Lethal effects of high intensity violet 405-nm light on Saccharomyces cerevisiae, Candida albicans and on dormant and germinating spores of Aspergillus niger

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Abstract

This study assessed the effects of high intensity violet light on selected yeast and mould fungi. Cell suspensions of Saccharomyces cerevisiae, Candida albicans and dormant and germinating spores (conidia) of the mould Aspergillus niger were exposed to high intensity narrow band violet light with peak output at 405 nm generated from a light emitting diode (LED) array. All three fungal species were inactivated by the 405nm light without a requirement for addition of exogenous photosensitiser chemicals. Of the fungal species tested, S. cerevisiae was most sensitive and dormant conidia of A. niger were most resistant to 405nm light exposure. Five $\log_{10}$ CFUml^{-1} reductions of the tested species required exposure doses of 288 J cm^{-2} for S. cereviaiae, 576 J cm^{-2} for C. albicans and a much higher value of 2.3 kJ cm $^{-2}$ for dormant conidia of A. niger. During germination, A. niger conidia became more sensitive to 405 nm light exposure and sensitivity increased as germination progressed over an 8 hour test period. Light exposure under aerobic and anaerobic conditions, together with results obtained using ascorbic acid as a scavenger of reactive oxygen species, revealed that 405-nm light inactivation in fungi involved an oxygen-dependent mechanism, as previously described in bacteria. The inactivation results achieved with yeast cells and fungal spores together with operational advantages associated with the use of a visible (non UV) light source highlights the potential of 405-nm light for fungal decontamination applications.

1. Introduction

There have been extensive studies carried out on the effects of light on the biology of fungal organisms with numerous reports published on effects on growth, metabolism and differentiation. There has also been considerable interest in the application of light-based technologies, using specific light wavelengths and intensities, for the inactivation and control of problematic fungal organisms. Ultraviolet light (UV) in particular has been extensively studied as an inactivation technology for destruction of pathogenic and spoilage microorganisms including fungi and yeasts (Begum et al. 2009). Although UV-light can be effective, it has limitations as a decontamination technology due to its low penetration into opaque liquids and solids, photodegradation of plastics, and human exposure safety issues (Elmnasser et al., 2007).. These limitations associated with UV-light radiation create potential opportunities for alternative light-based decontamination technologies that are safer to use and cause less degradation of photosensitive materials.

Novel technologies utilising visible wavelengths of light, most notably in the violet/blue region of the electromagnetic spectrum that induce so-called photodynamic inactivation (PDI) of microorganisms have been developed. Traditionally PDI has involved the use of dyes and other exogenous photosensitiser molecules coupled with light exposure to induce inactivation, but more recently natural photosensitiser molecules, particularly porphyrins endogenous within microbial cells have been targeted. Exposure of microorganisms to visible light particularly at wavelengths of 405 nm, has been shown to be effective in inactivating a range of bacteria, including Gram positive and Gram negative bacterial species and antibiotic resistant microorganisms such as Methicillin-resistant Staphylococcus

aureus, and its use has been suggested for a range of medical and environmental decontamination applications (Guffey & Wilborn 2006; Enwemeka et al. 2008; Maclean et al. 2008a; Maclean et al. 2009; Maclean et al, 2010; Murdoch et al. 2012, Dai et al, 2012; Dai et al, 2013). However use of 405-nm light for inactivation of fungal organisms and the inactivation process involved has not been previously reported.

In prokaryotes, 405-nm light inactivation has been shown to be an oxygen-dependent process that is thought to involve absorption of the 405-nm light by endogenous porphyrin molecules. Light at 405-nm wavelength excites these molecules and leads to transfer of energy. This induces generation of highly reactive singlet oxygen ($^1O_2$) and other reactive oxygen species (ROS) that cause cellular damage and ultimately cell death (Hamblin & Hasan, 2004; Maclean et al. 2008b; Lipovsky et al. 2010). Studies into broadband light inactivation of fungi have shown the sites of damage caused by singlet oxygen are typically the plasma membrane and mitochondria (Donnelly et al. 2008). This is likely to be where endogenous porphyrins reside in fungi as singlet oxygen can only diffuse a short distance and has a relatively short life-span (10^{-6} s) (Bertoloni et al. 1987; Dougherty et al. 1998; Kalka et al. 2000).

Fungi in general are considered to be more difficult to inactivate than bacteria by some decontamination procedures because of their larger size and complex structures. UV light studies have shown that fungal spores in particular require higher UV light dosage to achieve the same level of inactivation seen in bacterial cells. A study by Anderson et al. (2000) achieved 7-8-$\log_{10}$ reductions in Escherichia coli, Salmonella enteritidis, and Bacillus cereus cells upon exposure to 1000 pulses of high-UV

content broadband light, whereas fungal spores from Fusarium culmorum and Aspergillus niger were only inactivated by 3-4-$\log_{10}$ after treatment. Fungi are also protected from external ROS-induced damage by a thick cell wall containing components such as chitin, mannan, glucan, and various lipids (Donnelly et al. 2008). In addition to the thick outer wall, fungi have a plasma membrane separated from the cell wall by a periplasmic space. This reduces the diffusion of photodynamic sensitising agents, used in PDI, into cells thereby limiting singlet oxygen cell damage.

The present study sets out to assess the effects of 405-nm light, without the addition of exogenous photosensitisers, on cells of S. cerevisiae and C. albicans and on dormant and germinating conidia of A. niger. These fungal species were chosen due to their recognised significance as test organisms in scientific studies and because of their applied importance in medical, industrial and food mycology (Straus, 2009; Mean et al. 2008; Luksiene et al. 2004; Blacketer et al. 1993). This study also aims to determine whether the 405-nm light inactivation of fungi involves an oxygen-dependant mechanism as has been established in similar studies on bacterial species.

