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Enhancement of Biodegradation and Detoxification of Methylene Blue by Preformed Biofilm of Thermophilic Bacilli on Polypropylene Perforated Balls

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Abstract: Microbial degradation represents an eco-friendly alternative to traditional physicochemical treatments in removing persistent and toxic environmental pollutants, including synthetic dyes (i.e., methylene blue, MB) employed in different industries. The exploitation of thermophilic bacilli, such as those isolated from the shallow hydrothermal vents of the Eolian Islands (Italy), could provide valuable resources for the treatment of warm, dye-containing wastewater. In this study, we evaluated the ability of preformed biofilms on polypropylene perforated balls (BBs) of fifteen thermophilic bacilli, to decolor, degrade, and detoxify MB in aqueous solutions. Among them, BBs of *Bacillus licheniformis* B3-15 and *Bacillus* sp. s7s-1 were able to decolorize MB more than 50% in saline solution (NaCl 2%), incubated in static conditions at 45 °C for 48 h. At optimized initial conditions (10 mg L^{-1} MB, pH 5.2 for B3-15 or pH 4 for s7s-1), the two strains enhanced their decolorization potential, reaching 96% and 67%, respectively. As indicated by ATR-FTIR spectroscopy, the treatment with BB B3-15 was the most efficient in degrading the Cl–C and –NH groups of MB. This degraded solution was 40% less toxic than undegraded MB, and it has no impact on the bioluminescence of *Vibrio harveyi*, nor the growth of the marine diatom *Phaeodactylum tricornutum*. Biofilm formed by strain B3-15 on polypropylene perforated balls could be proposed as a component of bioreactors in the treatment of warm, dye-containing wastewater to concomitantly remediate MB pollution and simultaneously counteract harmful effects in aquatic environments.

Keywords: biofilm; bioremediation; decolorization; degradation; methylene blue; thermophiles

1. Introduction

Synthetic dyes are widely used in many economic activities, mainly for the textile industry and their products, to add color to cosmetics, food, paper, plastics, and in medical industries [1–3]. Thiazine dyes, including methylene blue (MB), are a class of electron-rich

tricyclic nitrogen–sulfur heterocycles with a low oxidation potential and a high propensity to form stable radical cations, and they are recalcitrant compounds able to resist light, chemicals, and microbial degradation. MB, a heterocyclic aromatic chemical compound, cationic, and thiazine-type dye, is widely applied in the textile industry, as a fibber-coloring agent, and also in the field of medicine, for prophylactic and therapeutic purposes [4,5]. Synthetic dyes persist for a long time once large amounts are released into environments, representing a threat to both environmental and human health [5–10]. Although there is no international consensus on the release of dye-containing wastewaters and their textile effluents, it is known that untreated synthetic dyes may decay water quality, reducing photosynthesis and negatively affecting the crop primary productivity and fertility of soils. Hence, industrial dye-containing wastewaters must be treated before their final discharge into the environments [11]. Traditional treatments to remediate industrial effluents include physical, chemical, and biological methods, such as adsorption, coagulation, flocculation and oxidation, and photolysis [12]. However, these methods can generate a large number of secondary pollutants, highly toxic and mutagenic in nature [13], during the treatment processes, posing the need to search for new and more efficient strategies to remediate water bodies from hazardous synthetic dyes and their metabolites.

Bioremediation operated by microorganisms, considered as not hazardous, eco-friendly, and ecologically sustainable, represents a profitable alternative to traditional treatments for the removal of thiazine dyes from environments [14–16]. The biological decolorization of dyes can occur in two different ways: through the adsorption on microbial biomass, and the degradation by enzymatic activity, leading to the production of simple and non-toxic species [17]. Biosorption may occur on living or dead microbial biomasses (such as of microalgae, fungi, or bacteria) due to the presence of extracellular products (such as exopolysaccharides and surfactants) that provide sites (such as carboxyl and hydroxyl groups) to bind dyes [18]. In contrast, the degradation of dyes is related to the presence of intracellular or extracellular enzymes, such as laccase [2], manganese peroxidase [19], lignin peroxidase [20], and reductases [21].

Under proper physical and nutritional conditions, bacteria are capable of developing faster and degrade dyes more efficiently than fungi or algae [22]. Several bacterial species, such as those belonging to the genera of *Aeromonas*, *Bacillus*, *Pseudomonas*, *Shewanella,* and *Serratia* were reported to decolorize, degrade, and detoxify different synthetic dyes [18,23].

The decolorization and biodegradation of MB have been previously reported for several *Bacillus* strains, including *B. subtilis* MTCC 441 [24], *B. thuringiensis* 016 [25], *B. thuringiensis* F5 [26], and *B. albus* MW 407057 [27]. Degraded metabolites were usually less toxic than the parent compounds, as reported for seeds of model plants (i.e., *Vigna radiata*, *Triticum aestivum*, *Sorghum bicolor*) ([28] and references therein) and microbes (i.e., *Photorhabdus luminescens*) [29] relevant in agriculture.

However, the use of bacteria is often limited by environmental conditions, since pH values, high salinity, temperature, and the concentration of dyes may affect the removal efficiency, inhibiting enzymatic activity. In spite of these problems, dye removal by biofilm-forming bacteria has gained great attention [30,31]. Biofilms are complex structures of bacterial cells encased in an extracellular self-produced matrix, mainly composed by exopolysaccharides (EPSs), firmly attached to biotic or abiotic surfaces [32]. Biofilm matrix may interact with different substances, including pollutants (such as hydrocarbons and heavy metals), through different mechanisms including sorption, binding, emulsification, solubilization, precipitation, ion exchange, and complexation [33–37]. In biofilm, bacteria can produce a different quantity of enzymes and exopolymers that are usually under-expressed during their free-living state ([32], and references therein). Therefore, dyes can be decolorized more efficiently by cells immobilized in their own biofilms than cells in free-living style. Natural or synthetic matrices are particularly suitable to entrap bacterial cells able to decolor dyes, since they create a local anaerobic microenvironment also favoring anaerobic degradation [38]. Biofilms developed on different substrates were evaluated to remove dyes in bioreactors, for example *B. licheniformis* on polyurethane foam [29], *Bacillus*

sp. on sodium alginate [39], *B. subtilis* MTCC 441 on calcium alginate [24], *Bacillus* sp. MH587030.1 on plastic carriers [40], and *B. amyloliquefaciens* DT on filter paper [41].

