Photodynamic biocidal action of Methylene Blue and Hydrogen Peroxide on the cyanobacterium *Synechococcus leopoliensis* under visible light irradiation.

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Abbreviations:

MB: Methylene Blue

S. leopoliensis.: *Synechococcus leopoliensis*

Abstract

Biofilm growth on stone surfaces is a significant contributing factor to stone biodeterioration. Current market based biocides are hazardous to the environment and to public health. We have investigated the photodynamic effect of Methylene Blue (MB) in the presence of hydrogen peroxide (H_2O_2) on the destruction of the cyanobacterium *Synechococcus leopoliensis* (*S. leopoliensis*) under irradiation with visible light. Data presented in this paper illustrate that illumination of *S. leopoliensis* in the presence of a photosensitiser (MB) and H_2O_2 results in the decomposition of both the cyanobacterium and the photosensitiser. The presence of MB and H_2O_2 affects the viability of the photosensitiser and the cyanobacterium with the fluorescence of both decreasing by 80 % over the irradiation time investigated. The photo-dynamic effect was observed under aerobic and anaerobic conditions indicating that oxygen was not necessary for the process. This novel combination could be effective for the remediation of biofilm colonised stone surfaces.

1. Introduction

Scientific approaches to the safeguarding of stone monuments have evolved over the years to reach a high level of sophistication. Progress has also been made on the analysis and treatment of detrimental biofilms [1, 2]. One factor influencing this progress is the awareness that damage of stonework occurs via biologically and chemically initiated reactions [3]. Micro-organisms play a significant contributing role in the biodeterioration of stone monuments [4]. A considerable number of investigations have started to elucidate the essential role biological agents play in the deterioration of stone [5, 6]. What is becoming clear is that many factors affect the durability of stone. Physical chemical and biological agents act in co-association, ranging from synergistic to antagonistic effects, to deteriorate stone [7]. The economic impact of materials deterioration is one of the main problems in many developed countries causing losses of 2-4 % of the European Union G.D.P., with microbial biodeterioration being responsible for 30 % of these losses [8]. Biological deterioration is even more evident in those materials exposed to an outdoor environment, construction materials being the main target of this phenomenon. Stone surfaces have traditionally been treated using physical or chemical methods such as sand blasting or the application of chemical biocides. In the past 20 years, chemical biocides have become increasingly banned because of the environmental and health hazards associated with these toxic substances [9, 10]. Several external pressures including the approval of the European Directive 98/8/EC [11] concerning placing biocidal products on the market, and the 7th Amendment to Directive 67/548/EEC (Directive 92/32/EEC) [12] have accelerated the search for more environmentally and toxicologically safe, selective and effective biocides.

Photodynamic therapy (PDT) is a method that utilises chemicals that require the application of light for their activity [13]. PDT has most commonly been used in medical applications where PDT agents are site non-specific drugs, i.e. they do not target a specific enzyme or receptor [14]. Photodynamic therapy has been applied to the fields of cancer research [14-18] and as a potential method for the treatment of anti-biotic resistant microbial species [19, 20]. Methylene blue (MB), from the phenothiazine family, is a photosensitiser [21, 22] that has been used for a variety of applications, including solar energy conversion and PDT [19, 23].

In this paper we describe the application of MB and Hydrogen Peroxide (H_2O_2) as a photodynamic reagent for the destruction of *Synechococcus leopoliensis* (*S. leopoliensis*), a cyanobacterium that colonises stone. The photo-activity of a range of concentrations of MB was investigated to determine the optimum concentration for use in the photo-destruction of *S. leopoliensis*. The combination of MB and H_2O_2 was studied under argon to determine the mechanism of photo-destruction of *S. leopoliensis*. The photo-dynamic effect of MB and hydrogen peroxide (H_2O_2) towards the photo-destruction of *S. leopoliensis* could provide an environmentally non-toxic method towards the remediation of colonised stone surfaces.

2. Materials and Methods

2.1 Chemicals

Methylene blue, ~ 85 %, (remaining 15 % primarily salt), was purchased from Aldrich and used in aqueous solution (Milli Q water). Further purification of MB considered unnecessary as it would add cost to the commercial application. Hydrogen peroxide (> 30 % w/v) was purchased from Fisher. All chemicals were used as received. *Synechococcus leopoliensis* was purchased from Sciento and was cultured in Blue-green medium BG11 broth, incubated with continuous illumination at 21 ºC. The cyanobacterium was sampled in the stationary phase of growth.

