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Trypan blue dye decolorization by *Aeromonas caviae* isolated from water sewage in Jakarta, Indonesia

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Abstract. *Pinontoan R, Susanto TSR, Lucy J, Angelina C, Soentoro SE, Purnomo JS, Cornelia M. 2024. Trypan blue dye decolorization by* Aeromonas caviae *isolated from water sewage in Jakarta, Indonesia. Biodiversitas 25: 1631-1637.* The textile industry generates a substantial amount of hazardous chemical waste, which requires proper treatment to mitigate negative environmental and health consequences if left untreated. Studies on the removal of Trypan Blue (TB), a widely used commercial diazo textile dye, by indigenous bacteria are limited. Therefore, this study aimed to isolate, identify, and characterize microorganisms capable of decolorizing TB in textile dye-contaminated wastewater from Jakarta. Microorganisms were initially screened for decolorization activity in solid media containing TB. Among the isolated strains, TB2 isolate exhibited the highest decolorizing potential and was selected for further analysis. The morphological, biochemical, molecular, and phylogenetic characteristics of the TB2 isolate revealed that it belonged to the species *Aeromonas caviae*. To examine the ability of the isolate to remove TB, various culture conditions, such as pH, temperature, and agitation, were tested. The results demonstrated that the *A. caviae* TB2 strain could reduce up to 77.10% of TB (0.0025% (w/v)) under static conditions, pH 7.0, and 27°C over six days. To our knowledge, this is the first study to report the ability of the *Aeromonas* genus to decolorize TB. These results imply the potential use of *A. caviae* for decolorizing dye-bearing industrial wastewater.

Keywords: *Aeromonas caviae*, bacterial isolation, decolorization, textile dye, trypan blue

INTRODUCTION

The textile industry continues to expand globally to fulfill the demands of the ever-growing world population and technological advancements. Although industry contributes significantly to the global economy, the disposal of excess dyes used in the manufacturing process poses a major environmental problem. In recent years, an estimated 700,000 tons of dyes have been utilized by the textile and dye community each year, with 15% remaining unfixed and carried out as effluent, ultimately being discharged into surrounding water bodies (Katheresan et al. 2018; Velusamy et al. 2021). The release of textile effluents into the environment negatively affects water quality, soil fertility, and aquatic life, potentially disrupting ecosystems. For example, a study has reported that the discharge of effluents poses genotoxic, mutagenic, and carcinogenic risks, leading to potential hazards to both human and environmental health (Desai et al. 2021).

Currently, chemically derived synthetic dyes dominate the textile industry because of their stability and superior coloration qualities, such as resistance to detergents, sunlight, and temperature, as well as offering a wide range of color choices. Azo dyes are widely used in textile dyeing, accounting for approximately 70% of all colorants (Benkhaya et al. 2020). One example of an azo dye is Trypan Blue (TB), a dark blue dye extensively used in the textile and bioscience industries as a staining reagent. Despite its usefulness in various industries, TB has raised environmental concerns due to its toxicity (Marshall et al. 2021). Its release into the surrounding environment poses significant human health concerns due to its mutagenic properties and resistance to microbial breakdown, attributed to its fused aromatic ring structure (Al-Tohamy et al. 2022).

There is a significant interest in removing textile dyes from wastewater through physical, chemical, or biological processes. Physical and chemical methods include adsorption (Marshall et al. 2021), ion exchange, filtration, coagulation/ flocculation, the Fenton reagent technique, ozonization, and photocatalytic methods (Slama et al. 2021; Amalina et al. 2022). These methods have shown promising results, but the complete breakdown of secondary toxic waste was difficult and costly for each breakdown technique (Khandare et al. 2023).

In recent decades, various microorganisms, including bacteria, fungi, algae, lichens, and plants, have demonstrated the potential to degrade structurally different dye compounds such as azo dyes (Ngo and Tischler 2022; Santana et al. 2023). This process, known as bioremediation, is an alternative, cost-effective, and eco-friendly treatment method that removes and detoxifies dyes, mineralizes dye molecules into CO_2 and H_2O, and produces low amounts of sludge (Jamee and Siddique 2019). Examples of agents used for bioremediation of dyes include water hyacinth biomass (*Eichhornia crassipes*) for Malachite Green (MG) decolorization (Pinontoan et al. 2019) and microorganisms such as bacteria *Ralstonia mannitolilytica* (Michelle et al.

2020; Purnomo et al. 2023), *Pichia* sp. (Victor et al. 2020), *Acinetobacter* sp. (Irawati et al. 2022), and *Bacillus cereus* (Irawati et al. 2023). Furthermore, microbial consortia, a combination of microbes, activated sludge, and biofilms, has also been used to remove dye (Vikrant et al. 2018). Notably, *Bacillus subtilis*, *Pseudomonas resinovorans* (Mandragutti et al. 2021), *Vibrio* sp. (Khandare et al. 2023), and *Pseudomonas* sp. (Abubakar et al. 2023) have also been reported for their ability to remove TB.

