

Original Research

**BIOLOGICAL EFFECTS OF EVANS BLUE AND ITS DEGRADATION PRODUCTS
ON GROWTH AND VIABILITY OF THE GREEN MICROALGA**

***Chlamydomonas acidophila* PM01**

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Abstract

This study investigates the degradation of Evans Blue (EB) under UV irradiation and oxidative conditions, as well as the impact of its transformation products on the growth and viability of Chlamydomonas acidophila PM01. UV–Vis analysis showed that EB is stable under direct UV exposure, while the presence of Fe³⁺ and H₂O₂ significantly enhanced its degradation via photo-Fenton processes. Cellular response analysis showed that EB alone had no notable effect on algal growth. However, its degradation products containing EB, Fe³⁺, and H₂O₂ caused reduced cell density and increased physiological stress, while products formed under UVB irradiation significantly decreased viability. These findings suggest that although EB itself is relatively stable and non-toxic, its degradation products may exert considerable ecological impact.

Keywords: photodegradation, Evans Blue, toxicity, viability, *Chlamydomonas acidophila* PM01.

INTRODUCTION

The environmental fate and biological effects of dye degradation products remain insufficiently understood, particularly in aquatic systems where such compounds may interact with primary producers. While EB is widely used as a biological stain, its transformation under UV irradiation in oxidative conditions can lead to the formation of reactive intermediates with altered biological activity [1]. Green microalgae, such as *Chlamydomonas acidophila*, represent sensitive model organisms for assessing the impact of such compounds,

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as this extremophilic species exhibits high metabolic plasticity and tolerance to various environmental stressors, including heavy metals [2]. In this context, the present study investigates the effects of Evans Blue degradation products on the growth and cell viability of *C. acidophila* PM01.

MATERIALS AND METHODS

Reagents

The Evans Blue was purchased from Serva GmbH. Hydrogen peroxide (H_2O_2 , 30%) was obtained from Sigma-Aldrich, while $\text{FeCl}_3 \times 6\text{H}_2\text{O}$ was supplied by Centroprom. All experiments were carried out using bidistilled, deionized ultrapure water (18 M Ω). Stock solutions of EB (1 mM), FeCl_3 (1 mM), and H_2O_2 (10 mM) were prepared in deionized water. The pH of the solutions was adjusted to pH 5 using NaOH or HCl.

UV-Vis spectroscopy

The UV-visible (UV-Vis) spectra of samples were recorded from 200 to 800 nm using UV-Vis spectrophotometer Shimadzu UV-i1900 with a spectrometric quartz cell (1 cm path length). Samples were prepared in deionized water pH 5 (sample volume 1 mL), placed in a quartz cuvette (1 cm $\times$ 1 cm $\times$ 5 cm), and exposed to UV radiation up to 60 minutes using a lamp equipped with a filter for visible light (Carl Roth GmbH, Karlsruhe, Germany, H469.1), and UV tubes (Sankyo Denki, Tokyo, Japan). Measurements were carried out immediately after sample preparation (for non-irradiated systems), or 30 s after the end of UV irradiation. The UV A and UV B lamps emitted maximum radiation at wavelengths of 312 nm and 352 nm, respectively. The irradiation took place at 25 °C in a temperature-controlled chamber, and the irradiance was set at 17 J m⁻²s⁻¹ for UV A and 7.7 J m⁻²s⁻¹ for UV B.

Algal culture growth and viability assessment

The *Chlamydomonas acidophila* strain PM01 was originally isolated from acid mine drainage at Parys Mountain (Anglesey, North Wales, UK). Cultures were maintained under photoautotrophic conditions in modified MAM medium enriched with nutrients and prepared according to Olaveson and Stokes [3]. The medium was adjusted to an initial pH of approximately 3. For cultivation, microalgal inocula were transferred into 50 mL of MAM medium in 100 mL Erlenmeyer flasks at a starting density of 5×10^5 cells/mL. Cultures were incubated at 22 °C on an orbital shaker (120 rpm) under continuous illumination (120 $\mu\text{mol m}^{-2} \text{s}^{-1}$; MST TL-D Reflex 36W840 1 SLV/25, Philips, Amsterdam, The Netherlands). Growth progression was followed by measuring optical density at 750 nm and 680 nm (OD₇₅₀ and OD₆₈₀) until the early stationary phase was reached (15 days). Following cultivation, cultures were exposed for 24 h to degradation products of EB under defined conditions (10 μM EB, 20 μM Fe^{3+} , and 100 μM H_2O_2), alongside corresponding untreated controls. Biomass was estimated via OD₇₅₀, while OD₆₈₀ served as a proxy for chlorophyll a content [4]. Turbidity correction was performed using OD₇₅₀, and the OD₆₈₀/OD₇₅₀ ratio was used to estimate relative chlorophyll a content per biomass unit [5]. Absorbance measurements were carried out using a UV–Vis spectrophotometer (Jenway Genova Plus, Staffordshire, UK), with samples appropriately diluted to keep absorbance values below 1. Cell viability after exposure to EB degradation products was assessed using EB staining [6].