2. Materials and Methods

2.1 Yeast Preparation

Saccharomyces cerevisiae MUCL 28749 and Candida albicans MUCL 29903 cultures were obtained from the Mycotheque de l'Universite catholique de Louvain (MUCL) (part of The Belgian Co-ordinated Collections of Microorganisms (BCCM)). Saccharomyces cerevisiae and C. albicans were inoculated into 100 ml Malt Extract Broth and Malt Extract Broth with 0.1 % yeast extract, respectively (Oxoid, UK). After incubation for 18-24 hours at 30°C and 37°C for S. cerevisiae and C. albicans,

respectively, broths were centrifuged at 3939 × g for 10 minutes and the resultant cell pellet was re-suspended in 100 ml phosphate buffered saline (PBS; Oxoid, UK). Yeast suspensions were serially diluted to give an approximate starting population of ~2.0×10^5 CFU ml^{-1} for experimental use.

2.2 Aspergillus niger Spore and Mycelia Preparations

Aspergillus niger MUCL 38993 was obtained from the Mycotheque de l'Universite catholique de Louvain (MUCL). Aspergillus niger spores were inoculated onto Malt Extract Agar slopes and grown at 30°C for five days to produce sufficient conidial production (spore production) for use in experimentation. Slopes were flooded with PBS containing one drop of Tween 80 (Sigma, UK) (added to prevent aggregation of spores) and the end of a plastic L-shaped spreader was used to gently remove spores from the surface of the agar slope. One-ml of the resultant solution was then pipetted into 9 ml of PBS before the spores were counted using an Improved Neubauer haemocytometer (Weber Scientific International, UK) to ascertain the population density of the spore suspension. Spore suspensions were then diluted to ~2.0×10^5 CFU ml^{-1} for experimental use. A. niger mycelia were cultured by inoculating 100 µL A. niger spores into 100 mL Malt Extract Broth and incubating under rotary conditions (120 rpm) for 24 hours at 30°C. Mycelia were then dispersed by stomaching (Don Whitley Scientific, UK) in PBS.

2.3 High Intensity 405-nm light source

High-intensity 405-nm light was produced by an indium-gallium-nitride (InGaN) 99-DIE light-emitting diode (LED) array (Opto Diode Corp, USA), with a centre

wavelength (CW) of around 405 nm and a bandwidth of ~10 nm at full-width half-maximum (FWHM). In effect although the light source produced light of 405 nm (± 5 nm), this will, for convenience, be referred throughout the text as 405-nm light. The LED array was powered by a DC power supply, and a cooling fan and heat sink were attached to the array to dissipate heat from the source, which also served to minimise heat transfer to the sample throughout treatment. The LED array was mounted in a PVC housing designed to fit a 12-well plate (NUNC, Denmark), with the array positioned directly above one of the central sample wells. Irradiance levels of 40 and 63 mW cm^{-2} at the surface of the fungal suspensions were used for exposure of the yeast and spore samples, respectively, and the applied light dose was calculated as the product of the irradiance (W cm^{-2}) multiplied by the exposure time (s).

2.4 High-intensity 405-nm Light Inactivation Method

A 3 ml volume of yeast or fungal spore suspension was pipetted into a sample well giving a liquid depth of approximately 10 mm. A micro-magnetic follower was placed in the well, and the plate placed onto a magnetic stirring plate for continuous agitation of the sample. The LED array was placed directly above the sample well, with a distance of approximately 2 cm between the light source and the liquid surface, and samples were exposed to increasing durations of high-intensity 405-nm light. Control samples which received no high-intensity 405-nm light exposure were also set-up. Temperature of fungal samples was monitored using a thermocouple to ensure there was no build-up of heat from the LED arrays during this exposure time.

2.5 Oxygen Dependence Experiments

175 Exposure of fungal populations in oxygen-depleted environments was carried out as
176 follows. Fungal suspensions were prepared in an anaerobic cabinet (Don Whitley
177 Scientific, UK) using PBS that had acclimatised in the anaerobic environment for at
178 least an hour before use. 405 nm light exposure of fungal suspensions was then
179 performed in the anaerobic cabinet with the applied doses used being selected based
180 on the lethal dose required to achieve inactivation of each organism in the aerobic
181 (benchtop) experiments. Anaerobic and aerobic non-exposed controls were also set-
182 up for each test sample. To further deplete the levels of available oxygen, exposure in
183 the anaerobic cabinet was repeated with a scavenger of reactive oxygen species added
184 to the fungal suspensions. The scavenger and concentration used was 30 mM
185 ascorbic acid (Sigma Aldrich, Dorset, UK), as used in previous studies (Feuerstein et
186 al., 2005; Maclean et al., 2008b). Exposure of the organisms in the presence of the
187 scavenger was also performed under aerobic conditions as an experimental control.
188
189 **2.6 Effect of 405 nm light on Germinating Aspergillus Spores**
190 A. niger spore suspensions were prepared as previously described but resuspended in
191 a chemically-defined germination medium (KH_2PO_4 1.0 g, $MgSo_4$ 0.25 g, $CuSO_4$
192 0.2 mg, $FeSO_4$ 6.0 mg, $ZnSO_4$ 1.0 mg, $MnCl_2$ 4.0 mg, $CaCl_2$ 50 mg, NH_4SO_4 2.0 g,
193 Sucrose 10.0 g, monosodium glutamate 5.0 g l^{-1}) rather than PBS. This germination
194 medium was used as it was clear and transparent thereby facilitating light exposure
195 experiments without light attenuation complications. Ten millilitre volumes were
196 dispensed into petri dishes and held at 30°C in a static incubator (to prevent clumping
197 during germination, an effect that occurs more readily under shaken flask cultivation
198 conditions) for up to 8 hours. At 2-hour intervals, 3 ml volumes of germinating spore
199 suspension were removed and exposed to a dose of 454 Jcm^{-2} 405 nm light. Non-

exposed control samples were set up simultaneously with test samples. Results then allowed comparison of the susceptibility of spores at different stages of germination to that of dormant spore populations.

