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Screening and cytotoxicological assessment of sulfur-spring *Bacillus* isolates for the bioremediation of methyl red and other azo dyes

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ABSTRACT

Azo dyes are persistent industrial colorants that contribute significantly to wastewater pollution, and biological remediation offers a chemical-free, cost-effective detoxification strategy. In this study, bacterial strains isolated from a sulfur spring were screened for methyl red decolorization, dye tolerance, activity against other dyes, and cytotoxicity of metabolites. Two strains, identified as *Bacillus spizizenii* and *Bacillus subtilis* via MALDITOF MS and 16S rRNA gene sequencing, achieved over 95% decolorization at 50 ppm, 90% at 250 ppm, and 60% at 1250 ppm. *B. spizizenii* further degraded trypan blue (55%) and methyl orange (42%), while *B. subtilis* showed limited activity. Cytotoxicity assays revealed higher IC_{50} values for metabolites from both strains compared to untreated dye, indicating reduced toxicity, with *B. spizizenii* producing the least harmful byproducts ($IC_{50=}$ 1236.45 µg/ml). These findings highlight *Bacillus* isolates, particularly *B. spizizenii*, as versatile candidates for sustainable eco-friendly bioremediation of azo dye-contaminated textile effluents, generating less hazardous byproducts.

Keywords: azo dye, bioremediation, methyl red, pollution, sustainability

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INTRODUCTION

Synthetic dyes have been long used in textile, plastics and paper industries for their high color intensity, possibility for generating a vast range of shades, color fastness and fading resistance. They are also more cost-effective and of great price value compared to natural dye alternatives. In pharmaceutical industry, many dyes were incorporated for their antibacterial, antifungal, antiviral antioxidant and anti-inflammatory activities, beyond coloration. Among the synthetic variants, azo dyes represent the largest and most versatile commercial colorants, which are principally produced by diazotization of aromatic amines to obtain the azo chromophore (N=N) followed by coupling with nucleophilic congeners yielding monoazo, disazo, trisazo, polyazo and azoic structures. Less employed synthesis methods include, but are not limited to, nitroaromatic compounds reduction, primary amines oxidation, primary amines reaction with nitroso compounds[1,2]. The azo chromophoric linkage is flanked by aromatic or heterocyclic rings building blocks, called chromogen and responsible for the color, in addition to water-solubilizing groups (e.g., sulfo SO_3H, carboxy COOH groups) (Figure 1). The functional moieties are modified depending on the target color and particle size that controls dispersibility, and more recently bestowing additional properties such as microbial resistance, thermal stability, etc.[1,3].

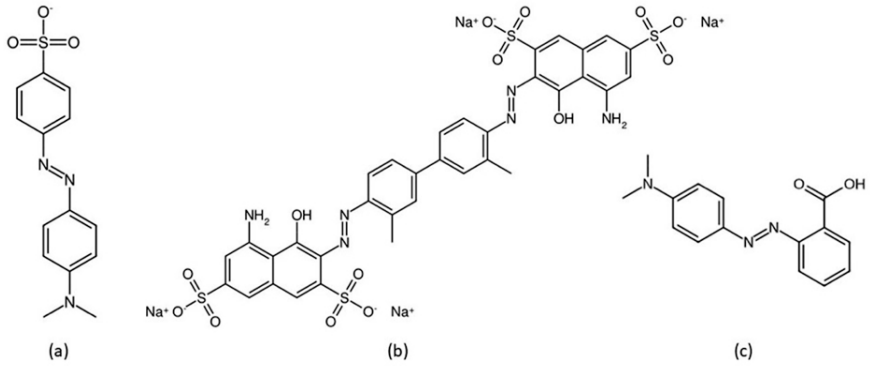

Figure 1. Chemical structure of some azo dyes examples: (a) methyl orange, (b) trypan blue, (c) methyl red