2.2 Photochemical reactions

A series of concentrations of MB (1 μM – 6 μM) were exposed to illumination from a 500 W tungsten halogen lamp in the presence and absence of H_2O_2. The cyanobacteria experiments were performed with a 20 % solution of *S. leopoliensis*, which was equivalent to 1.759 μg m^{-3} biomass, the fluorescence of *S. leopoliensis* is proportional to its biomass. MB (3 μM) was added to a volume of *S. leopoliensis*. In separate experiments the effect of 1 M H_2O_2 with MB on *S .leopoliensis* was investigated. The solutions (30 mls) were exposed to illumination from a 500 W tungsten halogen lamp in open pyrex flasks for a period of 240 minutes. Samples were taken at either 15 or 30 minute intervals. Each experiment was repeated 3 times and dark controls were carried out simultaneously. For the inert experiments the solutions were placed in a sealed vessel and were bubbled with argon for 15 minutes prior to commencing the experiment. The solutions were blanketed with argon for the duration of the experiment. The irradiated samples were analysed using a luminescence spectrometer (Perkin-Elmer LS B50). The excitation and emission

wavelengths for fluorescence monitoring for cyanobacteria and MB were 540nm:720nm and 667nm:691nm respectively.

3. Results

3.1 PDT of Synechococcus leopoliensis with Methylene Blue and H_2O_2

The concentration of MB (3μM) investigated in this study was that which was found to have the maximum absorption of visible light and optimal activity for the investigation. At lower concentrations the kinetics for the process were slower while at higher concentrations methylene blue formed dimeric compounds, also reducing the efficiency of the system. The viability of *S. leopoliensis* was monitored via the fluorescence of its pigment, phycocyanin. The decrease in fluorescence indicated a decrease in the amount of intact phycocyanin. As this is the pigment responsible for photosynthesis in cyanobacteria it is reasonable to suggest that a decrease in the fluorescence of the pigment would signify cell death. Dark control experiments were conducted with both MB alone, and MB and H_2O_2. In both dark control experiments, the fluorescence of *S. leopoliensis* remained within 5 % of the initial value (figure 1) demonstrating that MB and MB/H_2O_2 were non-toxic to the cyanobacteria in the dark. In addition a control solution of *S. leopoliensis* was exposed to illumination, in the absence of MB, over a period of 240 minutes to investigate the effect of light alone on the cyanobacteria (figure 2). Again the *S. leopoliensis* fluorescence level remained within 7 % of the initial value over the course of the illumination exposure time indicating that light alone had little effect on the cyanoabacterium. When a suspension of *S. leopoliensis* was irradiated in the presence of MB, in the absence of H_2O_2, a slight reduction in the cyanobacteria fluorescence (around 13 %) was observed after 240 minutes illumination.

The addition of H_2O_2 to the MB photosensitiser, however, had a marked effect on the fluorescence of *S. leopoliensis* following irradiation. In figure 2 it can be seen that

after 90 minutes illumination the fluorescence of *S. leopoliensis* began to decrease. The decrease in fluorescence continued with further illumination and after 240 minutes the signal had decreased to 20 % of the starting value. Whereas when the cyanobacteria were exposed to illumination in the presence of H_2O_2 alone, no decrease in the fluorescence of *S. leopoliensis* was observed (figure 2.)

In order to determine the extent to which oxygen was involved in the photosensitised destruction of *S. leopoliensis*, and whether a type I or a type II mechanism was likely to predominate, the effect of MB with H_2O_2 on the viability of *S. leopoliensis* was investigated in an oxygen free atmosphere. Figure 3 illustrates the change in fluorescence of *S. leopoliensis* with MB/H_2O_2 under an argon atmosphere upon illumination for 240 minutes. The pattern of decreasing fluorescence was similar to the aerobic case, there was an initial increase of fluorescence of *S. leopoliensis* suggestive of cell damage and the release of a fluorescing component. After 90 minutes illumination the fluorescence had decreased by 60 % of the initial fluorescence reading. The fluorescence decreased by a further 30 % after 240 minutes irradiation with the final reading being only 11 % of the original value.