To investigate whether 405 nm light exposure had an effect on morphological changes during germination, spores were incubated in germination medium for 6 hours at 30°C, exposed to 454 Jcm^{-2} 405 nm light, and re-incubated for a further 2 hours. Microscopic examination of the light-exposed fungal samples and the non-exposed control samples (which had been incubated for 8 hours with no light-exposure) enabled a visual comparison of whether the light exposure had an effect on morphological changes during germination. For photographic purposes, fungal samples were centrifuged and resuspended in $1/10^{th}$ of the volume to effect cell aggregation and increase the cell density in the field of view.

2.7 Plating and Enumeration

S. cerevisiae and C. albicans samples were plated onto Malt Extract Agar, and Malt Extract Agar containing 0.1 % yeast extract, respectively (Oxoid, UK). A WASP 2 spiral plater (Don Whitely Scientific Ltd, UK) was used to plate out samples (50 µl spiral plate and 100 µl spread plate samples), with each sample being plated in a minimum of triplicate. Sample plates were then incubated at 30°C and 37°C, respectively, for 18-24 hours before enumeration. Results were recorded as colony forming units per millilitre (CFU ml^{-1}). Samples of A. niger were, diluted if required, and manually spread plated (50 µl and 100 µl volumes) onto Malt Extract Agar in triplicate and incubated at 30°C for five days. Colonies were enumerated and

224 recorded as CFU ml^{-1}. Due to the plating techniques used the detection limit of these
225 experiments is <10 CFU ml^{-1}.
226
227 **2.8 Statistical Analysis**
228 In the 405-nm light exposure experiments, data points on each figure represent the
229 mean results of two or more independent experiments, with each individual
230 experimental data point being sampled in triplicate at least. Data points also contain
231 the standard deviation and significant differences attained from results. Significant
232 differences were calculated at the 95% confidence interval using ANOVA (one-way)
233 with MINITAB software release 15 and are highlighted with asterisks (*).
234
235 **2.9 Fluorescence Spectrophotometry**
236 The presence of endogenous porphyrins within the fungal species was determined by
237 fluorescence spectrophotometry. Cell cultures were twice washed with PBS, and the
238 resultant cell pellets were suspended in 1 ml of 0.1 M NaOH-1% sodium dodecyl
239 sulphate (SDS) for 24 hours in the dark. Cell suspensions were then centrifuged and
240 the supernatant was used for fluorescence measurements (RF-5301 PC
241 spectrofluorophotometre; Shimadzu, US). Excitation was carried out at 405 nm and
242 emission spectra were recorded between 500-800 nm.
243
244 **3. Results**
245 **3.1 High-intensity 405-nm Light Inactivation of Yeasts and Fungal Spores**
246 Results in Figure 1 demonstrate that C. albicans was inactivated by 5-$\log_{10}$ CFU ml^{-1}
247 upon exposure to 405-nm light at a dose of 576 J cm^{-2}. S. cerevisiae showed higher
248 susceptibility, with a 5-$\log_{10}$ CFU ml^{-1} reduction achieved in half the dosage required

for C. albicans (288 J cm^{-2}). Inactivation data for A. niger dormant spore suspensions is also shown in Figure 1. When dose levels similar to those used to achieve a 5-log_{10} $CFUml^{-1}$ reduction in population of the yeast populations (up to approximately 500 Jcm^{-2}) were used, no reduction of dormant A. niger spores was evident. However, with the application of higher doses there was a steady decrease in population, with a 5-log_{10} CFU ml^{-1} reduction of A. niger spores achieved with a dose of 2.3 kJ cm^{-2} – almost five times the dose required for the same 5-log_{10} reduction of the yeast cells. Control samples for both the yeast and fungal spore populations did not significantly change over the duration of the experiment. No significant increase in the temperature of the exposed fungal samples was recorded during light exposure, with the maximum temperature recorded being 29°C (±1 °C) after application of the highest dose (2.3 kJ cm^{-2}).

Figure 1

3.2 Oxygen Dependence Experiments

Fungal samples were light-exposed in an anaerobic cabinet to doses which achieved a 5-log_{10} $CFUml^{-1}$ reduction under normal aerobic atmospheric conditions (which were 288 J cm^{-2} for S. cerevisiae; 576 J cm^{-2} for C. albicans; 2.3 kJ cm^{-2} for A. niger). Results, which are shown in Table 1, demonstrate that the normally 'lethal' 405-nm light dose had no significant effect on A. niger spores exposed in the absence of oxygen, with only 0.1-log_{10} CFU ml^{-1} inactivated. Inactivation of C. albicans was also reduced in the anaerobic environment with only 1.8-log_{10} CFU ml^{-1} inactivated. Inactivation of S. cerevisiae did not appear to be as oxygen-dependent as the other two species, as a reduction of 4.4-log_{10} CFU ml^{-1} was achieved when light-exposed in

the anaerobic environment. Although the effect was less pronounced with S. cerevisiae, statistical analysis confirmed that for all three fungal species tested there was a statistically significant difference in the population reductions when exposed in the aerobic versus the anaerobic environments.