A significant environmental challenge arises from the inefficiency of dyeing processes, wherein approximately 15% of industrial dyes fail to bind to the target substrate and are consequently discharged into wastewater. Azo dyes, which are valued for their stability and durability against light and moisture,

are a major component of this effluent. However, these dyes and their metabolic byproducts, such as aromatic amines, are highly recalcitrant to natural degradation. Their persistence poses a serious environmental hazard, as recognized by the regulatory bodies like the U.S. Environmental Protection Agency (EPA), impacting aquatic ecosystems, agricultural land, fauna and human health[3-5]. In water bodies, azo dyes change the physicochemical parameters such as pH, chemical content, and impede sunlight penetration-disrupting plant photosynthesis. They also increase biological and chemical oxygen demand (BOD, COD) while exhibiting direct toxicity to aquatic biota. On land, their accumulation compromises soil fertility and plant germination rates. Furthermore, upon incorporating into the food chain, these compounds exert adverse health effects in humans, including carcinogenicity, mutagenicity, and genotoxicity, as well as associations with immunological and neurological disorders, and irritation of the skin, eyes, and respiratory tract[2,3,5,6].

Several physicochemical and biological methods have consequently been applied for treatment of azo dye waste. Physical techniques include membrane separation (e.g., reverse osmosis or ultrafiltration), sonication, photocatalytic degradation and adsorption, while chemical methods comprise flocculation/coagulation, electrochemical oxidation, ion exchange, ozonation and treatment with Fenton reagent. However, most of these processes are either inadequate alone or involve the use of expensive chemicals that may also constitute an environmental burden themselves or via production of secondary wastes[6,7]. In contrast, biological treatment has emerged as an effective alternative to the conventional processes as it requires less energy, generates less sludge that requires proper treatment and disposal, and due to its eco-safety and cost-effectiveness. Bioremediation has been reported both aerobically and anaerobically by bacteria, fungi, yeast, algae and lichens that have the capacity to adsorb dyes to the biomass or break it down through their enzymatic systems into non-toxic compounds. The enzymes involved in this process include laccases, peroxidases and azoreductases, which catalyze the breakdown of azo dyes into smaller intermediates that can be metabolized and ultimately mineralized into biomass, carbon dioxide and water[3,6]. Among microorganisms, bacteria are more commonly investigated and applied in pure cultures or mixed cultures, when possible, due to their superior adaptation, robust metabolic potential, ease of cultivation and fast growth rates. They can either entirely catabolize the dye molecule and primary amines or produce intermediates that can be readily treated, especially when found in coexisting consortia[6].

In this context, this research aimed to isolate bacterial strains with the ability to decolorize azo dyes *in vitro*. Their efficiency was evaluated under varying conditions, and the degradation into benign compounds was verified via toxicity studies.

METHODOLOGY

Unless otherwise specified, all chemicals and reagents utilized in this study were of analytical grade and procured from Sigma-Aldrich, employed without additional purification. Solution preparations were conducted using deionized water with a resistivity of no less than 18.2 MΩ.cm.

Microorganisms and maintenance

Water samples were collected from Eyoon Mousa, sulfur water spring, in Ras Sedr, South Sinai governorate in Egypt (29.5933°N, 32.7178°E) in June 2024. The sampling took place in sterile containers that were then firmly closed until processing. Later, ten-fold serial dilutions were prepared and spread over the surface of nutrient agar (NA) plates. After incubation for 24 h at 37°C, the plates were checked for colony growth, and single colonies were picked and streaked on the surface of NA plates and further incubated. A touch of the terminal growth of single separate colonies was transferred to NA slants to be preserved at 4°C and regularly subcultured every 2 months, and pure cells were preserved in 50% glycerol (v/v) for master cell bank storage at 80°C[8]. Other bacterial strains were obtained from the culture collection of the National Research Centre (NRC) in Egypt.

Growth media preparation and culture growth conditions

Inoculum was prepared by adding a complete overnight-grown slant to 50 ml nutrient broth medium (g/l: D-glucose, 1; peptone, 15; sodium chloride, 6; yeast extract, 3), and incubated overnight in a rotary shaker incubator (MaxQ 481 HP, Thermo Scientific, USA) at 37°C, 250 rpm. Grown bacteria were adjusted to optical density (OD_{600}) of 0.1 and inoculated at 37°C, 250 rpm in media containing (g/l: glucose, 10; yeast, 0.5, Na_2CO_3, 1; KH_2PO_4, 0.2; $MgSO_4.7H_2O$, 0.02), and adjusted at pH 7[5,9].