This assay identifies cells with compromised membranes, as non-viable cells accumulate the dye due to increased membrane permeability. Viability was expressed as the percentage of Evans Blue-negative cells, with at least 100 cells evaluated per sample.

RESULTS AND DISCUSSION

The UV-Vis spectra of EB exhibited characteristic absorption bands at 247, 311, 398, and 620 nm (Figure 1). These bands are associated with $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ electronic transitions of the aromatic rings, azo groups ($-\text{N}=\text{N}-$), and the extended conjugated chromophoric system [7]. The most intense band at 620 nm, located in the visible region, is responsible for the characteristic blue colour of the dye and serves as the main indicator of dye degradation during treatment.

Irradiation with UVA did not induce any changes in the UV-Vis spectrum of EB, while UVB irradiation caused only slight changes in absorbance intensity, indicating limited direct photolysis of Evans Blue under the applied conditions. These findings are in agreement with earlier studies demonstrating that azo dyes are generally resistant to degradation under UV irradiation [8].

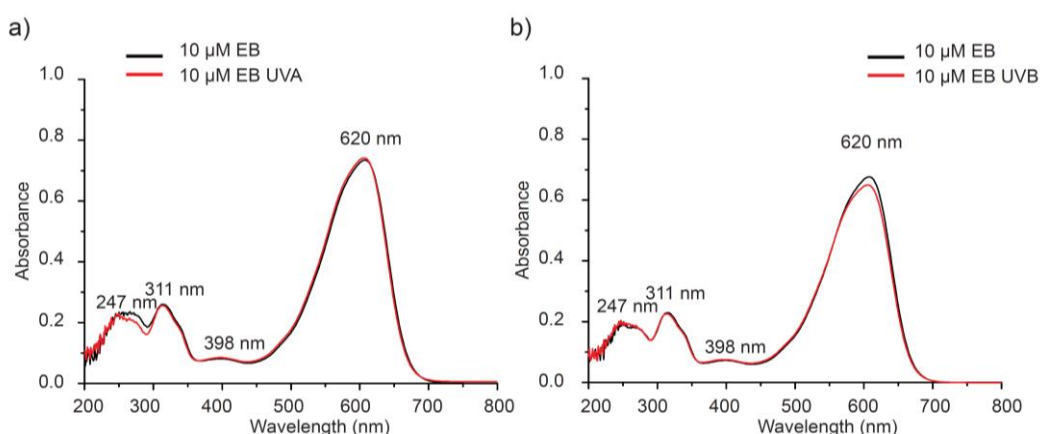


Figure 1 Effect of UV irradiation (30 min) on 10 μM EB in aqueous solution at pH 5 a) UVA irradiation; b) UVB irradiation

In the presence of Fe^{3+} ions, a gradual decrease in the absorbance at 620 nm was detected during both UVA and UVB treatment (20–60 min) confirming enhanced degradation compared with irradiation alone (Figure 2). This effect can be attributed to the formation of reactive oxygen species through iron-mediated photochemical reactions. Slightly higher efficiency under UVB irradiation was observed, likely due to its higher photon energy (Figure 2 b).

The highest degradation efficiency was achieved in the $\text{EB}/\text{Fe}^{3+}/\text{H}_2\text{O}_2$ systems under both UVA and UVB irradiation (Figure 2). A pronounced reduction of the 620 nm band with increasing treatment time indicated effective degradation of the azo chromophore. This behavior is associated with Fenton and photo-Fenton processes, where hydroxyl radicals are generated and rapidly react with the dye structure [8].