In order to further examine oxygen-dependence in the test yeasts, ascorbic acid, a scavenger of reactive oxygen species, was added to the cell suspensions, which were held in the anaerobic cabinet, as an additional measure to minimise the residual dissolved oxygen in the samples. As a definitive oxygen-dependent effect had been found with A. niger, no further scavenger testing was carried out on this organism. Results of exposing scavenger-supplemented suspensions of C. albicans and S. cerevisiae to 405-nm light under anaerobic and aerobic conditions are shown in Table 1. Ascorbic acid was effective at inhibiting inactivation with C. albicans, with no significant inactivation of C. albicans occurring when exposed anaerobically in the presence of ascorbic acid. This was significantly different to the 1.8 $\log_{10}$ reduction achieved when exposed anaerobically in the absence of ascorbic acid. Presence of ascorbic acid also reduced the level of inactivation of S. cerevisiae, with a 2.3 $\log_{10}$ difference between anaerobically-exposed cells in the presence and absence of the scavenger. Exposure of C. albicans and S. cerevisiae in the presence of ascorbic acid under aerobic conditions resulted in 3.2 and 2.7 $\log_{10}$ reductions, respectively; significantly less than the 5 $\log_{10}$ reductions achieved when exposed under the same conditions in the absence of the scavenger. Overall, although differences between the organisms were noted, results in Table 1 demonstrate that for both organisms, most inactivation was achieved when exposed in aerobic conditions in the absence of the

298 scavenger, and the most effective reduction of microbial inactivation was achieved
299 using the scavenger in the anaerobic environment.

300 **Table 1**

301

302 **3.3 Effect of 405 nm Light on Germinating Spores**

303 Results in Figure 1 demonstrated the high 405 nm light doses required to cause
304 inactivation of dormant A. niger spores. Experiments were carried out to determine
305 whether germinating spores were more susceptible to 405 nm light. Figure 2
306 demonstrates the results achieved when A. niger spores were incubated in a
307 germination medium prior to light exposure using a dose of 454 J cm-^{2}. A significant
308 reduction in the CFU count of the spore suspension was achieved when the spores
309 were light-exposed after a 2-hour incubation period in the germination medium.
310 When the period in germination medium, prior to light exposure, was extended,
311 reductions progressively increased from 0.8 to 2.5 $\log_{10}$ CFU ml^{-1} reduction over the 8
312 hour test period. No significant change in CFU count was recorded with dormant
313 conidia exposed to the same dose of 454 J cm-^{2}; therefore demonstrating that once
314 germination of conidia is initiated, conidia have an increased sensitivity to 405 nm
315 light, with their sensitivity increasing as germination progresses.

316

317 **Figure 2**

318

319 The effect of 405 nm light exposure on morphological changes during the
320 germination process was also investigated. Microscopic examination of conidia after
321 2, 4, 6 and 8 hours incubation in germination medium showed that the conidia
322 underwent the typical germination process previously described for A. niger conidia

(Anderson and Smith, 1971). Dormant conidia (3.5 µm. mean diameter), which possessed a rough dark pigmented spore coat (Fig 3a), increased in diameter by a process of both imbibitional and spherical growth swelling to 6.5 to 7.0 µm. before germ-tube outgrowth. The majority of conidia produced one or occasionally two germ tubes after 6 hours. The effect of 405 nm light exposure when applied during the germination process can be clearly seen in Figures 3b-c which illustrates the significant difference in the extent of germination between light-exposed and non-exposed spores.

Figure 3

3.4 Fluorescence Spectrophotometry

Figure 4 shows the fluorescence emission spectra of suspension preparations of the three fungal species dissolved in NaOH-SDS. Excitation of the cell supernatants at 405 nm displayed emission peaks at 608 and 611 nm for C. albicans and S. cerevisiae, respectively. No peaks were observed for A. niger spores when excited under the same conditions, however analysis carried out using A. niger mycelia demonstrated a peak at 607 nm.

Figure 4

4. Discussion

The results of this study have demonstrated that fungal organisms can be inactivated by exposure to high intensity light from within the visible spectrum and specifically violet light of wavelength 405 nm (± 5 nm). Of the two yeast species tested,

S. cerevisiae was more sensitive to 405-nm light exposure than C. albicans with the dose levels required to achieve an approximate 5 $\log_{10}$ reduction being ~288 J cm^{-2} for S. cerevisiae and ~576 J cm^{-2} for C. albicans. By contrast, dormant conidia of the filamentous mould A. niger were most resistant, requiring a dose of approximately 2.3 kJ cm^{-2}.

Although the dormant conidia of A. niger required relatively high levels of 405 nm light exposure before inactivation was achieved, results demonstrated that upon germination, the susceptibility of spores significantly increased. Inactivation data over the germination period (Figure 2) showed that an increased susceptibility of the conidia to 405 nm light was evident after only a 2-hr germination period, indicating that the initial changes undergone by the germinating spores are enough to increase their susceptibility. It can only be speculated whether this increase in susceptibility is related to morphological changes, e.g. increased light penetration associated with stretching or fracture of the dense pigmented spore coat, or to an increased metabolic vulnerability to light induced reactive oxygen species or indeed to a combination of these effects.