Screening assay for methyl red (MR) dye degradation/decolorization

The growth media was supplemented with 50 ppm MR dye and incubated with the different bacterial strains. A positive control consisted solely of the dye without bacterial inoculation, while the negative control lacked both dye and bacterial strains. Dye degradation was evaluated after 24 and 48 h. Cultures were centrifuged at 10,000 rpm for 10 min at 4°C (Z 326 K, HERMLE Labortechnik

GmbH, Germany), and the absorbance of the clear supernatant was measured at 430 nm using a UV/Vis spectrophotometer (Jasco V630, Tokyo, Japan). The efficiency of the different strains was assessed using equation 1, and the strain with the highest decolorization potential was used for further studies[5,10]. The experiments were done 3 times, and the data is represented as the mean ± standard deviation.

$$\% \text{ Degradation/Decolorization} = \frac{\text{Initial absorbance - Final absorbance}}{\text{Initial absorbance}} \text{ X100} \quad \text{[Eq 1]}$$

Dye load decolorization capacity test

The most active strains, 3 and 8, were tested for their MR dye decolorization capacity against different concentrations thereof. Therefore, the bacteria were incubated with different concentrations, calculated at 5 times increments of MR for 6 days. The decolorization was evaluated spectrophotometrically after centrifugation, as described before.

Efficiency against different azo dyes

The MR-degrading strains, 3 and 8, were tested for their decolorization capacity for different azo dyes, trypan blue and methyl orange were used as examples (Figure 1). The bacteria were incubated with 50 ppm of each dye, the decolorization was evaluated spectrophotometrically at 600 nm for the earlier[2,7] and 465 nm for the latter[4,9], as previously described. The experiments were done 3 times, and the data is represented as the mean ± standard deviation.

Cytotoxicity assay

The efficiency of azo dye clearance was assessed by testing the treated dye (50 ppm in 50 ml culture) on normal human skin fibroblast (HSF cell line). Cell viability was evaluated by mitochondrial-dependent reduction of 3(4,5dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) to insoluble purple formazan. Cells were suspended in Dulbecco's Modified Eagle Medium (DMEM) containing 1% antibiotic-antimycotic mixture (10,000 U/ml penicillin G, 10,000 µg/ml streptomycin, 25 µg/ml amphotericin B), 1% L-glutamine and 5% fetal bovine serum (FBS), and incubated at 37°C in a humidified 5% CO_2 incubator (Sartorius Stedim Biotech, France) until confluence. Cells were then seeded at 10×10^3 cells/well in fresh growth medium in 96well plates for 24 h, either alone (negative control), or with different concentrations of methyl red dye (positive control) or microbially treated dye. After 48 h, the medium was discarded and 20 µl MTT (2.5 µg/ml) was added then plates were incubated for 4 h after which 200 µl of 10%

sodium dodecyl sulphate in 0.01 M HCl were added to dissolve the formazan crystals and incubated overnight[11,12]. The absorbance was measured using a UV microplate reader (Bio-Rad Laboratories Inc., Hercules, California, USA) at 595 nm wavelength. Cell viability was calculated as the percentage of drug absorbance to control absorbance, and IC_{50} denoted the sample concentration which causes the death of 50% of cells in 48 h.

$$\% \text{ viability} = \frac{\text{Absorbance of drug}}{\text{Absorbance of control}} \text{ X } 100 \quad [\text{Eq } 2]$$

$$\% \text{ cytotoxicity} = 100 - \% \text{ viability} \quad [\text{Eq } 3]$$

Strains identification

Bacteria were identified via matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDITOF MS), coupled with the Vitek® MS IVD v3.0 system (bioMérieux, France) in accordance with the manufacturer's protocol. The analysis was outsourced to the Children's Cancer Hospital Egypt 57357 (CCHE). For sample preparation, isolates were co-crystallized with α-cyano-4-hydroxycinnamic acid (CHCA) matrix solution on a designated target slide and subsequently air-dried. The slide was then introduced into the vacuum chamber, where laser irradiation facilitated ionization and protein release. The resulting mass spectra were used to generate species-specific profiles for microbial identification[13,14].