3.2 Fate of MB during PDT of Synechococcus leopoliensis

The photo-stability of the MB photosensitiser was also monitored. This is an important factor in its application as an environmental biocide. If MB has the ability to destroy biofilms with the concomitant breakdown of the dye itself then it will not pose a threat as a secondary toxin within the environment. The activation of MB upon illumination and in the presence of oxygen was anticipated to affect the stability of MB. Over the 240 minute irradiation period under visible light, however, very little

change in the MB fluorescence was observed, (figure 4). The presence of H_2O_2 and *S. leopoliensis* with illumination however, resulted in a significant decrease in the fluorescence of MB (figure 4) after 45 minutes illumination.

Irradiation of MB in the presence of H_2O_2 causes a decrease in the fluorescence of 90 %, of MB in the absence of *S. leopoliensis* (figure 5). The fluorescence of MB maintained in the dark did not decrease over the course of the experiment illustrating that the H_2O_2 in the dark did not have any affect on MB, figure 5.

Under the argon atmosphere the MB fluorescence, in the presence of *S. leopoliensis* and H_2O_2, decreased by 50 % after 90 minutes illumination, the decreasing fluorescence continued with increased illumination time, (figure 6). The trend of decreasing fluorescence follows the same pattern as the decrease in fluorescence of *S. leopoliensis (*figure 3), suggesting that the species responsible for the photo-dynamic effect attacks both the photosensitiser and the cyanobacterium.

4. Discussion

Data presented in this paper illustrates that illumination of *S. leopoliensis* in the presence of MB and H_2O_2 affects the fluorescence of both the cyanobacteria and the photosensitiser. The fluorescence of the cyanobacteria decreases with exposure to illumination in the presence of MB.

Reports on photodynamic therapy have been extensively reported in the past, however activity at the molecular level has only recently been elucidated. Many reports describe the appearance of mitochondrial photodamage [24-27]. Mitochondria play a central role in different cell death pathways leading to either apoptosis or necrosis [28, 29]. The first evidence that mitochondria play an important role in cellular death mechanisms came from the observation that '*in vitro*' models required an organelle fraction enriched in mitochondria to induce apoptosis [30]. Initial reports of cell death through PDT ascribed the mechanism of necrosis however recently apoptosis has been implicated [31, 13, 32-35]. It has been suggested that cytochrome c, a mitochondrial component has the ability to trigger apoptosis. Release of cytochrome c following mitochondrial photodamage could therefore represent a primary event in apoptotic cell death [36-38].

In figure 2 during the first 60 minutes irradiation, the fluorescence of *S. leopoliensis* increased which is indicative of initial cell breakdown with the release of a fluorescing pigment. Balk *et al*. [39] reported the release of cytochrome c from the mitochondria of cucumber plant cells following 10-minutes heat treatment. After 3 hours of heat treatment they reported that cytochrome c could no longer be detected in the mitochondria. The presence of H_2O_2 causes the fluorescence of *S. leopoliensis* to

increase to a greater extent when compared to the increase observed with MB alone. The presence of superoxide has been reported to potently trigger mitochondrial swelling and the release of proteins involved in activation of postmitochondrial apoptotic pathways in the absence of mitochondrial depolarisation [40].

Previous studies on the use of phenothiazines in the PDT of cancer cells have concentrated predominantly on MB and its demethylated analogues azure C, thionine and toluidine blue [21]. Toluidine blue is phototoxic in human submaxillary gland epidermoid carcinoma cells [23], however the activity of toluidine blue was found to be lower than that of MB. MB is therefore one of the most commonly used photosensitisers for *in-vitro* PDT [41]. Upon illumination with visible light the oxidation of cells proceeds via two competitive mechanisms. The type I mechanism produces a radical intermediate by direct interaction of the light excited photosensitiser (MB*) with the substrate via electron transfer. The type II mechanism involves energy transfer from the photo-excited state of the photosensitiser to oxygen with the production of singlet oxygen [42]. In order to assist in determining what mechanism type the current process followed, the system was investigated in an inert atmosphere. The results in figure 3 indicate that under an oxygen free atmosphere an increase in the photo-dynamic effect of the MB/H_2O_2 towards the viability of *S. leopoliensis* resulted. It is therefore unlikely that this process is mediated by singlet oxygen generated by a type II process. In fact the presence of oxygen marginally reduces the efficiency of the system, probably through competition with the peroxide for reaction with the photoexcited MB species. It is therefore most likely that the photoexcited MB reacts in a type I mechanism with the transfer of an electron to the H_2O_2 generating either hydroxyl or more likely hydroperoxide radicals. Either of

these species could then attack the *S. leopoliensis* cells resulting in the subsequent decomposition of the cyanobacteria. This hypothesis is supported by the work of Gak *et al* [43] who investigated the quenching of triplet excited MB by H_2O_2 using laser and steady-state photolysis. They suggested three possible mechanisms for triplet quenching by H_2O_2:

(i) Electron transfer from the excited dye to H_2O_2 with the formation of a dye radical cation and OH,

(ii) Electron transfer from H_2O_2 to the dye molecule with the formation of the dye radical anion and $HO_2^{\cdot}$ radical or

(iii) the quenching of the triplet excited state by H_2O_2 without the formation of any products.

The results presented in this paper illustrate that illumination of *S. leopoliensis* in the presence of an MB photosensitiser and H_2O_2 results in the degradation of both the cyanobacteria and the photosensitiser. Under an oxygen free atmosphere an increase in the activity of the MB/H_2O_2 system was observed and it was proposed that the process involves a type I mechanism with electron transfer from MB methylene blue to H_2O_2 generating active radical species. The process of cell death also appears to involve the release of a fluorescing component from the plant cell in the initial stages of the destruction of the cyanobacteria. The results presented here support the proposal that PDT could be applied to plant cells for the removal of algae or cyanobacteria on building surfaces.

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List of captions for Figures

Figure 1: Dark control experiments on the effect of MB in combination with H_2O_2 on the fluorescence of *S. leopoliensis*. The results are reported as % fluorescence vs. time with the initial reading recorded as 100%. (□ MB ♦ No biocide Δ H_2O_2 x MB + H_2O_2).

Figure 2: The effect of illumination, MB and MB with H_2O_2 on the fluorescence of *S. leopoliensis* under aerobic conditions. (Δ H_2O_2 ■ MB ◊ No biocide x MB + H_2O_2).

Figure 3: Plot of % fluorescence of *S. leopoliensis* under aerobic and anaerobic conditions, in the presence of MB and H_2O_2. [♦ MB + H_2O_2 (light/air), □ MB + H_2O_2 (Light/Argon)]

Figure 4: Fate of MB during photo-activation of *S. leopoliensis*, in the presence of H_2O_2. (▲H_2O_2 ◊ No *S. leopoliensis*, ■ *S. leopoliensis*, x *S. leopoliensis* + H_2O_2).

Figure 5: Comparison of photodegradation of MB in the presence and absence of H_2O_2 and under dark and illuminated conditions. (♦ Dark, □ Dark + H_2O_2, Δ Light, x Light + H_2O_2).

Figure 6: Plot of % fluorescence of *S. leopoliensis* under aerobic and anaerobic conditions, in the presence of MB and H_2O_2. [♦ *S.Leopoliensis* + H_2O_2 (light/air), ■*S. Leopoliensis* + H_2O_2 (Light/Argon)]

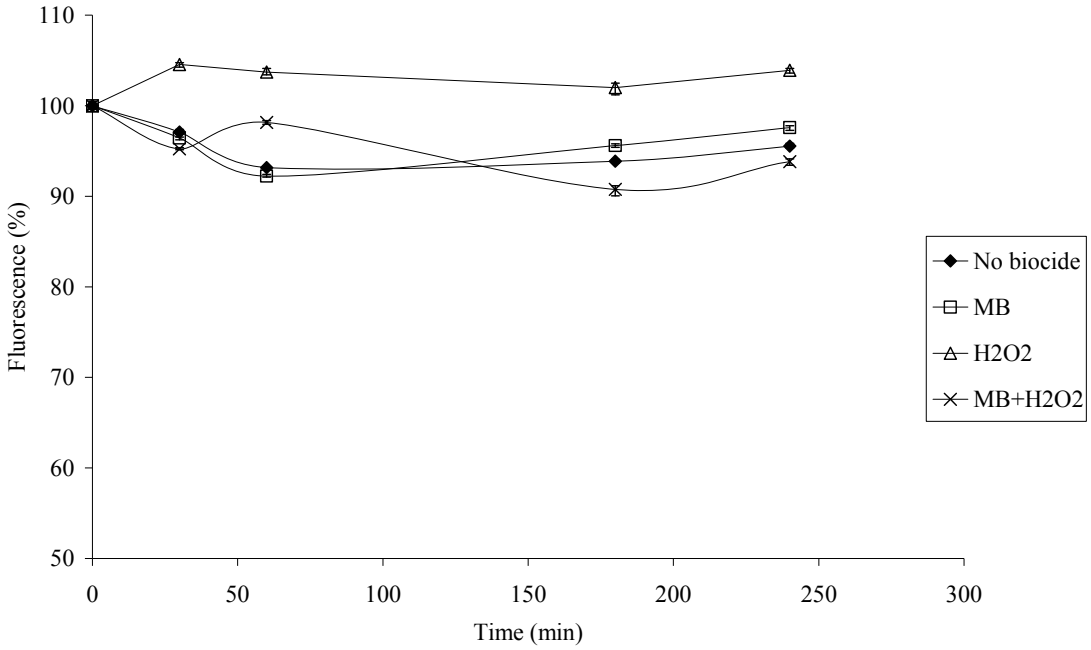
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Figure 1