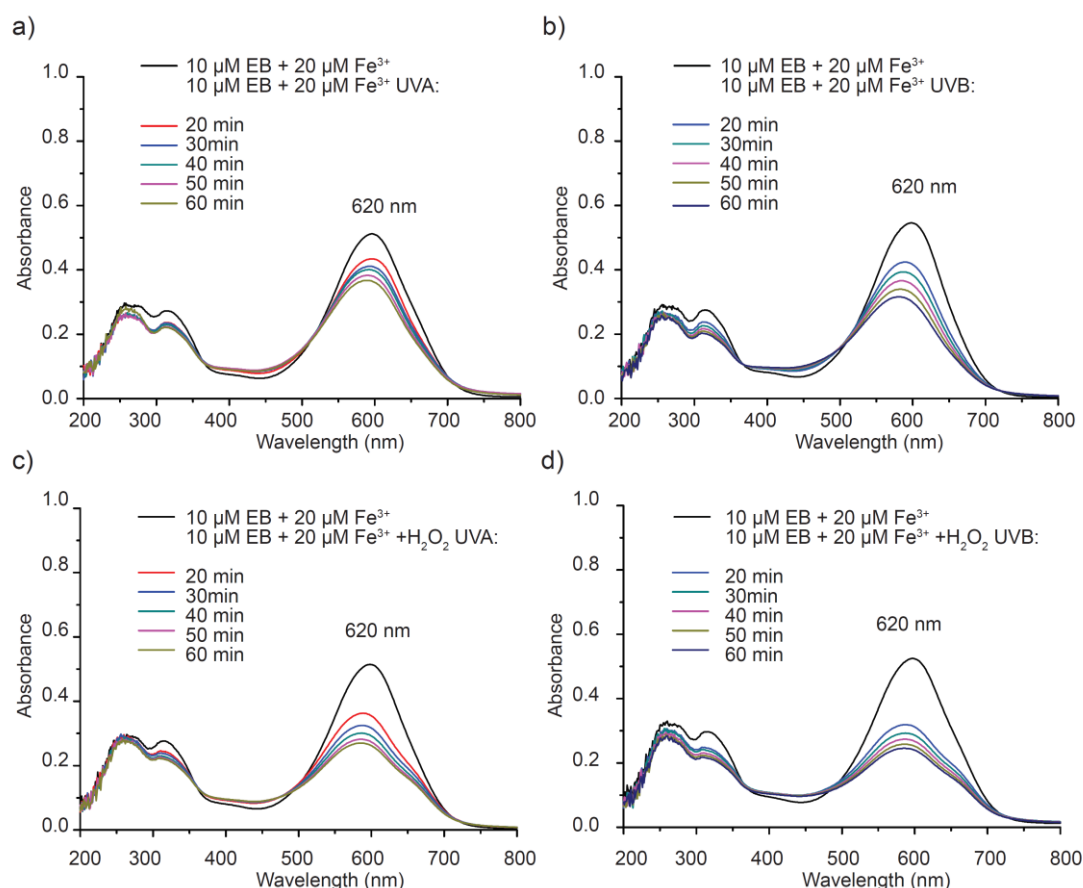


Figure 2 Effect of UV irradiation on a 10 μM EB aqueous solution in the presence of 20 μM Fe^{3+} at pH 5 under: a) UVA irradiation; and b) UVB irradiation, as well as in the presence of 20 μM Fe^{3+} and 100 μM H_2O_2 under: c) UVA irradiation; and d) UVB irradiation

Overall, the degradation efficiency followed the order:

$$\text{UV} < \text{UV}/\text{Fe}^{3+} < \text{UV}/\text{Fe}^{3+}/\text{H}_2\text{O}_2$$

These results demonstrate that UV-assisted Fenton-based processes significantly improve the removal of Evans Blue from aqueous solution compared with direct photolysis alone.

The effect of EB and its degradation products on the viability and growth of *Chlamydomonas acidophila* PM01 was further investigated. OD values displayed a consistent pattern independent of wavelength (Figure 3a). Although treatment with EB alone had no effect on OD, its degradation products did (Figure 4a). Fe^{3+} alone, as well as Fe^{3+} combined with EB and UVB or with EB, H_2O_2 , and UVB, modestly increased both OD_{680} and OD_{750} in *Chlamydomonas acidophila* PM01 cultures. Since the $\text{OD}_{680}/\text{OD}_{750}$ ratio remained comparable to the control, this indicates a proportional increase in biomass and chlorophyll content, rather than major changes in cellular physiology [9] (Figure 3a). On the other hand, treatment with EB in combination with Fe^{3+} and H_2O_2 , in the absence of UV radiation, led to a sharp decrease in both OD values, accompanied by an increase in the $\text{OD}_{680}/\text{OD}_{750}$ ratio (Figure 3a). This pattern suggests that this treatment primarily affected cell density rather than pigment integrity, resulting in a higher proportion of chlorophyll relative to biomass [5]. Iron availability and oxidative stress associated with H_2O_2 can influence pigment metabolism in microalgae, potentially inducing adaptive changes in chlorophyll and accessory pigment