RESULTS and DISCUSSION

With the industrial expansion in several fields, effluent waste subsequently increased, constituting a great environmental burden. Discharge from textile and paper industries contains a high percentage of persistent colorants that pose an ecotoxicological threat to both aquatic and terrestrial ecosystems, as well as endangering human health if left untreated. Bioremediation is an important strategy to break down the dye pollutants into non-toxic substances without generating an extra concern regarding the hazards of the formed sludge. Motivated by the ecological challenge and given that microbial biofactories are still bioprospected via conventional lab experiments beside advanced omics and strain engineering endeavours for economically valuable products, this study investigated the capacity of bacterial cultures to degrade MR azo dye.

Four different pure bacterial strains were isolated from the sulfur water spring, designated 3 to 6, and used for screening for their azo dye decolorization, along with three strains from the culture collection of the National Research Centre

(NRC) that were preliminarily classified as *Bacillus* spp. *Bacilli* were particularly screened due to their resilience in extreme niches, robust enzymatic machinery and well-documented bioremediation potential[15-18].

Screening assay for methyl red dye degradation/decolorization

Among the 7 tested bacteria, organisms 3 and 8 showed the highest color reduction capacity (Figure 2), reaching 76% after 24 h and 95% after 48 h for the former, and 94% after 24 h and 98% after 48 h for the latter. Strains 7 and 9 exhibited similar moderate potential where strain 7 could degrade 20% of the dye after 24 h and 54% after 48 h, while strain 9 cleared 27% of the color after 24 h and 55% after 48 h. Strain 4 managed 7% of the dye decolorization on the first day, followed by 20% on the second while strain 5 scored 1% on the first day and 7% the following day. Strain 6 has no decolorization capacity beyond 1% after 48 h incubation. Therefore, bacterial strains 3 and 8 were chosen for further investigations.

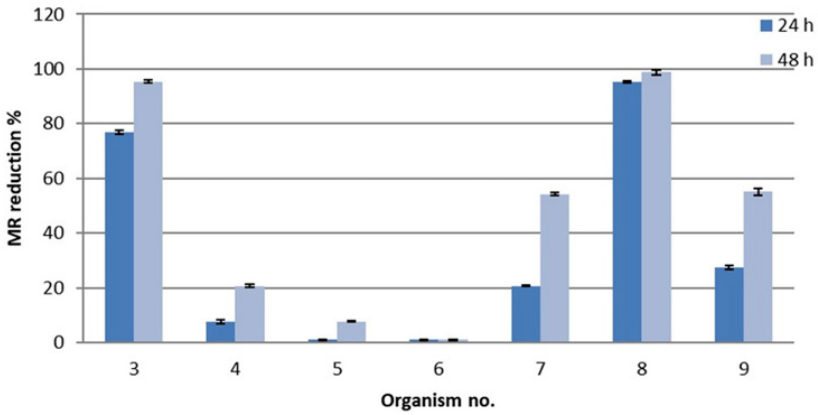

Figure 2. Methyl red dye degradation/decolorization by different bacterial strains

Bacterial strain 8 was identified using rRNA gene sequencing within the framework of a concurrent project and was found to belong to the species *B. spizizenii*, submitted to the GenBank® under the accession number PV591015.1. Strain 3 was identified based on its fingerprint unique ribosomal proteins spectrum, detected by the MALDI-TOF technique, as *B. subtilis*. MALDITOF MS has been established as a prominent highthroughput platform for microbial identification, leveraging the analysis of conserved ribosomal proteins to generate unique species-specific profiles. Its high sensitivity, speed and cost-efficiency, vis-à-vis conventional biochemical and molecular techniques, have increased its popularity in modern clinical microbiology laboratories[19,20].

The most active candidates belonged to *Bacillus spp*., in accordance with previous reports that enlist *Bacilli* as the most frequently isolated genus from extreme environments, such as sulfur-rich thermal aquatic vents, cold seeps, hot springs, near-space regions up to 100 km above sea level[16,21,22]. *B. subtilis*, isolated from the sulfur hot water spring, had a high capacity to decolorize/degrade 50 ppm of the model MR azo dye (>95%) within 48 hours, and hence, conforming to the objective of microbial bioprospecting in harsh niches. The indigenous microorganisms that tolerate stressful high sulfur concentration and temperature produce stable enzymes that also function under extreme conditions of xenobiotic contamination[21-23]. Moreover, as sulfur springs are characterised by reducing conditions, dwelling microbial metabolism often occurs via anaerobic or facultative anaerobic pathways comprising electrons' transfer to sulphate or elemental sulfur as terminal acceptors. Such environmental background might pre-adapt the microorganisms for the azo-dye degradation whose initial step is the reductive cleavage by azoreductases. Notably, both strains demonstrating the highest dye-degradation potential in the screening experiment were members of the *Bacilli* genus. In general, *Bacilli* are tolerant to hostile selective pressure due to spore-formation enabling them to survive adverse conditions with metabolic dormancy and scarce nutritional requirements. They also possess high chromosomal G+C content and produce secondary metabolites that adapt them to the surrounding conditions[18]. Further, *Bacilli* have been long regarded as a robust producer of a wide variety of secondary metabolites of important biotechnological value and hence, exhibiting several bioactivities. For instance, the market value for its industrially relevant products was estimated by a value of $18 billion in 2020. These include enzymes, biosurfactants, phytohormones and antibiotics[17,18], and therefore, other *Bacilli* spp. were incorporated in the initial screening.

