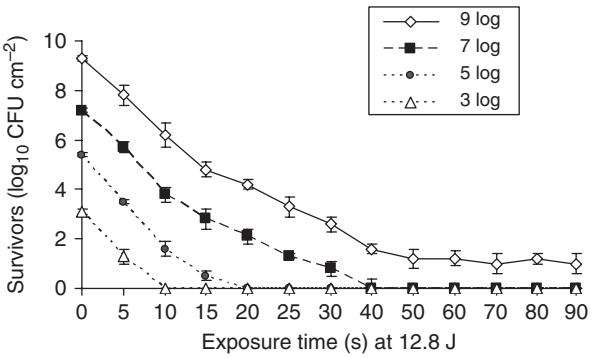


**Fig. 4.** Effect of initial population size (expressed in $\log_{10}$ CFU cm$^{-2}$) and pulse number on the inactivation of *Candida albicans* 6250 using a lamp discharge energy of 12.8 J at an 8-cm distance from a xenon light source. Bars indicate ± SD.

illustrates changes in the level of inactivation as a function of the initial number of *C. albicans* 6250 cells present on the surface of agar that were flashed using a lamp discharge energy of 12.8 J. A clear pattern emerged where the lower the initial starting cell population treated, the shorter the time taken to achieve surface sterilization. However, the rate of microbial inactivation was similar for the differently started cell populations investigated in this study. Inactivation data for yeast treated using the highest population size in this study (*c.* 9 $\log_{10}$ CFU cm$^{-2}$) resulted in a distinct tailing or a possible tolerance effect. Figure 5 shows that *C. albicans* 10231 was more sensitive to the lethal action of PL treatment at 12.8 J during the exponential (14 h) phase of growth compared with similarly treated 24-h cultures enumerated on SDA plates.

## Influence of possible microbial photoreactivation repair and surface heating on PL decontamination efficacy

To test the possibility that photoreactivation occurs, inoculated plates of *C. glabrata* 2001 were separately pulsed at 3.2, 7.2, 12. 8 and 20 J for 75, 50, 20 and 10 s, respectively, and were then exposed to 4 h of sunlight. Treated control plates (wrapped in an aluminium foil for an equivalent 4-h photoreactivation period) were incubated and enumerated post-treatment. A clear pattern emerged where photoreactivation was evident from samples treated at the lower discharge energy of 3.2 J, while no photoreactivation was observed using lamp discharge energies of ≥ 7.2 J (Fig. 6). Photoreactivation relates to the ability of PL-treated microorganisms to enzymatically repair molecular damage to cells using sunlight. Interestingly, the inclusion of organic matter (i.e. use of foetal calf serum at ≤ 20% w/v) in inoculated agar surface samples did not affect the *in vitro* inactivation performance of PL treatment nor did it impact on photoreactivation studies described above (data not shown) ($P < 0.05$).

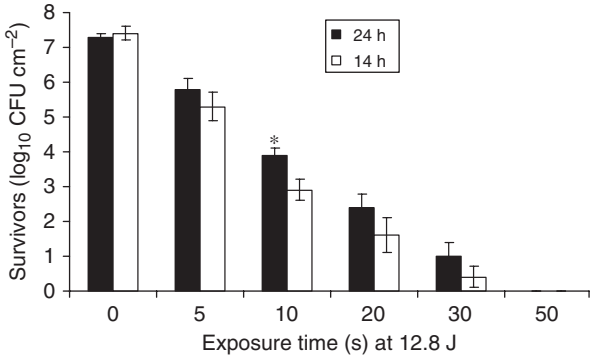


**Fig. 5.** Effect of population age on the sensitivity of *Candida albicans* 10231 to the lethal action of PL at 12.8 J at 8 cm from light sources. (■) 24-h and (□) 14-h cultures. Significant differences found in the data are highlighted with * ($P < 0.05$). Bars indicate ± SD.

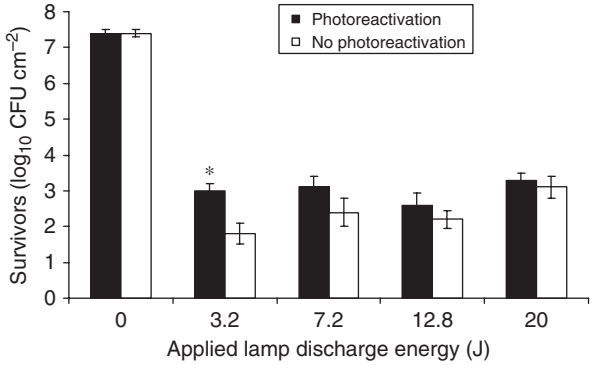


**Fig. 6.** Effect of photoreactivation and repair for *Candida glabrata* 2001 treated using different lamp discharge energies at an 8-cm distance from the light source. Reductions in populations expressed in $\log_{10}$ CFU cm$^{-2}$ with (■) and without (□) provision for conditions inducive to photoreactivation. Significant differences found in data are highlighted with * ($P < 0.05$). Bars indicate ± SD.