Results of this study also demonstrated that conidial sensitivity increases as the germination process progresses. Microscopic analysis of conidia which had been light-exposed for a period during germination, demonstrated that the 405 nm light exposure arrested the development of the germinating conidia, with hyphal development appearing stunted compared to non-exposed germinating conidia (Figure 3b-c), indicating that light exposure during germination can negatively affect both the germ tube and hyphal growth processes.

373

374 The resistance to 405-nm light exhibited by dormant A. niger conidia is not surprising
375 given the requirement of these structures to survive periods of exposure to solar
376 radiation during aerial dispersal. Strong resistance to UV exposure has been
377 demonstrated by F. culmorum and A. niger spores during inactivation studies
378 involving use of pulsed ultra-violet light radiation (Anderson et. al., 2000). A. niger
379 spores are particularly difficult to inactivate by light, as they possess multi-layered
380 pigmented spore coats containing aspergillin, a black coloured melanin-like
381 compound (Ray & Eakin, 1975).

382

383 An observation made whilst studying the inactivation effects of 405-nm light on
384 dormant A niger conidia was that some of the spores that survived the light treatment
385 germinated and gave rise to colonies that did not produce spores even after prolonged
386 incubation (Figure 5). A study by Hatakeyama et al. (2007) found that conidiation of
387 Aspergillus oryzae was repressed upon exposure to white light. Interestingly they
388 also found that blue light completely suppressed colony formation, which supports the
389 A. niger results of this study. However, Zhu & Wang (2005) found that A. niger
390 conidiation actually increased upon exposure to blue light, which is contrary to the
391 aforementioned results. It is likely that variations in the effects of light on conidiation
392 also depend on other factors such as the growth media employed and the light
393 wavelengths and intensities used. In Aspergillus nidulans it has been reported that
394 conidiation can be regulated by light (Ruger-Herreros & Rodriguez-Romero, 2011)
395 and toxin production and morphogenesis can be controlled by interactions between
396 light and glucose regulation (Atoui et. al., 2010).

397

398 **Figure 5**

399

400 Light from the red and blue regions of the spectrum have also been shown to have
401 important effects on spore germination, hyphal growth and mycotoxin formation in
402 the food relevant Aspergillus and Penicillium fungi (Schmidt-Heydt et. al., 2011). At
403 the molecular level there has been considerable interest in light-mediated regulation in
404 fungi which is primarily conferred by blue-light receptors referred to as WHITE
405 COLLARs which are responsible for both light-dependent and –independent
406 processes such as induction of sporulation, carotenoid biosynthesis and circadian
407 rhythms (Corrochano, 2007). Whether or not blue light receptors are involved in any
408 of the morphogenetic or inactivation responses reported in the current study is outwith
409 the scope of this investigation,

410

411 Regarding the susceptibility of the fungi to 405-nm light inactivation, it is interesting
412 to compare the results of the current study to those of previously published work on
413 bacteria. The prokaryotic bacteria also exhibit considerable variability in
414 susceptibility with values, to achieve similar 5-$\log_{10}$ order reductions, as low as 18
415 J cm^{-2} with Campylobacter jejuni (Murdoch et al., 2010) but most typically around 50
416 - 300 J cm^{-2} , with Gram positive species being generally more susceptible than Gram
417 negatives (Maclean et al., 2009). Comparison with the values for fungi reported in the
418 current study indicates that fungal organisms may be somewhat more resistant to 405-
419 nm light than bacteria. Interestingly it has been previously observed that C. albicans,
420 and other yeasts, are slightly more difficult to kill by photodynamic antimicrobial
421 chemotherapy (PACT) than Gram positive bacterial cells, thereby necessitating higher
422 drug and light doses (Zeina et. al., 2002). This has been attributed to the presence of a

423 nuclear membrane in the yeasts, the greater cell size and the reduced number of
424 targets for singlet oxygen per unit volume of cell (Donnelly et. al., 2008).

425

426 It is also interesting that the relatively high levels of 405 nm light, as reported in the
427 current study, required to inactivate dormant A. niger spores are not dissimilar to
428 those required for the inactivation of bacterial endospores. We report here that a 5-log
429 order reduction of dormant conidia of A. niger required a dose of 2.3 kJ cm^{-2} whereas
430 Maclean et al (2012) reported that approximately 2 kJ cm^{-2} was required to achieve an
431 approx 4-log order reduction of Bacillus species endospores.

432

433 Due to the irradiance output of the light sources used in this study, inactivation of the
434 fungal organisms required relatively long exposure periods, i.e. 2 and 4 hours at an
435 irradiance of 40 mW cm^{-2} for S. cerevisiae and C. albicans, respectively, and 10 hours
436 at 63 mW cm^{-2} for fungal spores (for 5-log_{10} reduction). Microbial inactivation by
437 405 nm light exposure has been found to be dose-dependent (Murdoch et al, 2012),
438 and in applications where more rapid fungal inactivation is desirable then use of a
439 much higher power light source would significantly reduce the exposure times
440 required for effective treatment.