Dye load decolorization capacity

Bacterial strains 3 and 8 were incubated with increasing concentrations of MR for up to 6 days (Figure 3). At 50 ppm MR concentration, both strains managed to eliminate 95% of the dye after 48 h, with no residual color in the culture flasks. At 250 ppm, strain 3 reached its highest decolorization potential of 90% after 48 h, while strain 8 reached its maximum 88% score after 72 h. At 1250 ppm, both strains recorded 60% dye degradation after 24 h which did not increase any further. Testing higher dye concentrations of 6250 and 31250 ppm was attempted, but the analysis was not quantitatively feasible due to the poor solubility of MR. It is also worth noting that the two *Bacilli* strains, selected for further experiments, were wild strains that decolorized >95% of the MR

dye without any necessary cultivation parameters or genetic modification. The activity plateau after 60% dye decolorization is perceived as a sign of microbial inhibition attributable to the toxicity threshold of the high initial dye load or that of any of the degradation products throughout the bioremediation process. This establishes a functional limit for the strains. The bacterial treatment of MR results in several compounds such as anthranilic acid, anthranilic acid amide, 3-acetylephenylamine, 1,2-benzenedicarboxylic acid, 4-hydroxybenzamidine and N,Ndimethyl-p-phenylenediamine, some of which may cause acute toxicity at high exposure levels, even when completely mineralized at lower doses[10,15]. Beside the direct bacterial toxicity, they might be exerting their inhibition through compromising the action of the involved enzymes, such as laccase, veratryl alcohol oxidase, tyrosinase, lignin peroxidase and azoreductases, namely FMN-dependent NADH-azoreductase, FMN-dependent NADHazoreductase and NAD(P)H-azoreductase[7,10,24].

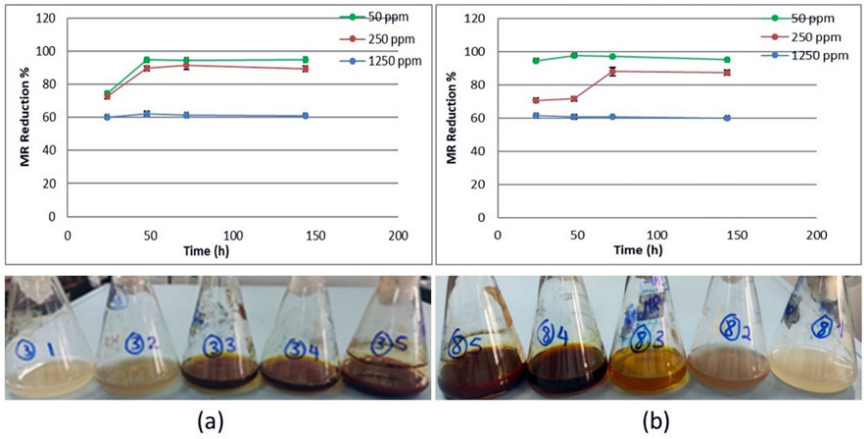

Figure 3. Testing for the dye load decolorization capacity of test strains (a) Strain 3 and (b) Strain 8

The results, herein reported, fall remarkably in comparison to previous reports, where bacterial isolates from textile and industrial effluents could decolorize 80-92% MR at the same concentration[25,26], while different fungi decolorized azo dyes at a maximum of 89% also at the same dye load[27]. Whereas *B. thuringiensis* strain could break down 90% MR in static conditions vs. 60% in shaking conditions, like the herein employed[28], *Bacillus* strain from oil refinery waste sludge could similarly degrade 98% of 100 ppm dye[29], while *Bacillus circulans* from sludge effluent samples removed 75% of 50 ppm MR[15]. In general, Gram-positive bacteria were observed to be better azo dyes' decolorants than Gram-negative species[10].