Wastewater discharges can have high temperatures (50–80 °C) and a high salinity that can inhibit cell viability and reduce enzyme activity [42,43]. Extremophilic microorganisms, such as those from the shallow hydrothermal vents of the Eolian Islands (Italy), could represent valuable resources to overcome the major bottlenecks in the treatment of dye-containing wastewater. Thermophilic *Bacillus* and *Geobacillus* spp., isolated from shallow hydrothermal vents of the Eolian Island (Italy), are able to produce different enzymes [44] and exopolymers (i.e., exopolysaccharides) with interesting physical, chemical, and rheological properties, such as thermostability and bioactivities, that could be useful in several industrial applications [34–37,45–49]. In an ongoing search for a valuable strategy to mitigate MB pollution, in this study, we evaluated the ability of thermophilic, biofilm-forming bacilli on polypropylene perforated balls (BBs), as easily available, cost-effective and reusable substrata, providing a larger surface area than regular balls, for the development of biofilms, to decolor, degrade, and detoxify MB in aqueous solution. To these purposes, BBs of thermophilic bacilli were investigated for their ability to: (i) decolorize MB, as the sole carbon and energy source, at different concentrations and pH values; (ii) degrade MB by enzymatic activities, and the preliminarily characterization of MB degradation products, using spectroscopic technique (ATR-FTIR); and finally, (iii) detoxify MB by the inhibition of *Vibrio harveyi* bioluminescence and of *Phaeodactylum tricornutum* viability.

2. Materials and Methods

2.1. Chemicals

Methylene blue was purchased from Sigma-Aldrich (St. Luis, MD, USA). The perforated polypropylene balls, with the dimensions 23.8 × 20 × 9.2 mm and a weight equal to 1.21 ± 0.01 g, were purchased from Spectrum Brands (Manchester, UK). All the other chemicals were purchased from Sigma Aldrich (Milan, Italy).

2.2. Bacterial Strains

Thermophilic bacterial strains (15) used in this work were previously isolated from hydrothermal fluid and sediment samples collected from the vents of Eolian Islands (Italy) [46,47,50] (Table 1) and are part of the collection of the "Research Centre for Extreme Environments and Extremophiles" at the Department of Chemical, Biological, Pharmaceutical, and Environmental Sciences of the University of Messina (Italy). All strains are routinely grown on plates of Tryptic Soy Agar (TSA, Sigma Aldrich, Milan, Italy), plus 1% NaCl (1.5% NaCl final concentration, TSA1.5), and maintained frozen at 80 °C in 40% (*v*/*v*) glycerol for long-term storage. Partial 16S rRNA gene sequences of B3-15 and s7s-1 were submitted to GenBank under accession numbers KC485000 and PQ037032, respectively.

Table 1. Characteristics of samples collected from the hydrothermal vents of Eolian Islands (Italy), and designation of the 15 isolated bacilli.

Island	Site	Station and Sample Type (F, S)	Depth (m)	Temp (°C)	pH	Isolate
Vulcano	Levante Harbor	A (F)	0.3	43	5.2	A1-3
		B3 (F), T3(S)				B3-15, B3-18, B3-24, B3-28, B3-75, T1-3, T3-2
Panarea	Pta Conigliara	4 (F)	15	45	6.1	md4-1-1, md4-1-2
	La Calcara	7 (S)	19.8	95	5.1	s7s-3ng, s7s-1, s7s-3g, s7s-5
	Black point	BP (S)	23	130	3.3	SBP3

2.3. Screening for MB Decolorization

To screen the ability of the isolates to decolor MB, the method described by Kishor et al. [2] was performed with some modifications. MB was added at a final concentration of 10 mg L^{-1} to TSA1.5. Strains were inoculated in three replicates and, after incubation

at 45 °C for 24 and 48 h, the formation of a clear halo around each colony was considered positive for MB decolorization.

2.4. Selection of Biofilm-Forming Strains on Polypropylene Balls

To evaluate the selected strains' biofilm formation on polypropylene perforated balls, aliquots of each strain culture (2 mL), grown overnight in Tryptic Soy Broth with 1.5% NaCl final concentration (TSB1.5) (OD_{600nm} = 0.1), were added to 20 mL of fresh TSB1.5 in a 200 mL flask containing ten balls, sterilized by autoclaving. The flasks were incubated for 12 h at 45 °C without shaking to allow stable biofilm formation. After the incubation, each ball was placed on a sterile Petri plate and washed five times with distilled sterile water to remove non-adherent bacteria. Adherent bacteria were stained with 0.1% crystal violet solution (*w*/*v*) for 25 min, as reported by O'Toole [51]. To remove the stain in excess, the biofilm fixed on balls (BBs) were washed (five times) with sterile distilled water and airdried for 15 min. The crystal violet in the stained biofilms was solubilized with absolute ethanol (Sigma Aldrich, Milan, Italy). The determination of the biofilm mass, as the level of crystal violet present in each de-staining solution, was estimated spectrophotometrically (OD = 585 nm) using a microtiter plate reader (Multiskan Go, Thermo Scientific, Waltham, MA, USA). Average and standard deviation (SD) of six replicates were calculated.

To confirm, the biofilm formation sections of perforated balls were washed five times and then stained with SYTO9 (Sigma Aldrich) and observed under a fluorescent microscope (Olympus BX60, Oaza-Odakura-Aza-Okamiyama, Japan).