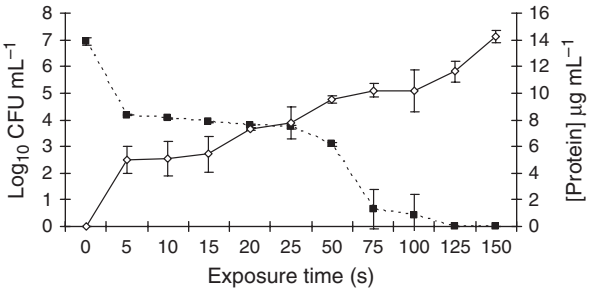


**Fig. 7.** Relationship between eluted protein concentration (■) (expressed in µg mL$^{-1}$) and $\log_{10}$ reductions in the population of *Candida albicans* 90028 (◇) suspended in PBS using a lamp discharge energy of 7.2 J at an 8-cm distance from the light source. Bars indicate ± SD.

Because of the presence of visible and infrared light (together with UV light) in the pulsed spectrum emitted by the xenon lamp, test samples and surrounding surface media can warm up during flashing. A significant change in surface media temperature can contribute in part to inactivation performance achieved by the PL system. Findings from this study showed that limiting the pulse number to ≤ 90, 80, 60 and 40 pulses at lamp discharge energies of 3.2, 7.2, 12.8 and 20 J, respectively, did not significantly alter the surface temperature beyond 4.6 ± 0.2 °C. The possible contributing role of a photothermal effect on the cellular destruction of treated yeast is described below.

### Mechanisms of cellular destruction induced by PL irradiation

SEMs of *C. albicans* 90028 cells treated at 20 J for 90 pulses revealed irreversible cellular damage. This is in agreement with findings from tandem protein release assay investigations, which revealed that increasing amounts of proteins were eluted into the surrounding aqueous PBS solution in parallel with increased durations of exposure to light flashing of *C. albicans* 90028 at 7.2 J (Fig. 7).

## Discussion

This present study demonstrated that varying electrical and operational factors could significantly affect the decontamination efficacy of PL against a range of medically important yeasts that were artificially seeded and subsequently treated on agar surfaces. These findings corroborate and further augment developments in this particular field of nonthermal processing technologies, where other researchers have reported on the efficacy of using fixed single operational parameters from PL sources to kill nonclinically significant yeast on food surfaces and packaging. For example, the food-spoilage yeasts *Candida lambica* and *Rhodotorula mucilaginosa* isolated from mixed lettuce at 7 °C were reported to be reduced by 3 log on agar media after PL treatment using a lamp discharge energy of 7 J (Gómez-López *et al.*, 2005), while Fine & Gervais (2004) compared the effects of food-spoilage yeast *S. cerevisiae* seeded on black pepper and wheat flour, and noted that rapid modification of product colour occurred before the microbial decontamination threshold was achieved: this finding was more rapid for black pepper than for wheat flour. The authors attributed this PL-induced colour modification to overheating combined with an oxidation process, and also explained that dark products absorb more light energy than pale products, which may account for differences in the colour change observed. While no other comparative study exists in the literature on the comparative sensitivity of medically important *Candida* species to UV irradiation, other researchers have reported that each *Candida* species has a different degree of susceptibility to common antifungal agents, with *C. krusei* exhibiting stronger resistance to latter lethal stress (Piemonte *et al.*, 1996; Orozco *et al.*, 1998). Interestingly, this particular *Candida* species was also shown to be the most resistant of all the yeasts challenged to PL exposure in this study.

This study demonstrated that rapid *in vitro* reductions in populations of *Candida* spp. and in control test bacteria occurred using the combination of a high lamp discharge intensity (20 J), greater number of pulses and at shorter distances from the light source (8 cm) to the treatment agar surface. In contrast, it took on average four times the number of light pulses to kill similar levels of seeded test microorganisms if lower lamp discharge energies (e.g. 3.2 J) were used at a greater distance (18 cm) from the treatment surfaces. The latter findings were expected because the intensity of the light is inversely proportional to the square of the distance from the light source (Beveridge *et al.*, 2004). Although all test microorganisms were reduced to nondetectable levels using the full spectrum of test PL parameters (*c.* 7 log order reduction in cell numbers), pronounced shoulder and tailing sections in death rate kinetic plots were evident for many *Candida* species (and for the Gram-positive test bacterium) using lower levels of pulsing at weaker lamp discharge energies. This finding is in agreement with reports from previous researchers who demonstrated that the initial shoulder effect can be possibly attributed to a sublethal injury phase and once the maximum amount of injury has been surpassed, minimal additional UV exposure would be lethal for food-related and potential pathogenic microorganisms with commensurate rapid reductions in survivor numbers (Gómez-López *et al.*, 2007). This tailing effect was also noted by other researchers investigating the efficacy of continuous UV light (Elmnasser *et al.*, 2007; Gómez-López *et al.*, 2007). Previous researchers have put forward several explanations to account for this tailing phase that include lack of a homogenous population, multi-hit killing phenomena, varying abilities of cells to repair DNA damage and shading effect that may have been