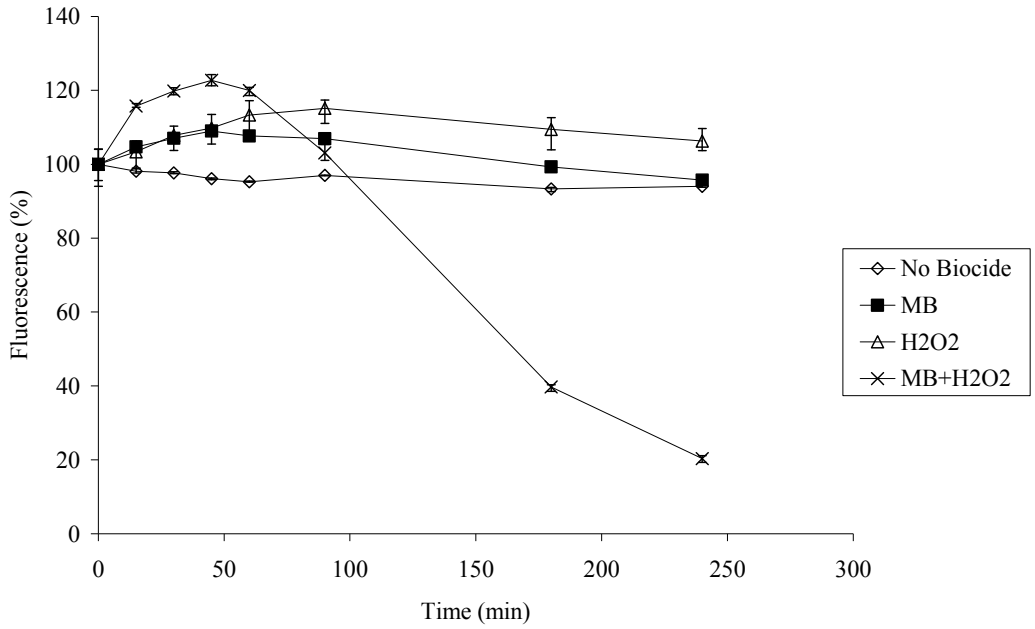
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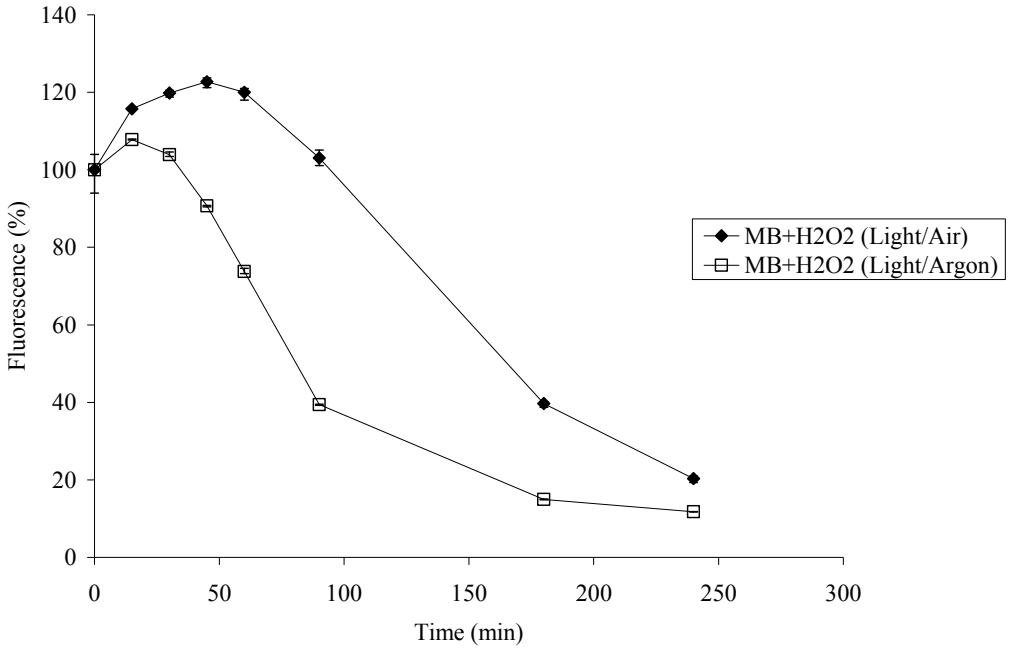
Figure 2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MB+H2O2 (Light/Argon)