2.5. MB Decolorization of Bacterial Biofilm Fixed on Ball (BBs)

To evaluate the MB decolorization ability of preformed biofilm on balls, each BB was incubated in 20 mL of 2% NaCl solution supplemented with MB at different concentrations (1, 10, and 100 mg L^{-1}) and incubated at 45 °C up to 48 h. MB solutions without BBs were used as control. After 48 h, an aliquot of each solution (1 mL) was centrifuged 8000× *g* for 10 min, and the absorbance (OD_{664nm}) of each supernatant was measured. The MB content was estimated according to Bharti et al. [30] as follow:

$$\text{MB (\%)} = \text{OD}_{664\text{nm}} \text{ sample}/\text{OD}_{664\text{nm}} \text{ control} \times 100 \quad (1)$$

2.6. Optimization of Physico-Chemical Conditions to Decolor MB

In order to determine the effects of the initial MB concentration (from 1 to 100 mg L^{-1}) and the pH value (from 4 to 10) on the overall MB decolorization, expressed in percentage (MB%), the Response Surface Methodology—Central Composite Design (RSM-CCD) was employed, using R software (v.4.3.2) package rsm. A set of 15 experiments were carried out as a batch decolorization assay for each selected strain, and the interactions among the variables, expressed as quadratic model equations, were used to predict the optimal operational conditions to decolor MB. Analysis of variance (ANOVA) was used to establish the proposed model's suitability.

2.7. Laccase and Lignin Peroxidase Activities

After the MB solution treatment with biofilms on polypropylene perforated balls of selected strains, 2 mL from each decolorized solution was collected at different times, centrifuged at 12,000× *g* at 4 °C for 20 min, and in each supernatant the laccase and lignin peroxidase activities were determined over the time (48 h), as described by Wu et al. [26].

To assay the laccase activity [52], 1.0 mL of each supernatant was added to 1 mL of a solution containing 0.6 mL of citrate buffer (pH 5.0, 100 mM), 0.2 mL of distilled water, and 0.2 mL to of 2,2′-azino-bis 3-ethylbenzothiazoline-6-sulfonic acid (ABTS, 1.0 mM).

To assay the lignin peroxidase activity [53], 0.5 mL of each supernatant was added to 1.5 mL of reaction solution containing 500 µL of veratrole (10 mM), H_2O_2 (2 mM), and tartaric acid buffer (pH 3.0, 125 mM). The laccase and lignin peroxidase was determined

spectrophotometrically, measuring absorbance (OD) at 420 nm and 310 nm, respectively. The enzymatic activity was expressed as U mL^{-1} in the following formula:

$$\text{Laccase}\left(\text{U mL}^{-1}\right) = \Delta abs420 \times Vt/(\varepsilon \times l \times Vs) \quad (2)$$

$$\text{Lignin peroxidase}\left(\text{U mL}^{-1}\right) = \Delta abs310 \times Vt/(\varepsilon \times l \times Vs) \quad (3)$$

where Δabs represents the difference between the OD of each tested supernatant and each control (without supernatant); ε indicates the extinction coefficient of ABTS (ε = 36,000 M^{-1} cm^{-1}) or veratrole (ε = 93,000 M^{-1} cm^{-1}), Vt is the total volume of reaction mixture (mL), Vs represents the volume of the sample (mL), whereas l is the length of the cuvette (1 cm).

2.8. Preliminarily Characterization of MB Degradation Products by Attenuated Total Reflectance Fourier Transform Infra-Red (ATR-FTIR)

The degradation of MB after the treatment of each strain with BBs was evaluated according to Haque et al. [54]. Preliminarily, aliquots (10 mL) of each decolorized solution were centrifuged (10,000× *g*, 20 min, at room temperature) and filtered through nitrocellulose membranes (pore size 0.45 µm) to remove bacterial cells. The cell-free supernatants were freeze-dried and analyzed in triplicate by Attenuated Total Reflectance Fourier Transform Infra-Red (ATR-FTIR) performed with VERTEX 70 V (Bruker Optics GmbH&Co., KG, Ettlingen, Germany) in the mid-IR region of 400–4000 cm^{-1}, as described in Caccamo et al. [45,55]. The analysis of FTIR spectra was carried out using OMIC software v.9.8 (Origin Lab Co., Northampton, MA, USA).

2.9. Toxicity Assays

The toxicity of untreated MB and decolored solutions after treatment with BBs was tested using inhibition assays of bioluminescence and viability of the marine diatom *Phaeodactylum tricornutum*.

Preliminarily, each decolored solution was centrifuged (10,000× *g* for 20 min, at room temperature) and the cell-free supernatants were freeze-dried and finally dissolved in distilled water to give 10 × stock solutions.

The bioluminescence inhibition assay was performed as previously described in Spanò et al. [34]. Briefly, *Vibrio harveyi* G5 was inoculated in the standard medium Sea Water Complete and incubated at 25 °C overnight. Each well of a 96-well microtiter plate was filled with 80 µL of the *V. harveyi* overnight culture (OD_{600nm} = 0.5, equivalent to 5 × 10^8 bacteria mL^{-1}) and 20 µL from each decolorized 10 × stock solution. After 15 min of incubation at 25 °C, the luminescence of the bacterial cell suspension was evaluated by Luminoskan™ Ascent™ Microplate Luminometer (Thermo Scientific) and expressed as a relative luminescence unit (RLU), calculated as follows: RLU = luminescence/OD_{600nm}. The Effective Concentration (EC_{50}), as 50% of RLU reduction, was used to indicate the toxicity of decolorized solutions after treatment with the BBs.

Phaeodactylum tricornutum Bohlin NIVA BAC 2 was purchased from the Norwegian Institute for Water Research (Oslo, Norway). The strain was maintained in f/2 medium, containing 983.5 mL of synthetic sea water (NaCl 22 g L^{-1}, $MgCl_2$ 6 H_2O 9.7 g L^{-1}, Na_2SO_4 3.7 g L^{-1}, $CaCl_2$ 1 g L^{-1}, KCl 0.65 g L^{-1}, $NaHCO_3$ 0.2 g L^{-1}, H_3BO_3 0.023 g L^{-1}), 15 mL of micronutrient solution A ($FeCl_3$ 6 H_2O 48 mg L^{-1}, $MnCl_2$ 4 H_2O 144 mg L^{-1}, $ZnSO_4$ 7 H_2O 45 mg L^{-1}, $CuSO_4$ 5 H_2O 9.8 mg L^{-1}, H_3BO_3 1140 mg L^{-1}, Na_2EDTA 1000 mg L^{-1}), 0.5 mL of vitamin solution (thiamin hydrochloride 50 mg L^{-1}, Biotin 0.01 mg L^{-1}, vitamin B12 0.1 mg L^{-1}), and 1 mL of solution B (K_3PO_4 3 g L^{-1}, $NaNO_3$ 50 g L^{-1}, Na_2SiO_3 5 H_2O 14.9 g L^{-1}), according to ISO10253 [56].

