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A novel photosensitization treatment for the inactivation of fungal spores and cells mediated by curcumin

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ABSTRACT

The global concerns regarding the emergence of fungicide-resistant strains and the impact of the excessive use of fungicidal practises on our health, food, and environment have increased, leading to a demand for alternative clean green technologies as treatments. Photosensitization is a treatment that utilises a photosensitiser, light and oxygen to cause cell damage to microorganisms. The effect of photosensitization mediated by curcumin on *Aspergillus niger*, *Aspergillus flavus*, *Penicillium griseofulvum*, *Penicillium chrysogenum*, *Fusarium oxysporum*, *Candida albicans* and *Zygosaccharomyces bailii* was investigated using three methods. The viability of spores/cells suspended in aqueous buffer using different concentrations of curcumin solution (100–1000 μM) and light dose (0, 24, 48, 72 and 96 J/cm^2) were determined. Spraying curcumin solution on inoculated surfaces of agar plates followed by irradiation and soaking spores/cells in curcumin solution prior to irradiation was also investigated. In aqueous mixtures, photosensitised spores/cells of *F. oxysporum* and *C. albicans* were inhibited at all light doses and curcumin concentrations, while inactivation of *A. niger*, *A. flavus P. griseofulvum*, *P. chrysogenum* and *Z. bailii* were highly significant ($P < 0.001$) reduced by 99%, 88.9%, 78%, 99.7% and 99.2% respectively. On the surface of agar plates, spores/cells exposed to a light dose of 360 J/cm^2 sprayed with curcumin at 800 μM showed complete inhibition for *A. niger*, *F. oxysporum*, *C. albicans* and *Z. bailii*, while *A. flavus P. griseofulvum*, and *P. chrysogenum* reduced by 75%, 80.4% and 88.5% respectively. Soaking spores/cells with curcumin solution prior to irradiation did not have a significant effect on the percentage reduction. These observations suggest that a novel photosensitization mediated curcumin treatment is effective against fungal spores/cells and the variation of percentage reduction was dependent on curcumin concentration, light dosage and fungal species.

1. Introduction

Fungi can be destructive for agricultural crops, causing significant losses, and is a health concern for humans and animals. Fungi are also associated with food spoilage, causing odour, rancidity, flavour changes and loss of nutrients [1]. Pathogenic moulds can produce mycotoxins which are secondary metabolites, and have been associated with human disease such as gastroenteric conditions and liver damage [2]. Filamentous moulds produce many types of asexual spores, which is critical in the life cycle, as the spores contribute to survival under unfavourable environmental conditions and remain dormant until suitable environmental conditions occur [3]. Unlike vegetative phase (or mycelia), the spores are more resistant to external stresses, and harsher treatments are required to prevent germination [4]. Failure of conventional fungicides and the concern globally of the emergence of fungicide-resistant microorganisms have increased the demand for safe and environmentally friendly alternatives.

Photosensitization is an effective approach *in vitro* and *in vivo* for significantly reducing bacteria [5–12], viruses [13], protozoa [14] and fungi [15–21]. Photosensitization employs the photochemical interaction between non-toxic photosensitiser and visible light at an appropriate wavelength. In the presence of oxygen, the interaction generates cytotoxic substances, which cause localised oxidative photodamage to microbial cells. This reaction can occur by two pathways (Type I and Type II). When photosensitiser is subjected to an appropriate wavelength of light, becomes an excited sensitiser (Sen*) and reacts directly with the substrate, producing radicals these radicals react with oxygen molecules and generate cytotoxic superoxide radicals (O_2^-) (type I pathway). While in type II pathway, the excited sensitiser reacts with an oxygen molecule forming cytotoxic singlet oxygen ($^1O_2$). These reactive oxygen species (ROS) can cause structural disintegration and inhibition of the functional activity of subcellular structures leading to cell death

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[21–25]. This promising approach has a dual selectivity advantage by selecting the appropriate photosensitiser to target the affected tissue or microbial cell and delivering the light accurately to the appropriate area [26]. In the last few decades, photosensitization has been used to cure cancer and skin infection diseases, it has achieved good results in controlling cellular multiplication by apoptosis or necrosis of cells and does not have an effect on healthy cells [4,27–29]. Development of resistant microorganisms after photosensitization treatment has not been reported to date, and is very unlikely to occur [4]. On the other hand, using effective natural, plant based photosensitisers is a challenge. Many of the photosensitisers that have been investigated for photosensitization, were synthetic substances [17–19]. Curcumin commonly known as diferuloyl methane, the bioactive non-toxic natural photosensitiser in turmeric (*Curcuma longa* L.), has been shown to have broad-spectrum bactericidal [30] and fungicidal activities [31]. The mode of action of this polyphenolic compound has been demonstrated to change the cell membrane integrity and affect membrane-bound proteins indirectly [32]. A few studies have been done utilising curcumin as a photosensitiser on Gram negative, Gram positive bacteria [33–36] and yeast (*Candida albicans*) [31,32,37–39]. However, studies on fungal spores are limited, and very few of them have reported the effects of photosensitization mediated curcumin [40]. The mould spores of common genera like *Aspergillus*, *Penicillium* and *Fusarium* as well as yeast cells have been selected as the model for this study. The aim of the present work was to investigate (i) the effect of photosensitization mediated curcumin on fungal spores/cells using different concentrations of curcumin solution and light doses, (ii) the effect of soaking prior to irradiation on spores/cells reduction and (iii) the effect of photosensitization mediated curcumin on germinating spores/cells inoculated on agar surface.