441

442 It is considered highly likely that the inactivation mechanism initiated within the
443 fungal species tested involves the photoexcitation of free porphyrins within the
444 exposed cells, as is indicated for bacterial inactivation (Dai et al, 2012). Fluorescence
445 spectrophotometry was used to confirm the presence of porphyrins within the test
446 organisms. The fluorescence emission spectra of the three species indicated similar

447 peaks in the region 605-615 nm, and although the results are qualitative, there was
448 distinct differences in the intensity of the detected peaks. These peaks indicate that
449 the three test species are likely to contain the same predominant intracellular free-
450 porphyrin, possibly coproporphyrin, due to the similarity in its emission peaks when
451 excited at 405 nm (Dai et al, 2013; Hamblin et al, 2005), however further analysis
452 would be required to provide a full identification.
453
454 Most probably due to limitations of the extraction methodology employed, analysis of
455 A. niger spores produced no detectable porphyrin excitation peaks. This may well
456 have been due to the persistence of some of the dark spore pigment extract in the
457 supernatant thereby masking the fluorescence detection of porphyrins. For this reason
458 A. niger mycelia was used as an extraction source to allow a comparative analysis
459 with the yeast species. Exposure of A. niger mycelia to 405 nm light showed that
460 complete inactivation of a 3.5 $\log_{10}$ CFU population of dispersed mycelia was
461 achieved upon exposure to a dose of 1080 J cm^{-2}, a result which demonstrated the
462 increased susceptibility of mycelia compared to the resilient spores.
463
464 Comparing the fluorescence emission and the inactivation rates of the three species, it
465 is interesting to note that S. cerevisiae, which was the most susceptible to inactivation,
466 had the greatest intensity of intracellular porphyrin, and A. niger, which was least
467 susceptible, had the lowest. This suggests that the intracellular levels of the detected
468 porphyrin may have a direct influence on the susceptibility of the organisms.
469
470 The current study has also demonstrated that oxygen plays an important role in the
471 405-nm inactivation mechanism in fungi, further supporting the hypothesis that

472 inactivation involves the photoexcitation of endogenous porphyrins – an oxygen-
473 dependent reaction (Hamblin & Hasan, 2004). The use of anaerobic exposures and a
474 ROS scavenger demonstrated that reducing the oxygen and ROS significantly
475 decreased, or in some cases completely inhibited, 405-nm light induced inactivation.
476 The use of ascorbic acid as the scavenger also provided some information on the
477 nature of the ROS important in the 405-nm light inactivation mechanism. Ascorbic
478 acid can be taken up by the cell and act as a singlet oxygen quencher and radical
479 scavenger (Granot et al, 2003; Maclean et al, 2008b), thus indicating that singlet
480 oxygen and other radicals are key contributors to the inactivation process.
481
482 Inactivation of A. niger spores was almost completely inhibited when exposed within
483 an anaerobic environment. The yeasts, particularly S. cerevisiae, still displayed a
484 degree of inactivation when exposed under anaerobic conditions, which was then
485 further reduced when combined with the scavenger, however a notable level of
486 S. cerevisiae inactivation (2-$\log_{10}$ CFU ml^{-1}) was still observed: thus indicating either
487 the increased susceptibility of the organism, or the potential involvement of other
488 factors in the inactivation mechanism for this organism. Although fluorescence
489 spectroscopy results demonstrated that there were similarities in the likely porphyrin
490 content of the three organisms, albeit at different levels, it is unlikely that this is the
491 sole reason for the differences between their inactivation kinetics, and it is possible
492 that additional chromophores contribute to the inactivation of these eukaryotic cells,
493 however elucidation of this was out-with the scope of this study.
494
495 Other groups studying the effect of light on yeasts have obtained results that are
496 compatible to those reported here. Fraiken et al. (1996) investigated the use of visible

497 light for the inactivation of three strains of S. cerevisiae and C. guillermondii, via a
498 "porphyrin-type compound" bound to the plasma membranes of the yeasts. Upon
499 exposure to the 400-600 nm light, reductions in cell numbers of 93-95% in C.
500 guillermondii and S. cerevisiae were achieved (10^6 CFU ml^{-1} starting population).
501 They also noted inactivation was greatly reduced when yeasts were exposed to visible
502 light under anoxic conditions and hypothesised that inactivation in these yeast species
503 was most likely driven by singlet oxygen damage. Therefore the results of this
504 current study correlate well with the previous findings that blue light inactivation of
505 microorganisms is an oxygen-dependent process (Fraiken et al., 1996; Feuerstein et
506 al., 2005; Maclean et al., 2008b).
507
508 The use of light to control fungal and other spoilage microorganisms, has traditionally
509 involved the use of light from the UV region of the spectrum which is much more
510 biocidal than 405-nm light. A study by Begum et al. (2009) treated different fungi
511 including A. niger using a UV-C lamp emitting light at 254 nm. They found that after
512 an exposure time of 180 seconds, a 2-log_{10} reduction in A. niger spores was observed
513 at a total dose of around 0.5 J cm^{-2}. Anderson et al. (2000) studied the effects of
514 pulsed UV-light (PUV) on Fusarium culmorum and A. niger. They exposed agar
515 plates seeded with fungi to light pulses produced by a Xenon flashlamp with high UV
516 content resulting in around 3 or 4-log_{10} reductions in A. niger and F. culmorum
517 respectively after 1000 pulses.
518
519 Although UV-light is highly biocidal there are problems with the application of this
520 technology including inactivation tailing effects, poor penetrability, and
521 photoreactivation responses with sub-lethally damaged populations. Also human

health exposure concerns and problems associated with UV-light damage to photosensitive materials are disadvantages associated with the use of UV light as a decontamination technology (Elmnasser et al.2007). Because of the ongoing requirements to control pathogenic and spoilage fungi and because of limitations associated with the use of other decontamination technologies, potential applications may be found for the use of 405-nm light for the inactivation of problematic yeast and mould fungi.