Figure 3

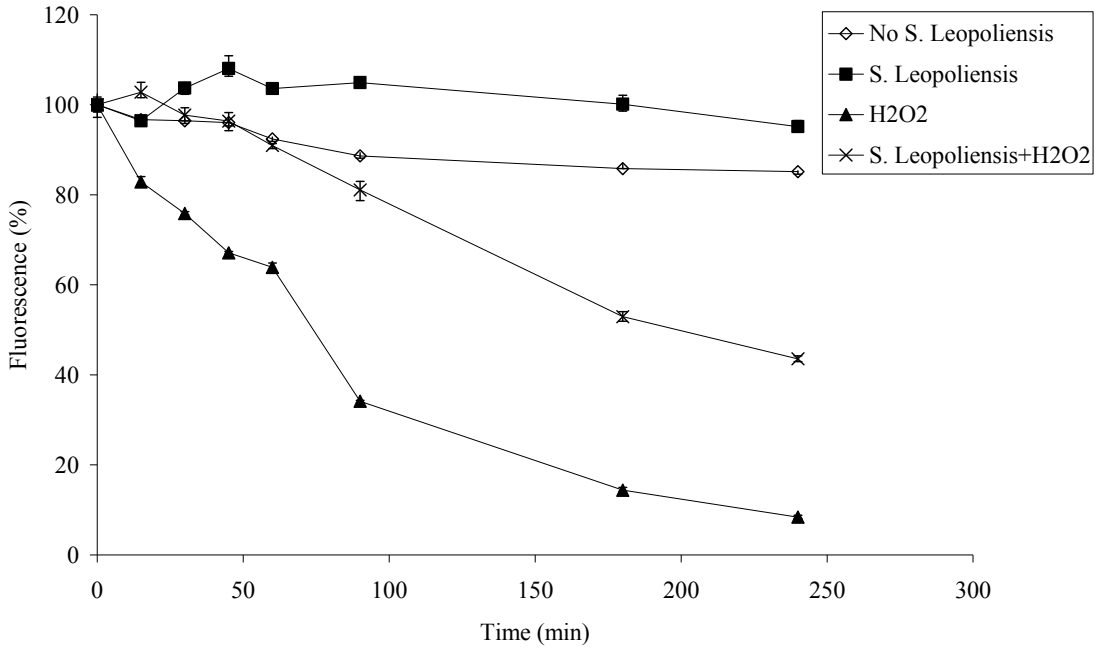
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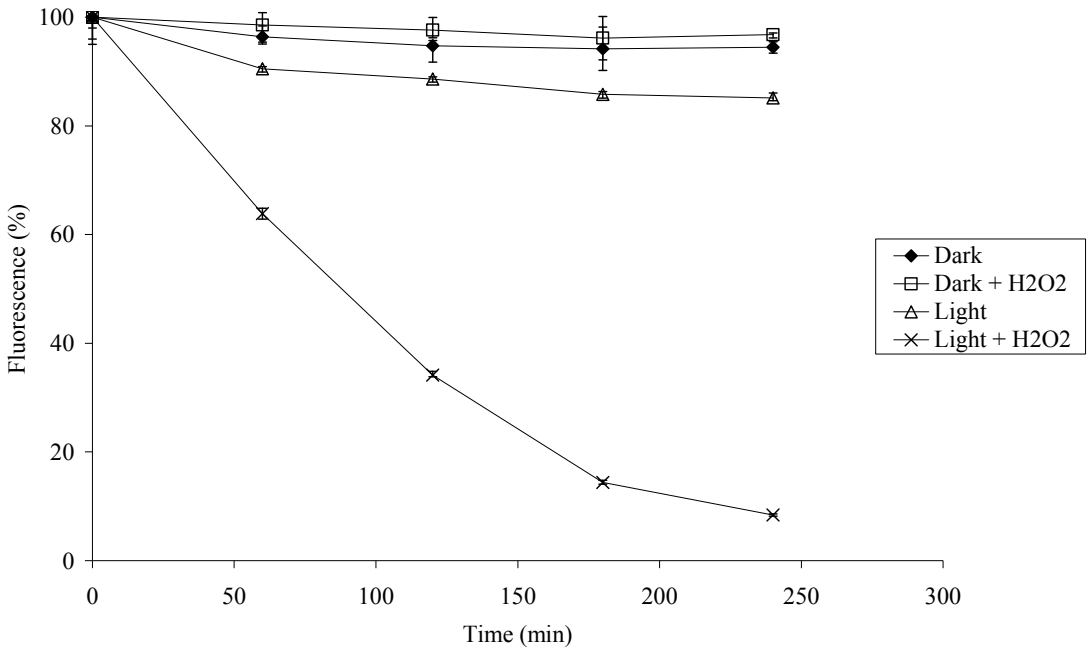
Figure 4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