produced by the edge of the Petri dishes used in some experiments (Yaun *et al.*, 2003). That said, complete inactivation of microorganisms and absence of tailing have also been reported (Otaki *et al.*, 2003; Wang *et al.*, 2005); however, Gómez-López *et al.* (2007) noted in their review of these findings that the effect on the detection limit of the enumeration method should have been better assessed. However, from a combined clinical practice and operational viewpoint, the effectiveness of using PL will be limited to treating soiled surfaces contaminated with microbial populations at *c.* $\leq 10^5$ CFU cm$^{-2}$. Previous published studies investigating the thoroughness of terminal disinfection and cleaning in a patient's immediate environment revealed marked variations in decontamination efficacy depending on the object category (Carling *et al.*, 2008). While the latter authors did not provide data on specific microbiological quality levels for each object surface sampled, this study corroborated other related work highlighting the need for improved cleaning or use of other decontamination practices in critical areas such as oncologic hospital departments (Floridia *et al.*, 1999), while Perdelli *et al.* (2006) recently revealed that the concentration of fungi in the clinical environment varied depending on the location of sampling: the lowest mean values ($12 \pm 14$ CFU m$^{-3}$) were recorded in operating theatres and the highest ($45 \pm 37$ CFU m$^{-3}$) were in kitchens. However, these studies did not discuss the possibility that surfaces can occasionally become contaminated with patient body fluids or faeces at microbiological levels (i.e. $\geq 10^5$ CFU cm$^{-3}$) beyond the capability of PL to surface sterilize properly. In such situations, use of conventional chemical biocide disinfection practices would be the preferred option over the use of PL.

Interestingly, the Gram-negative *E. coli* test organism was devoid of a shoulder effect in PL-induced inactivation data plots and was more susceptible to lethal action of PL compared with other test microorganisms investigated. The latter finding is in agreement with Anderson *et al.* (2000), who also reported that Gram-negative spoilage and potential bacterial pathogens are more susceptible to PL irradiation than are Gram-positive bacteria.

Microorganisms treated in their exponential growth phase were shown to be more susceptible to PL-decontamination treatments compared with older cultures. This is possibly attributed in part to the greater likelihood of inflicting irreversible damage to critical genetic information that governs key metabolic pathways (such as single-strand breakages and formation of pyrimidine dimers) along with damaging vital membranes, proteins and other macromolecules, where there is an increased demand for these activities during the exponential phase of microbial growth. Without sufficient repair mechanisms, such damage will result in irreversible events culminating in microbial cell death. Previous researchers have demonstrated that microorganisms treated when growing actively are more susceptible to a range of conventional and novel food-processing conditions (Anderson *et al.*, 2000; Elmnasser *et al.*, 2007). Considerable research has been performed to elucidate the different mechanisms underpinning microbial inactivation by PL, all of them emphasizing the pivotal role of the UV component of the pulsed spectrum along with intimating other minor photochemical and photothermal effects (Anderson *et al.*, 2000; Wekhof *et al.*, 2001; Takeshita *et al.*, 2003; Wuytack *et al.*, 2003; Wang *et al.*, 2005). Moreover, Takeshita *et al.* (2003) and Wang *et al.* (2005) demonstrated that no microbial inactivation occurs if a filter is included to remove the UV wavelength region $< 320$ nm. Elmnasser *et al.* (2007) attributed the holistic cidal effects of PL decontamination to the rich UVC content, its short pulse duration, high peak power and the ability to regulate both the pulse duration and the frequency output of the flash lamps.