Briefly, each well of the 24-well plate (three replicates) was filled with 1800 µL of *P. tricornutum* culture (10^4 cell mL^{-1}), grown in f/2 medium for 72 h, and 200 µL of each decolorized stock solution was added to each well. Sterile f/2 medium was used as a

negative control. The 24-well plate was incubated at 20 ± 2 °C and 6000–10,000 lux for 72 h. After 72 h of exposure, algal cell density was spectrophotometrically determined (OD_{670nm}).

2.10. Statistical Analysis

The experiments were performed in triplicate and data are expressed as averages and standard deviations or relative errors. The two-way ANOVA and Tukey's test was used for post hoc analysis (GraphPad Software Inc., La Jolla, CA, USA) to compare different experimental groups. All statistical values were considered significant at $p \leq 0.05$.

3. Results

3.1. Screening for MB Decolorization

The ability of the tested strains to grow in the presence of MB (10 mg L^{-1}) and to decolor it after incubation at 45 °C for 48 h is reported in Table 2.

Table 2. Growth of 15 thermophilic strains on TSA1.5 containing MB (10 mg L^{-1}). The ability to decolor MB is expressed as diameter of haloes around each colony: ≥3 mm (++), 1–2 mm (+), and none (-).

Strain	Growth	Halo
Bacillus sp. A1-3	+	++
B. licheniformis B3-15 [a]	+	++
Bacillus sp. B3-18	+	+
Bacillus sp. B3-24	+	+
Bacillus sp. B3-28	+	+
Geobacillus thermodenitrificans B3-72 [b]	+	++
Bacillus sp. B3-75	+	+
Bacillus sp. B3-76	+	+
B. licheniformis md4-1-1	-	-
Bacillus sp. md4-1-2	-	-
Bacillus sp. T1-3	+	+
G. thermodenitrificans S1	+	-
Bacillus sp. S1-1	+	-
B. licheniformis s7s-1	+	++
B. horneckiae SBP3 DSM 103063 [c]	+	++

[a] Maugeri et al. [47]; [b] Nicolaus et al. [57]; [c] Zammuto et al. [50].

Almost all strains (13/15) grew in the presence of MB, and five of them exhibited clear haloes ≥3 mm in diameter (Table 2). Moreover, the five most promising strains showed phenotypic mucoid on the agar plate, suggesting potential in biofilm formation.

3.2. Selection of Biofilm-Forming Strains on Polypropylene Balls

The ability of the selected five strains to form biofilm on polypropylene balls (BBs) is shown in Figure 1.

All strains were able to produce biofilms on BBs, although with different capabilities (Figure 1), as also confirmed by microscopic observations (Figure S1). *Bacillus licheniformis* B3-15 (OD_{585nm}= 0.985 ± 0.044) and *Bacillus* sp. s7s-1(OD_{585nm} = 0.822 ± 0.09) produced the highest biofilm mass on BBs after 12 h of incubation at 45 °C.

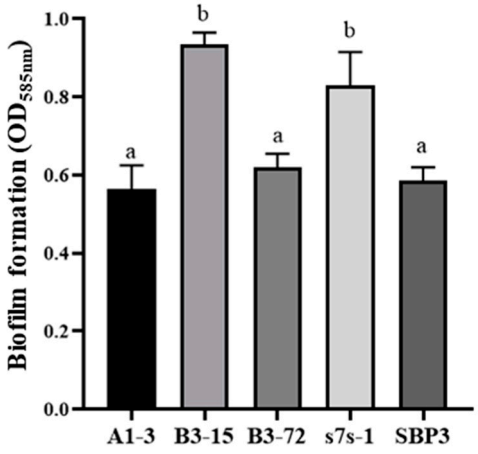

Figure 1. Biofilm formation by five selected strains on perforated polypropylene balls after 12 h of incubation at 45 °C. The bars represent the mean ± standard deviation for six replicates. Statistical differences were evaluated using a two-way ANOVA with Tukey's multiple comparisons test. Different lowercase letters above the bar graph indicate significant statistical differences ($p \leq 0.05$).

3.3. MB Decolorization by Biofilms on Polypropylene Perforated Balls (BBs)

The decolorization of MB (10 mg L^{-1}) by the preformed biofilms of five selected strains on polypropylene perforated balls (BB) in saline solution (2% NaCl) is shown Figure 2.

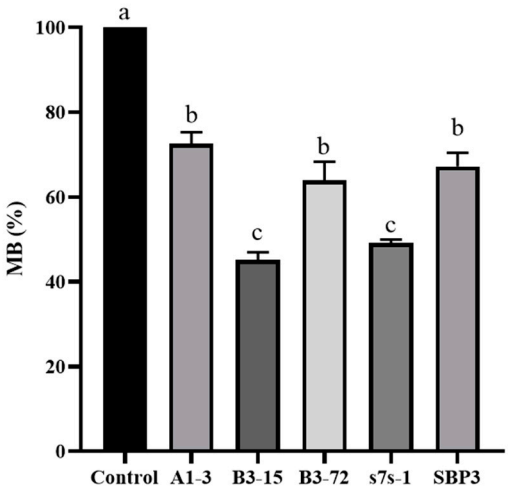

Figure 2. MB decolorization (expressed in percentage) after treatment with biofilms on polypropylene perforated balls of five strains, after 48 h incubation at 45 °C. Statistical differences were evaluated using a two-way ANOVA with Tukey's multiple comparisons test. Different lowercase letters above the bar graph indicate significant statistical differences ($p \leq 0.05$).