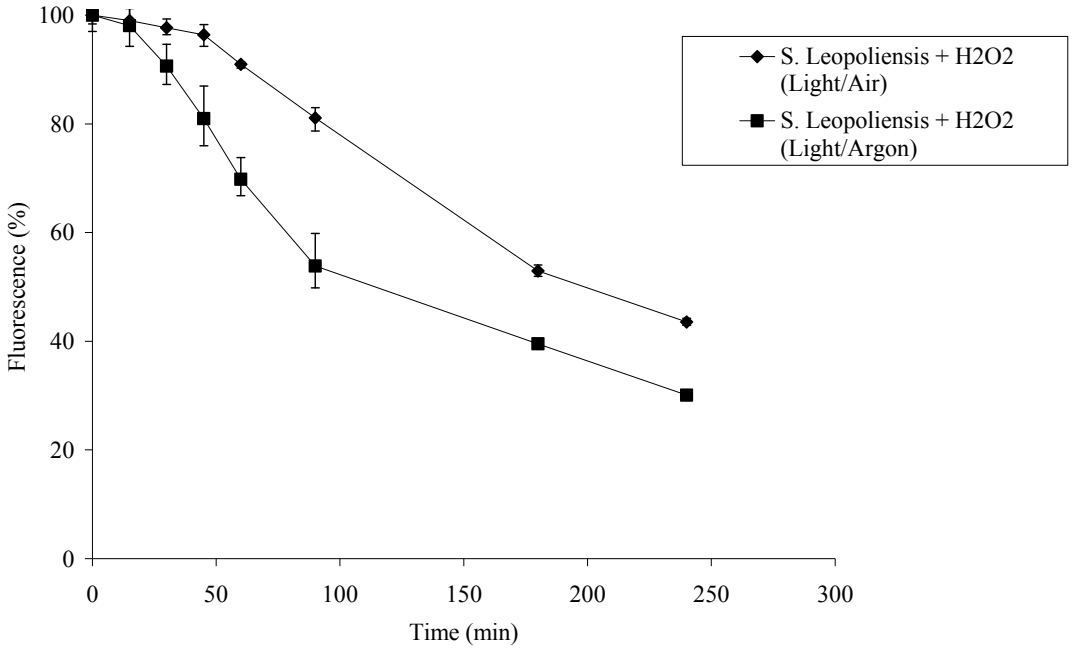
Light + H2O2

Figure 5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Time (min)
S. Leopoliensis + H2O2 (Light/Air)
S. Leopoliensis + H2O2 (Light/Argon)

Figure 6