While it was not feasible to implement the full battery of sophisticated testing to elucidate the exact cascade of molecular and cellular mechanism(s) underpinning both photochemical and photothermal effects attributed to PL killing of medically important yeast, limited studies were carried out to determine the impact of PL treatments on yeast cellular structure and possible loss of critical intracellular proteins due to associated permeabilization of the cellular membrane. The findings of this study corroborated related work carried out previously by Takeshita *et al.* (2003), who compared the inactivation of *S. cerevisiae* cells by PL and also found that the concentrations of eluted protein from yeast cells after PL treatment were higher than those observed under continuous UV treatments. These authors also attributed this effect in part to irreversible cell membrane damage induced by PL treatment. Takeshita *et al.* (2003) specifically revealed distinct structural changes that included expanded vacuoles, cell membrane distortion and a change to circular morphological shape, which were attributed to a PL-mediated photothermal effect. This is in marked contrast to similar samples that were treated with continuous UV light, where *S. cerevisiae* cells were almost the same as in the case of nontreated cells. The results reported by Takeshita *et al.* (2003) also supported violation of the principle of Bensen–Roscoe, which infers that the total number of photons delivered is the important factor for cidal action and not the number of photons delivered per unit time (peak power). For example, the authors compared the effect of peak power on *S. cerevisiae* cells using 4655 and 2473 kW and found that a greater killing effect and a higher concentration of eluted protein occurred under high peak power conditions.

Although the increase in the surface temperature of PL-treated SDA plates seeded with *C. albicans* 90028 did not exceed $4.6 \pm 0.2\,^{\circ}$C after full treatment regimes in the present study, localized overheating of the internal cellular

constituents cannot be dismissed. For example, Wekhof (2000) proposed that with a fluence exceeding 0.5 J cm$^{-2}$, the disinfection is achieved through rupture of the bacteria during their momentous overheating caused by absorption of all UV light from a flash lamp. Wekhof *et al.* (2001) later illustrated electron-micrographs of deformed and ruptured PL-treated *Aspergillus niger* spores that provided evidence of the escape of overheated contents of these fungal spores, which would appear to agree with the scanning electron microscopy images showing structure changes occurring in *C. albicans* 90028 cells as a consequence of the PL treatment. Interestingly, the ability of treated *Candida* species to elicit repair post-exposure to PL treatments was shown to be negligible using lamp discharge energies $> 3.2$ J. The ability of microbial organisms to photorepair molecular damage induced by PL exposure at 3.2 J possibly relates to the fact that treated cells were not lysed using this lamp discharge energy, and therefore capable of subsequent recovery when exposed to sunlight. This is in agreement with findings from other researchers who observed the absence of a photoreactivation effect in food spoilage microorganisms using PL approaches and concurrently reported on the occurrence of this repair effect in similar cultures exposed to conventional low-pressure lamps (Elmnasser *et al.*, 2007; Gómez-López *et al.*, 2007). Thus, greater information on the mechanistic destruction of yeast induced by PL exposure compared with less resistant bacterial pathogens is merited, and conducting a range of detailed holistic investigations such as focusing on clonogenic damage (such as the Comet assay), possible cellular apoptosis (such as Annex V staining and Tunnel assay) and on elucidating the potential involvement reactive oxygen species on microbial lethality are important.

While key electrical and biological factors affecting the efficacy of PL for inactivation medically important yeast (with emphasis on *Candida* spp.) were identified in this study, it must also be stated that the use of PL enriched in UV has operational limitations due to its potential detrimental effects on skin tissue and components of the eye. While such operational issues can be addressed, other research groups are alternatively exploring the potential of photodynamically inactivating bacterial pathogens using visible light at 400–420 nm (Ashkenazi *et al.*, 2003; Ganz *et al.*, 2005; Lukšiene, 2005). The mechanism of cellular destruction attributed to photosensitization involves using visible light where the blue wavelength component photoactivates endogenous porphyrins (photosensitizers) that accumulate within the bacterial cells in an oxygen-dependent process that ultimately kills the illuminated cells. However, a comparative analysis of PL studies (Wang *et al.*, 2005) with that using visible light at 400–420 nm (Guffey & Wilborn, 2006) for surface decontamination suggests that the latter photosensitization approach may require lengthy durations of exposure to achieve parity in terms of similar microbial inactivation performances. Because of the energy intensity associated with the use of visible light approaches, other critical factors such as soiling of surface with organic matter impacting on decontamination efficacy merit consideration due to possible limited penetrability. It must be recognized that operational use of both PL and visible light approaches for decontamination is limited to treating two-dimensional surfaces due to protective shading effects.

While the findings from the study demonstrate the effectiveness of using a xenon PL approach for the irreversible inactivation of medically important yeast, future studies investigating the efficacy of treating yeast on typical surfaces available in healthcare under a variety of abuse conditions (such as varying relative humidity and duration of exposure). Studies on elucidating the possible contributing role of microorganisms embedded in surface biofilms vs. less complicated planktonic microenvironments on PL decontamination efficacy of PL irradiation are also required. Findings from this present study will advance the field of nonthermal processing technologies.

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