After incubation at 45 °C for 48 h, BB B3-15 was the most efficient to decolor MB (57 ± 1.8%), followed by BB s7s-1 (51 ± 2.6%), BB SBP3 (39.9 ± 1.4%), BB B3-72 (38 ± 2.6%), and BB A1-3 (26 ± 1.2%). As the most active in the MB decolorization, the two strains B3-15 and s7s-1 were further investigated to optimize cultural conditions.

3.4. Optimization of Physico-Chemical Conditions to Decolor MB

The effects of the initial concentration of MB (from 10 to 100 mg L^{-1}) and pH (4–10) on decolorization by BBs of B3-15 and s7s-1 are reported in Table 3, as evaluated by a response surface methodology (RSM-CCD) model.

Table 3. Experimental setup designed by RSM-CCD with predicted and experimental responses (MB decolor).

Run	Initial Methylene Blue (mg L^{-1})	PH	BB B3-15 Decolor MB Predicted (%)	BB B3-15 Decolor MB Experimental (%)	BB s7s-1 Decolor MB Predicted (%)	BB s7s-1 Decolor MB Experimental (%)
1	55	4	60.1	62.3	10.3	0.5
2	55	7	75.0	72.3	25.1	21.2
3	10	4	86.1	86.3	64.6	4.6
4	100	7	94.7	91.9	38.8	5.8
5	55	4	95.4	91.3	29.5	1.5
6	55	10	81.7	80.3	29.2	0.2
7	10	7	52.5	57.8	59.1	51.1
8	100	4	96.7	96.3	29.6	40.6
9	55	7	85.6	81.8	18.5	20.5
10	55	10	32.9	30.6	28.0	2.1
11	100	7	90.8	88.3	65.8	78.9
12	10	10	40.1	36.8	1.5	4.6
13	55	7	53.1	51.8	21.6	22.6
14	10	7	51.8	59.5	59.5	51.5
15	100	10	50.181	44.1	41.4	38.4

Moreover, the obtained results based on the RSM-CCD model are shown in a 3D surface plot (Figure 3). The equations derived by the RSM-CCD model predicted the MB removal percentage by BB B3-15 (1) and BB s7s-1 (2) biofilms as function of the initial MB concentration (mg L^{-1}) and pH value in the ranges investigated. The models suitability can be assessed by the reasonable regression value coefficients R^2 equal to 0.920 and 0.890, respectively.

$$\text{MB removal (\%)} = 78.85 - 0.48\ \text{MB} + 9.5\ \text{pH} + 0.00284\ \text{MB} \times \text{MB} - 1.07\ \text{pH} \times \text{pH} + 0.002\ \text{MB} \times \text{pH} \quad (4)$$

$$\text{MB removal (\%)} = 26.4 - 1.497\ \text{MB} + 10.7 \times \text{pH} + 0.00601\ \text{MB} \times \text{MB} - 1.01\ \text{pH} \times \text{pH} + 0.0731\ \text{MB} \times \text{pH} \quad (5)$$

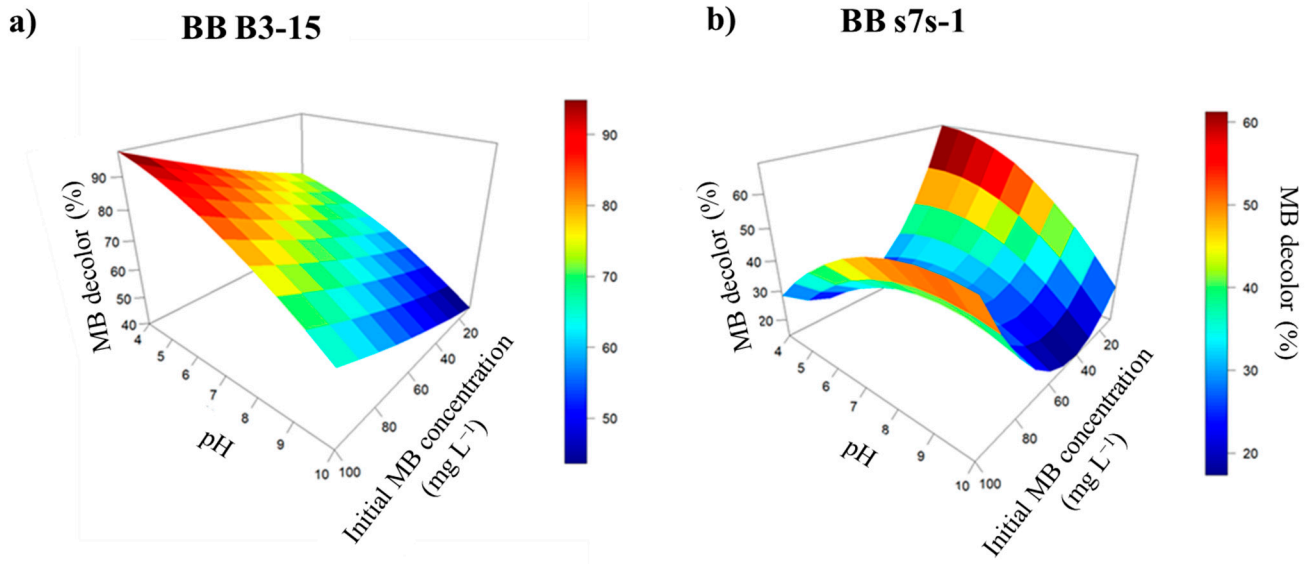

Figure 3. Response surface graph showing the effect of the initial methylene blue (MB) and pH in removing MB of BB B3-15 (**a**) and BB s7s-1 (**b**).

Based on RSM analysis (Figure 3), the BB B3-15 was able to decolor 96% of MB at the following operating conditions: 10 mg L^{-1} of initial MB concentration and pH 5.2. Meanwhile, that of BB s7s-1 decolored 67% of MB at a pH equal to 4, after incubation at 45 °C for 48 h (Figure 4). Moreover, BB B3-15 was able to decolor MB more rapidly than BB s7s-1, reaching more than a 50% removal rate after 24 h.