Decolorization of different azo dyes

Whereas both strains had similar decolorization/degradation potential towards MR, reaching above 95% in both cases, their behavior towards trypan blue and methyl orange was different. Strain 3 had barely any degradation potential against trypan blue (2.5% after 3 days), while its activity against methyl orange increased gradually to a maximum of 20% after 3 days. Strain 8 elicited gradual increase of activity towards both dyes, degrading 55% of trypan blue and 42% of methyl orange after 3 days (Figure 4). Evidently, methyl orange was less efficiently biodegraded than methyl red, though not to the same extent as the herein stated results, whereas trypan blue- a class-2B carcinogen as per the International Agency for Research on Cancer- was even more sturdy due to the double azo groups[5,7]. Interestingly, *B. spizizenii* was able to portray a broader substrate versatility spectrum by effectively decolorizing the more complex dyes trypan blue and methyl orange, opposite to *B. subtilis* which was specific for methyl red. This hypothesizes different mechanisms against dye pollutants where *B. spizizenii* can be linked to an enzyme system of higher substrate promiscuity, most notably short-chain dehydrogenase/reductase (SDR) family that are upregulated upon exposure to pollutants[24]. On a side note, biodegradation is reportedly worsened in aerobic conditions because of the competition between oxygen and azo bonds as electron acceptors for reduced carriers (e.g., NADH) and consequently, microaerophilic growth afforded better results[7].

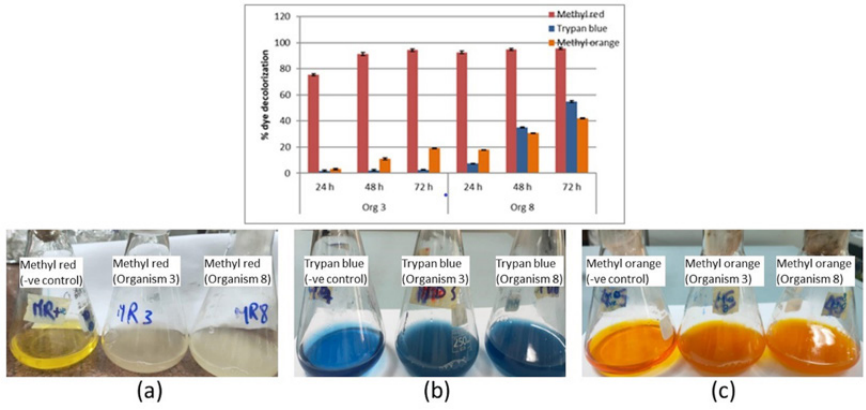

Figure 4. Efficiency of bacterial strains 3 and 8 to degrade/decolorize azo dyes (a) methyl red, (b) trypan blue and (c) methyl orange

Cytotoxicity assay of methyl red azo dye on normal dermal fibroblasts

The MTT assay revealed that both strains decreased the magnitude of cell toxicity with the increase in sample concentration (Figure 5). While IC_{50} of MR dye was measured at 290.03 µg/ml, that of strain 3 was 727.78 µg/ml and that of strain 8 was 1236.45 µg/ml. The normal cells cytotoxicity investigation also supports that both strains, although belonging to the same genus and showing more than 95% dye decolorization, function differently as mentioned before. *B. spizizenii* had the higher IC_{50} (i.e., less toxicity) which reflects less hazardous byproducts. This asserts that effective decolorization must be coupled with detoxification to constitute a truly successful bioremediation strategy and position *B. spizizenii* as a robust and promising biocatalyst for the development of sustainable wastewater treatment solutions. Therefore, a consortium of both strains could be an efficient strategy for real-world wastewater treatment where *B. spizizenii* serves for broad range cleanup of mixed contaminants, while *B. subtilis* aims for specialized targeted degradation. The cytotoxicity results also strongly suggest that the dye removal occurs by biodegradation rather than biosorption since the toxicity is not completely lost despite the near-complete color elimination, suggesting the transformation of the parent dye into less toxic, yet not fully innocuous, intermediates. In this accord, the initial step for azo dye degradation is proposed to be azoreductase-catalyzed azo bond cleavage, using NADH as electron donor, generating colorless aromatic amines (e.g., N, N-dimethyl-p-phenylenediamine, 2-aminonaphthalene, anthranilic acid, N,N-dimethylaniline or 3,5-diamino-4-hydroxynaphthalene-2,7-disulfonic acid in case of trypan blue). Those mutagenic and/or carcinogenic amines are further broken down by deamination and ring opening through oxidative enzymes (e.g., laccases, tyrosinases, peroxidases). This cascade eventually yields low-molecular-weight less toxic compounds (e.g., o-xylene, naphthalen-1-ol), aliphatic molecules or complete mineralization[5,7].