2. Materials and Methods

2.1. Fungal Spore/Cell in Aqueous Suspension

In this study, the spores/cells term refers to moulds/yeasts respectively. *Aspergillus niger* ATCC 6275, *Aspergillus flavus* ATCC 9643, *Penicillium griseofulvum* ATCC 48927, *Penicillium chrysogenum* ATCC 10106, *Fusarium oxysporum* ATCC 62606, *Candida albicans* ATCC 10231 and *Zygosaccharomyces bailii* ATCC 42476 were purchased from the American Type Culture Collection (ATCC, USA). The spores/cells of fungi were grown on potato dextrose agar (PDA) at 26 °C for 7 days. The mould spores were harvested by flooding the plate with 10–15 ml of 0.1% Tween 80 solution and rubbing the surface mycelium gently with a spreader to release the spores. Spore suspensions were then centrifuged to remove mycelial fragments and spore pellet suspended in sterile buffer solution. Spores/cells were counted by adjusting to approximate dilutions of (10^3 to 10^4 CFU per ml). The viable spores/cells reduction was enumerated on PDA.

2.2. Photosensitiser and Light Source

A stock solution (2000 μM, pH = 4.9) of curcumin powder (Sigma Aldrich, St. Louis, USA) was prepared by dissolving 73.8 mg in 30 ml of propylene glycol (99.5%, a colourless organic diol) and then diluted in 70 ml of sterile water, to obtain the different concentrations to be tested. This ratio of propylene glycol to water was selected, as preliminary studies showed no inhibitory effect on spores/cells (data not shown). The curcumin stock solution bottle was wrapped in aluminium foil and kept in a cool, dark place. From the stock solution, the desired serial dilutions were carried out using sterile water. The irradiation was conducted using a 500 Watt Xenon arc lamp (Polilight, PL 500, Rofin Australia Pty Ltd., Victoria, Australia) equipped with an optical fibre light over a range of 370–680 nm. The light doses or intensity delivered (J/cm^2) was calculated as: irradiation time (s) multiplied by the light power (w) divided by the area of irradiation (cm^2). Also, the maximum absorbance wavelength (λ_{max}) of curcumin was obtained using a spectrophotometer (Tecan, Infinite M200, Austria).

2.3. Determination of Spores/Cells Survival in Aqueous Mixture

The experiment was conducted *in vitro* to determine an efficient photosensitization mediated curcumin treatment on fungal spores/cells in aqueous suspension using a wide concentration range of curcumin from 100 to 1000 μM along with different levels of light doses (0, 24, 48, 72 and 96 J/cm^2) (equal to irradiation time of 0, 2, 4, 6 and 8 min respectively) and independent effect of light and curcumin. Aliquots (2 ml) of the fungal suspension was mixed with the same amount of curcumin solution (1:1 v/v), where each fungus was assessed separately. The light source was placed vertically at a distance of ~10 cm from the base of the Petri dish before irradiation. Meanwhile, during irradiation, the mixture (a combination of spores/cells and curcumin) was constantly stirred using a magnetic stirrer in order to spread the energy homogeneously to all spores/cells. After irradiation, aliquots (100 μl) of the treated mixture was transferred onto PDA and incubated at 26 °C for 4–5 days. Survival of fungal spores/cells following irradiation was calculated by Log reduction (LR) and percentage reduction as given below.

$$\text{LR (log reduction)} = \text{Log}_{10}(A) - \text{Log}_{10}(B) \quad (1)$$

$$\%\text{Reduction} = (A - B)/A * 100 \quad (2)$$

where:

A: the mean of spores/cells reduction before photosensitization.

B: the mean of spores/cells reduction after photosensitization.

2.4. Determination of Spores/Cells Survival in Agar Surface

Aliquots (100 μl) of suspended spores/cells was plated onto the surface of PDA plates and left for 15 min to absorb any excess liquid. The surfaces of agar were then sprayed with ~150 μl of curcumin (800 μM) using a spray bottle. The plates were then irradiated at three levels of light doses; 96, 240 and 360 J/cm^2 (equals to 8, 20 and 30 min), and then incubated at 26 °C for 4–5 days.

2.5. Soaking Spores/Cells Prior to Irradiation

The effect of soaking spores/cells with curcumin solution prior to irradiation was carried out according to Dovigo, Pavarina [38]. Aliquot (1 ml) of suspended fungal spores/cells was soaked in curcumin solution (based on the optimal concentration shown in Fig. 1) for 10, 20 and 30 min in the dark, followed by irradiation dose at 96 J/cm^2. Aliquots (100 μl) of photosensitised mixture was plated onto PDA and incubated at 26 °C for 4–5 days.

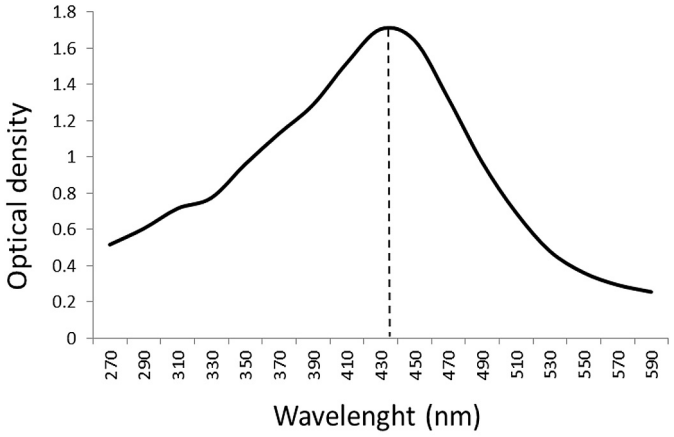

Fig. 1. Typical photosensitiser absorption (activation) spectra for curcumin solution.

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