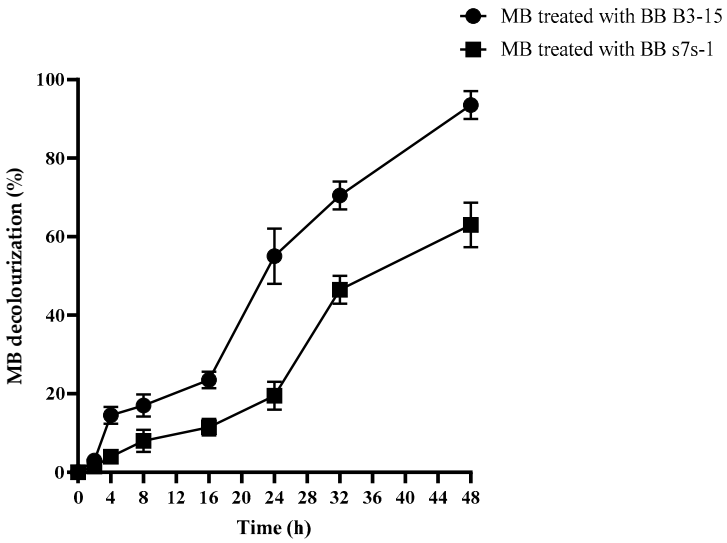

Figure 4. MB decolorization after treatment with BB B3-15 or BB s7s-1 at each optimal operating condition (MB 10 mg L^{-1}, pH 5.2 and 4, respectively). The data points are expressed as averages and standard deviations (n = 3).

3.5. Enzymatic Activity

The laccase and lignin peroxidase activities during the MB treatment with BB B3-15 and BB s7s-1 are reported in Figure 5.

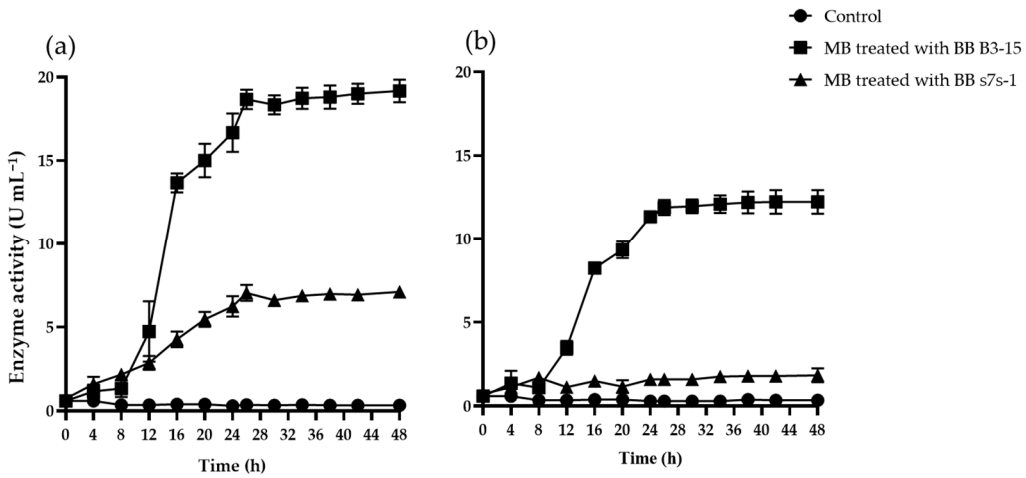

Figure 5. Activities of laccase (**a**) and lignin peroxidase (**b**) during the MB treatment with BB B3-15 and BB s7s-1.

The laccase activity of both strains increased significantly over the time, with the maximum levels reached after 26 h, which remained quite constant up to 48 h. The laccase activity of BB B3-15 was higher than that of BB s7s-1 over the whole period, with maximum levels of 19.16 ± 1.1 U mL^{-1} and 7.1 ± 0.3 U mL^{-1}, respectively (Figure 5a). Meanwhile, the lignin peroxidase activity of BB s7s-1 was negligible over time (Figure 5b), and that of BB B3-15 reached its maximum level (12.2 ± 0.4 U mL^{-1}) after 26 h and remained stable for

up to 48 h. These results indicate that BB B3-15 possess a greater enzymatic efficiency in degrading MB than BB s7s-1.

3.6. Preliminarily Characterization of MB Degradation Products by Attenuated Total Reflectance Fourier Transform Infra-Red (ATR-FTIR)

The ATR FTIR spectra of each MB solution collected before and after the treatment with each of the BBs and then lyophilized are shown in Figure 6.

