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Photo-dynamic biocidal action of methylene blue and hydrogen peroxide on the cyanobacterium *Synechococcus leopoliensis* under visible light irradiation

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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 photo-dynamic 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.

Keywords: Methylene blue; *Synechococcus leopoliensis*; Photosensitiser; Hydrogen peroxide; Cyanobacteria; Biofilm

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 GDP, 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.

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Photo-dynamic 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]. Photo-dynamic 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 photo-dynamic reagent for the destruction of *Synechococcus leopoliensis* (*S. leopoliensis*), a cyanobacterium that colonises stone. The photoactivity 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 °C. The cyanobacterium was sampled in the stationary phase of growth.

2.2. Photochemical reactions

A series of concentrations of MB (1–6 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 ml) were exposed to illumination from a 500 W tungsten halogen lamp in open pyrex flasks for a period of 240 min. Samples were taken at either 15 or 30 min intervals. Each experiment was repeated three 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 min 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 540:720 nm and 667:691 nm, respectively.

3. Results

3.1. PDT of Synechococcus leopoliensis with methylene blue and H_2O_2

The concentration of MB (3 μM) 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 (Fig. 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 min to investigate the effect of light alone on the cyanobacteria (Fig. 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

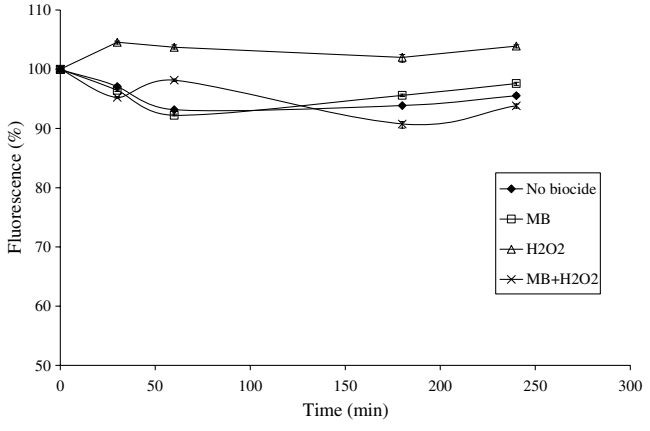

Fig. 1. Dark control experiments on the effect of MB in combination with H_2O_2 on the fluorescence of *S. leopoliensis*. The results are reported as % fluorescence vs. time with the initial reading recorded as 100% (□, MB; ◆, No biocide; △, H_2O_2; ×, MB + H_2O_2).