5. Conclusions

High-intensity 405-nm light has been successfully applied for the inactivation S. cerevisiae, C. albicans, and dormant and germinating spores of A. niger, in the presence of oxygen but without the requirement of exogenous photosensitiser molecules. Results indicate that in fungi, 405-nm light inactivation involves a violet light induced, oxygen dependent mechanism similar to that previously described in bacteria. Although 405-nm light is less fungicidal than UV-light, 405-nm light offers potential operational advantages over UV-light radiation, and development of higher intensity systems could find potential applications for surface, air or equipment decontamination in the medical, pharmaceutical and food industries or in any other setting in which reduction of fungal contamination is desirable or essential.

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Figure 1. Inactivation of S. cerevisiae, C. albicans and dormant A. niger spores in liquid suspension, by exposure to 405-nm light. Asterisks (*) represent light-exposed populations which were significantly different to non-exposed control populations ($P \leq 0.05$). No significant changes were observed in the control samples throughout the experiment.

720 **Figure 2.** Demonstration of the increasing susceptibility of A. niger conidia to
721 405 nm light when exposed at various stages of germination. Conidia were exposed
722 to a dose of 454 J cm^{-2}. Data for the exposure of dormant conidia suspended in PBS
723 is included for reference. Asterisks (*) represent light-exposed populations which
724 were significantly different to non-exposed control populations ($P \leq 0.05$).
725
726 **Figure 3.** Microscopic visualisation of (a) dormant conidia, (b) conidia exposed to
727 405 nm light (454 Jcm^{-2}) after a 6-hr germination period followed by a further 2-hr
728 post-exposure germination period, showing abnormal germination, and (c) non-
729 exposed conidia after an equivalent 8-hr germination period, showing normal
730 germination. Cells were viewed under ×400 magnification.
731
732 **Figure 4.** Fluorescence spectra of Saccharomyces cerevisiae, Candida albicans and
733 Aspergillus niger mycelia. Fluorescence emission spectra were detected from
734 suspension preparations of the three species dissolved in NaOH-SDS, using an
735 excitation wavelength of 405 nm.
736
737 **Figure 5.** A mixture of sporing and non-sporing A. niger colonies that have
738 developed from spores that were exposed to high doses of 405 nm light. Light
739 exposed spores were plated onto Malt Extract Agar and incubated for 10 days for
740 colony development.
741
742
743
744

Table 1. Mean population reductions of fungal test species after exposure to 405-nm light under aerobic and anaerobic conditions. Doses used for exposure were those required to achieve a 5-$\log_{10}$ reduction of each organism under aerobic conditions: 288 J cm^{-2} for S. cerevisiae, 576 J cm^{-2} for C. albicans; 2.3 kJ cm^{-2} for A. niger spores.

Microorganisms	Mean Reduction in Microbial Numbers (Log_{10} CFU ml^{-1})			
	Light-exposed		Light-exposed + Scavenger	
	Aerobic	Anaerobic	Anaerobic	Aerobic
C. albicans	5.02 (±0.1)	1.76 (±0.3)	0.03 (±0.0)	3.25 (±1.6)
S. cerevisiae	5.18 (±0.1)	4.37 (±0.8)	2.03 (±0.1)	2.72 (±0.3)
A. niger spores	5.24 (±0.2)	0.1 (±0.3)	N/A	N/A

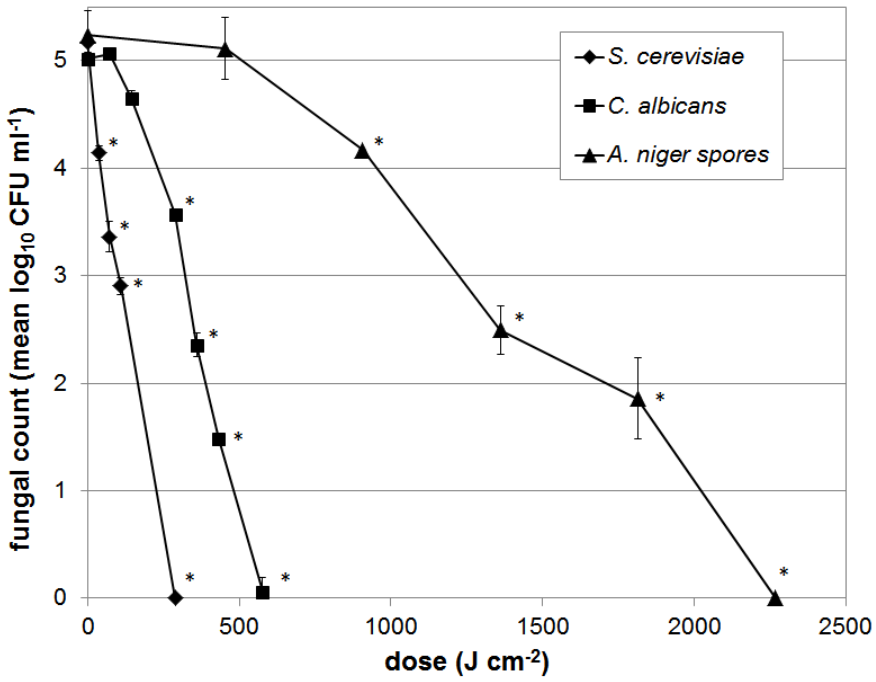
S. cerevisiae
C. albicans
A. niger spores
fungal count (mean log10 CFU ml-1)
dose (J cm-2)
0
1
2
3
4
5
0
500
1000
1500
2000
2500