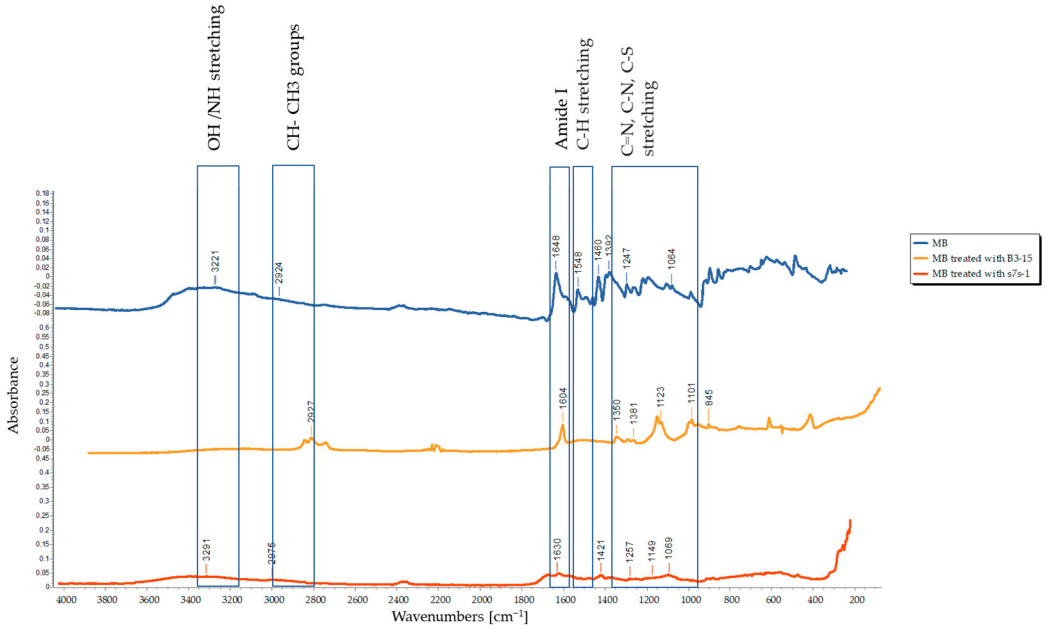

Figure 6. ATR-FTIR spectra of untreated methylene blue (MB), and MB treated with BB B3-15 or BB s7s-1.

Significant changes in the ATR-FTIR spectra of treated solutions compared to untreated ones indicated that biodegradation occurred. The untreated MB exhibits distinctive peaks near 3220 cm^{-1}, 2920 cm^{-1}, 1648 cm^{-1}, 1548 m^{-1}, 1440 cm^{-1}, and 802 cm^{-1}, attributed to MB dye (Figure 6) according to Wu et al. [26]. The peaks observed at 3220 and 2920 cm^{-1} were attributed to the overlapping of –OH and/or –NH stretching vibrations and CH_3 groups of aliphatic chains. The peaks at 1648 cm^{-1} and 1548 cm^{-1} belong to the stretching band of C–O, C–N from the amide I, and the symmetrical stretching band of carboxyl (–COOH), respectively. After the treatment with BB B3-15, the MB spectra showed notable alterations, and the peaks associated with NH stretching (3221 cm^{-1}), C–O/C–N groups, and C–Cl groups vanished, indicating that the heterocyclic structure was destroyed, which is consistent with the results of spectrophotometric analysis. Moreover, the peaks associated with C–O and N–H exhibited a shift towards lower wavenumbers, suggesting bond breakage. Differently, the spectrum of MB treated with the biofilm of s7s-1 showed shifts in the peaks attributed to N–H, CH_2, C–O, C–N, and Cl–C groups. Additionally, one peak corresponding to amide I disappeared, providing further evidence for the observed decolorization. The disappearance and the appearance of new peaks in the ATR-FTIR spectra explained that the MB was degraded, and new metabolites were produced after degradation. While the ATR-FTIR study indicates that MB molecules undergo a breakdown process, it is important to note that the resulting daughter compounds may also have harmful effects on some organisms.

3.7. Toxicity Assays

The bioluminescence inhibition assay is a fast and cost-effective test used to evaluate the harmful effects of different molecules even on higher organisms in different environ-

ments. The effects of BBs on MB and degraded solutions indicated using the luminescence of *V. harveyi* G5 are shown in Figure 7.

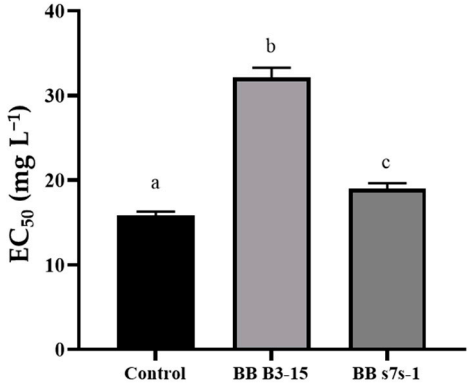

Figure 7. MB toxicity (expressed as EC_{50}) on *Vibrio harveyi* G5 luminescence after 15 min of incubation in the presence of undegraded MB (control) and BB B3-15- or BB s7s-1-degraded solutions. Statistical differences were evaluated using a two-way ANOVA with Tukey's multiple comparisons test. Different lowercase letters above the bar graph indicate significant statistical differences ($p \leq 0.05$).

The EC_{50} of the undegraded MB (control) solution was equal to 18.0 ± 1.1 mg L^{-1}, whereas it increased moderately after treatment with degradation products of BB s7s-1 (EC50 = 19.5 ± 1.2 mg L^{-1}). Toxicity significantly decreased after treatment with BB B3-15 (EC50 = 30.5 ± 2.5 mg L^{-1}).

The effects of treatment with BB B3-15 and BB s7s-1 on *P. tricornutum* growth of degraded and undegraded MB solutions for 72h are reported in Figure 8.

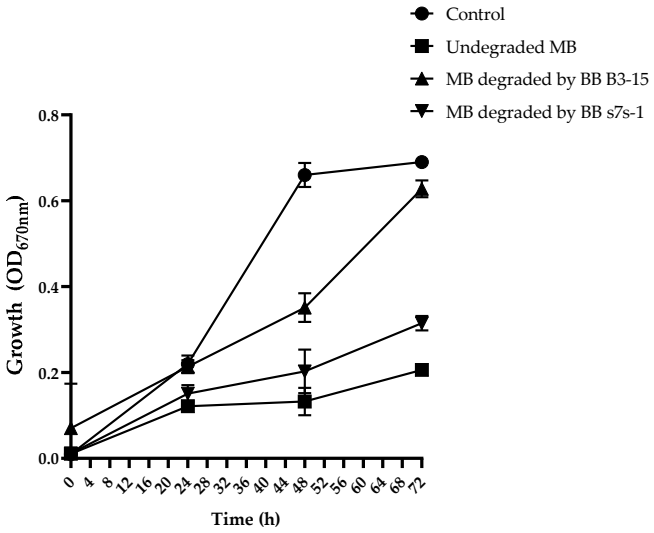

Figure 8. MB effects on the viability of *P. tricornutum* after 72 h of incubation in absence (control) and in the presence of undegraded MB and BB B3-15- or BB s7s-1-degraded solutions.