content as part of stress acclimation responses [10,11]. Although OD_{680} and OD_{750} values in the Fe^{3+} or H_2O_2 treatment were similar to the control, the decreased OD_{680}/OD_{750} ratio indicates reduced chlorophyll content per unit biomass, suggesting impairment of the photosynthetic apparatus, likely due to oxidative stress associated with Fenton-type reactions, where Fe^{3+} is reduced to Fe^{2+} , enabling hydroxyl radical generation [12] (Figure 3a). Although the photosynthetic apparatus of *Chlamydomonas* species is generally resistant to the harmful effects of H_2O_2 , the magnitude and direction of the response are highly species-dependent and require optimization for targeted metabolite production [13]. However, oxidative stress may also lead to reduced pigment yields [14]. A marked reduction in viability was observed in *C. acidophila* PM01 following 24 h treatment of product of EB photodegradation by UVB radiation (Figure 3b). The observed outcomes may be attributed to the formation of multiple aromatic intermediates, which can exhibit altered biological activity and enhanced cytotoxicity compared to the parent dye [1]. Further, likewise was evident in samples treated with EB in combination with Fe^{3+} and/or H_2O_2 (Figure 3b). This effect could be explained by elevated oxidative stress resulting from high concentrations of highly reactive hydroxyl radicals, which consequently lead to cell death [15].

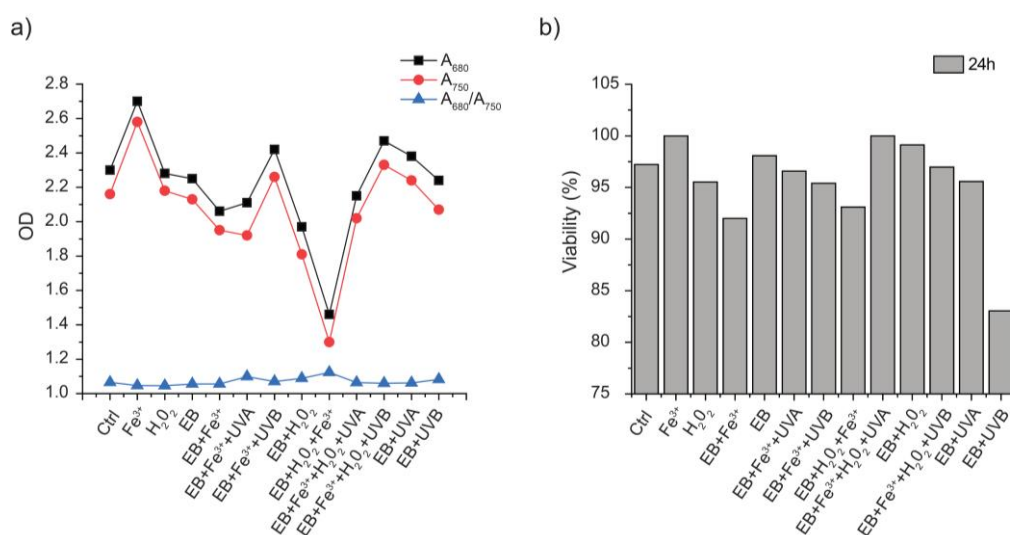


Figure 3 a) Growth of algal cultures, monitored by optical density at 750 and 680 nm, and b) cell viability evaluated using Evans Blue staining, 24 h after treatment with EB and its degradation products

CONCLUSION

The obtained results show EB stability in mildly acidic aqueous solutions under UVA irradiation, and its slight degradation under UV B irradiation. Additionally, EB/ Fe^{3+} / H_2O_2 processes significantly improve the removal of EB from aqueous solution compared with direct photolysis alone.

In *Chlamydomonas acidophila* PM01, most treatments caused only minor, wavelength-independent changes in OD_{680} and OD_{750} , indicating largely proportional changes in biomass and chlorophyll content without major physiological alterations. In contrast, EB combined with Fe^{3+} and H_2O_2 (without UVB) reduced cell density while increasing the

OD₆₈₀/OD₇₅₀ ratio, suggesting stress-induced shifts in pigment-to-biomass balance. These combinations also decreased viability, indicating that oxidative stress and reactive intermediates are the main drivers of cytotoxicity.

ACKNOWLEDGEMENT

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