Fig 1

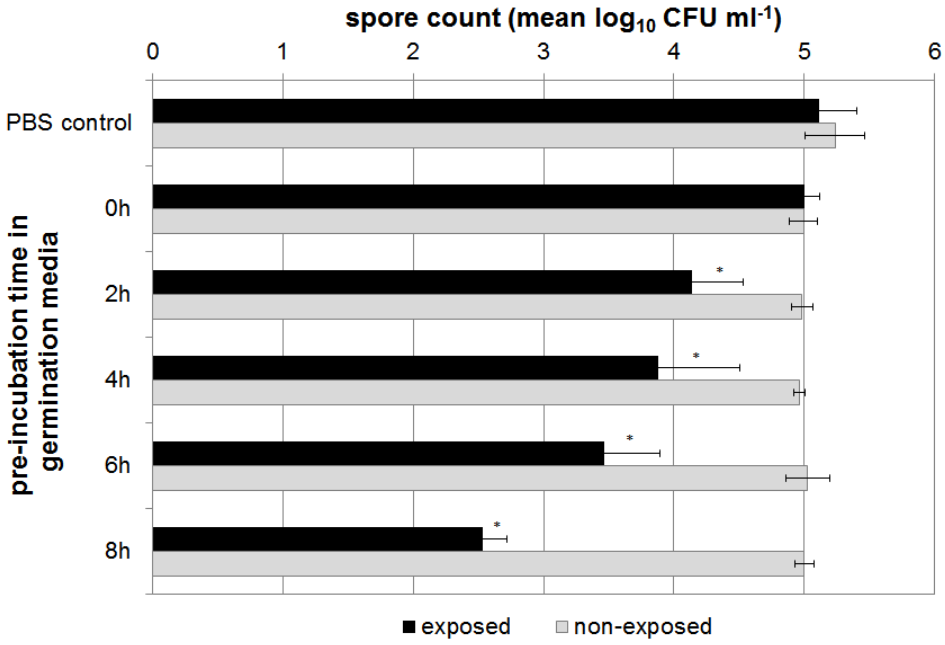
spore count (mean log10 CFU ml-1)
0
1
2
3
4
5
6
PBS control
0h
2h
4h
6h
8h
pre-incubation time in germination media
exposed
non-exposed

Fig 2


a
b
c

Fig 3

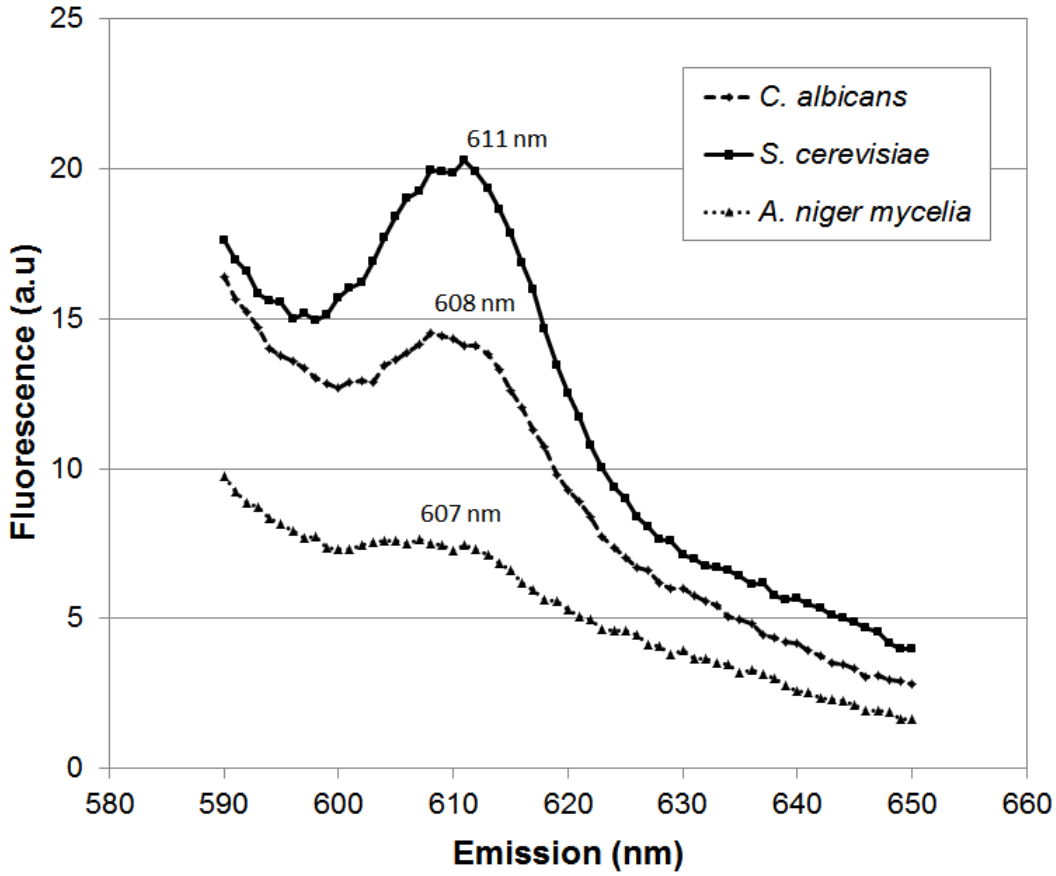
25
20
15
10
5
0
Fluorescence (a.u)
580
590
600
610
620
630
640
650
660
Emission (nm)
C. albicans
S. cerevisiae
A. niger mycelia
611 nm
608 nm
607 nm

Fig 4

783

784

785

786 Fig 5

787

788