Untreated MB inhibited the growth of *P. tricornutum* by more than 70% after 72 h (Figure 8). Differently, BB B3-15-degraded products from MB did not affect the growth of *P. tricornutum*, whereas those from BB s7s-1 reduced the viability of *P. tricornutum* by more than 40% after 72 h. These results indicate that the degraded products were less toxic than MB and that those of BB B3-15 were more harmless than those of BB s7s-1.

4. Discussion

Pollution from synthetic dyes is gaining increasing attention, owing to its enormous negative impact on terrestrial and aquatic life. To contrast the toxic effects of these pol-

lutants, microbes capable of efficiently degrading synthetic thiazides, such as methylene blue (MB), have been investigated as an eco-friendly alternative to traditional physical and chemical treatments. The decolorization of dyes by thermotolerant or thermophilic microorganisms, able to grow optimally at elevated temperatures, could be advantageous for the treatment of dye-containing effluents normally discharged at elevated temperatures (50–60 °C) [58,59].

Since microbes aggregated in their own biofilms can degrade dyes more efficiently than in free-living forms, in this study, thermophilic biofilm-forming bacilli, isolated from shallow hydrothermal vents of Eolian Islands (Italy), were evaluated for their ability to decolorize, degrade, and detoxify MB. Colonization of surfaces and biofilm formation are complex processes, starting with the initial adhesion of free-living bacteria, followed by the irreversible attachment, when the cells produce exopolymers, and the development of structured and stable biofilm architecture [35,36,60]. In this study, we used spherical, perforated polypropylene as support for the development of biofilms, since they are easily commercially available, cost-effective, and reusable, providing a large surface area. After the optimization of initial conditions, BB B3-15 in saline solution (NaCl 2%) was able to decolor 96% of MB (10 mg L^{-1}) at pH 5.2, whereas BB s7s-1 decolored 67% at pH 4, after incubation at 45 °C for 48 h. Although the MB decolorization after treatment with BB B3-15 appears to be comparable with those of biofilms formed on different supports after 9 weeks [61,62], the activity of the thermophilic B3-15 strain was remarkably faster, being registered after 48 h.

Donkadokula et al. [62] and Naresh Yadav et al. [61] suggested that the absorption by the bacterial biofilms was the main mechanism involved in MB removal, rather than any degradative activity, since they did not observe any modifications of MB's structure using FTIR. ATR-FTIR analyses of products released after treatments with BB B3-15 and BB s7s-1 indicated that BB B3-15 was the most efficient in degrading the MB structure (i.e., Cl-C and -NH groups), and the spectra also revealed the presence of different degraded products, structurally similar to those reported by Wu et al. [26] (i.e., 1,4-benzenediol, phenol, 4-(dimethylamino), N,N-dimethylaniline, 7-(dimethylamino)-3H-phenothiazin-3-one). These compounds could be produced by laccase and lignin peroxidases released by BB B3-15, similarly to other MB degrading strains, such as *B. albus* MW407057 [2], *B. thuringiensis* strain F5 [26], and *Bacillus* sp. React3 [63]. Compared with BB B3-15, BB s7s-1 possessed minor enzymatic activities, and fewer changes were observed in the spectrum after the treatment, mainly attributed to toxic amine intermediates [54,62,64].

Although the degraded products of MB were not further chemically characterized, it has been previously reported that some metabolic outcomes from the dye-degradation processes are frequently more toxic than their parent untreated dyes [22]. Moreover, acute or chronic/sub-lethal effects of undegraded and BB-degraded MB were evaluated using the inhibition of *Vibrio harveyi* luminescence [34,65] as an high-efficient and cost-effective method for evaluating the harmful effects of different substances on organisms in diverse environments, with the viability of the marine diatom *P. tricornutum* considered as a reference model in monitoring aquatic pollution [66]. As assessed by the luminescence inhibition assay, degraded products obtained after the treatment with BB B3-15 reduced the MB acute toxicity of about 40%, while negligible reduction was observed with BB s7s-1. Moreover, BB B3-15 treatment did not affect the viability of *P. tricornutum*, and confirmed that degraded products did not exert chronic toxic effects. Although the degraded products released after treatment with BB s7s-1 were less toxic than MB, they exerted moderate toxicity towards the vitality of *P. tricornutum*, suggesting the presence of toxic intermediates, different from those of BB B3-15.

Remarkably, it is urgent to guarantee the safety and toxic-free nature of dye-industrial wastewater effluents, since these could be used for different purposes, including the irrigation of non-edible crops, to overcome water deficiency problems.

5. Conclusions

In this study, thermophilic biofilm-forming bacilli from shallow hydrothermal vents of the Eolian Islands (Italy) were investigated for their ability to decolor, degrade, and detoxify MB. *B. licheniformis* B3-15 and *Bacillus* sp. s7s-1 biofilms formed on polypropylene perforated balls as supports were able to decolorize more than 50% of MB when incubated in saline solution (NaCl 2%) at 45 °C for 48 h in static conditions. After optimizing the initial conditions (10 mg L^{-1} MB, pH 5.2 for B3-15, and pH 4 for s7s-1), the BBs of the two strains showed enhanced decolorization potential, reaching 96% and 67%, respectively. As shown by ATR-FTIR analyses, each treatment produced different degradation products with distinct toxic effects, with those from BB B3-15 being not toxic, whereas those of BB s7s-1 being moderately toxic. Moreover, the use of assays based on the inhibition of bioluminescence and viability of *P. tricornutum* may be considered as a rapid test to assess the safety of MB degraded compounds in wastewater treatments.

BB B3-15 could be proposed as a component of bioreactors in the treatment of warm, dye-containing wastewater to concomitantly mitigate environmental pollution and reduce toxicity of MB.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/jmse12081248/s1, Figure S1: Micrograph images (x600) of biofilms from (a) *Bacillus licheniformis* B3-15 and (b) *Bacillus* sp. s7s-1 on perforated polypropylene balls stained with SYTO9.

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Conflicts of Interest: Author Vincenzo Zammuto was employed by the company ATHENA Green Solutions S.r.l. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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