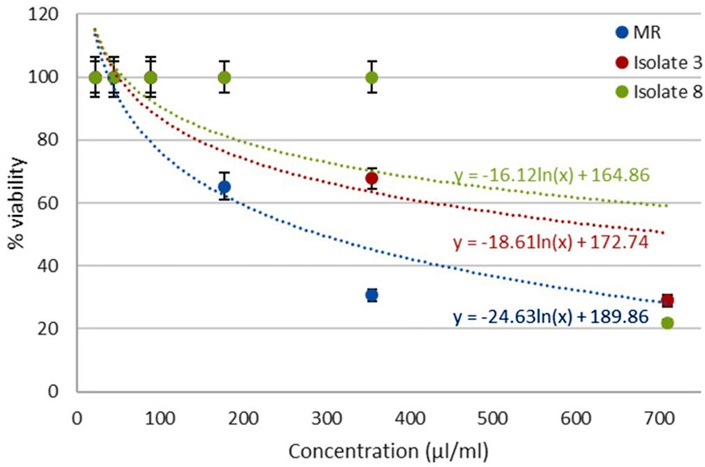

Figure 5. Effect of microbially treated-methyl red solution vs. methyl red on normal human skin fibroblasts

The results of this study indeed pose inexpensive and safe choices for dye waste treatment. Looking forward, the translation of these results to commercial economic application requires a multi-faceted approach. A follow-up of extracting the corresponding metabolites and structure elucidation via different spectroscopic techniques, primarily FTIR and GC-MS, would serve to characterize the mechanism of decolorization and outline the complete pathway. This would also ensure that no transient or toxic intermediates are released. Additionally, it allows evaluating the possibility of using and optimizing these organisms against other pollutants. Furthermore, identifying the enzyme cascade involved in such processes, through proteomic analyses, enables understanding the biological catalysts and modifying the extent of toxic metabolites' production and improve their activity towards higher concentrations. Scaling up the detoxification processes and validating the efficiency against real industrial effluents should also be investigated in the subsequent studies to conclude the strains' suitability against complex recalcitrant dyes pollutant milieus. Genomic and transcriptomic analyses could be used to unravel the underlying genetic basis, providing targets for metabolic engineering. Through techniques like CRISPR-Cas9, it is possible to amplify key biosynthetic genes or silence undesirable pathways, thus enabling strains' transformation into efficient, tailored cellular factories. The robust azoreductase in *B. spizizenii* point toward interesting future studies involving the cloning and heterologous expression of the responsible genes to engineer high-performance microbial strains or to produce enzyme

cocktails for enzymatic treatment processes. Other future prospective include evaluating the toxicity against soil and aquatic microflora, agricultural crops (phytotoxicity), animal models and more human cell lines to validate the practical utility in factual industrial scale. Finally, the economic and life-cycle assessment of employing these microbes in a circular bioeconomy model is highly desirable. In conclusion, by bridging conventional microbiology with modern biotechnological tools, bacteria can be upgraded into a high-value resource for different applications.

In summary, while this work provides a strong foundation by identifying highly effective native bacterial strains, translating these findings into a viable bioremediation technology requires an integrated approach combining microbiology, biochemistry, and process engineering.

STATEMENT OF ETHICS

This study does not require any ethical permission.

CONFLICT OF INTEREST STATEMENT

The authors have no conflicts of interest to declare.

AUTHOR CONTRIBUTIONS

The authors contributed equally to the article.

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