The study aimed to isolate, characterize, and identify potential dye-degrading bacteria from industrial dye-contaminated effluents in the administrative village of Jelambar, West Jakarta, Indonesia, where several textile industries are located within their proximity. This area was chosen for its indigenous bacteria primarily because its populations exhibit high adaptability to toxic environments. This location provides an optimal environment for selecting and developing dye-degrading bacteria within the local microbial population. Several analyses were conducted to identify bacteria from the consortium capable of dye degradation. Initially, potential dye-degrading bacteria were identified by growing effluent samples in a bacterial growth medium supplemented with TB. After successfully identifying and purifying the bacteria, their ability to remove TB dye and the effects of various culture conditions such as agitation, pH, and temperature on decolorization were analyzed.

MATERIALS AND METHODS

Procedures

The study was divided into three stages: (i) identification and purification of dye-degrading bacteria, (ii) evaluation of the decolorization activity of isolated bacteria, and (iii) optimization of the decolorization activity under various growth conditions.

Chemicals tested

Azo dye, Trypan Blue (TB) CI 23850 with 80% dye content, was purchased from MERCK, Germany.

Growth and selection of the textile dye-decolorizing bacteria

Water samples used in this study were collected from textile dye-contaminated sewage in the administrative village of Jelambar, West Jakarta, Indonesia. Sewage samples were centrifuged for 6 min at 1,500 × g to remove solid remnants. The obtained supernatant was plated on Nutrient Agar (NA) enrichment media (MERCK, Germany) supplemented with 0.005% (w/v) TB (MERCK, Germany) and incubated at 37°C for 6 d. Colonies with clear zones were selected and streaked further on NA supplemented with 0.005% (w/v) TB. Pure colonies were stored in enrichment media for further characterization.

The selected isolate morphological and biochemical characterization

Cultural characteristics, such as colony morphology on NA plates, were observed after incubation at 37°C for 24 h. The colony observations included shape, elevation, margin, size, texture, appearance, pigmentation, and optical properties. Gram and endospore staining was subsequently performed, and further morphological characterization was performed. Biochemical assays were further evaluated, as described by Bergey's Manual of Systematic Bacteriology, using the Microgen® GN-ID biochemical identification kit. Biochemical assays were performed following the manufacturer's instructions. These include catalase, carbohydrate fermentation, indole, and Voges-Proskauer (VP) assays.

Molecular identification and phylogenetic analysis of isolated bacteria

Identifying the bacterial isolates involved sequencing of the 16S rRNA gene. For that purpose, bacterial genomic DNA was extracted using the Wizard® Genomic DNA Purification Kit (Promega, USA), following overnight cultivation of the isolates. The extracted DNA was quantified using a BioDrop DUO UV/Vis spectrophotometer (BioDrop, UK) and used as a PCR template. Universal primers 27F (5′-AGA GTT TGA TCM TGG CTC AG-3′), 1492R (5′-GGT TAC CTT GTT ACG ACT T-3′), and the KAPA HiFi™ PCR Kit (Kapa Biosystems, USA) were used totaling 50 µL for each reaction. The PCR conditions included an initial denaturation for 3 min at 95°C, followed by 25 cycles of amplification by denaturation at 98°C for 20 s, annealing at 55°C for 15 s, and extension for 60 s at 72°C. The final extension step was performed at 72°C for 2 min. The amplified product was confirmed using agarose gel electrophoresis. The 16S rRNA PCR product was sent to First BASE Laboratories, Pte. Ltd., Malaysia, for DNA sequencing.

Qualitative decolorizing plate assay

The qualitative decolorizing plate assay was performed by spot inoculation of isolated bacteria (Wang et al. 2017) on NA plates containing 0.0025% (w/v) TB dye and incubating them at 37°C for 6 d. The plates were regularly monitored, and a reduction in color intensity around the bacterial colonies was observed over 6 d.

Quantitative colorimetric decolorizing assay

A quantitative colorimetric decolorizing assay was performed on selected isolates cultured in Nutrient Broth (NB) supplemented with 0.0025% (w/v) TB at 37°C for 6 d. Samples were centrifuged for 10 mins at 12,000 x g to remove cell debris, and then the supernatants were collected. The decolorization percentage was determined by measuring the color intensity changes using a BioDrop UV/Vis spectrophotometer DUO (BioDrop, UK) at 400-800 nm. A decrease in the absorbance at a wavelength of 590 nm indicates decolorization of TB, based on the experimental and theoretical maximum absorption spectra of TB (Graham et al. 2013). The following equation was used to calculate the decolorization percentage:

$$Decolorization\ percentage\ (\%) = \frac{A_0 - A_t}{A_0} \times 100\%$$

Where :

A_0 : Control or initial absorbance value at 590 nm

A_t : Absorbance value at 590 nm following treatment after t time

The effect of agitation, pH, and temperature on decolorization

The effects of agitation, pH, and temperature on decolorization were evaluated by culturing the selected isolates on NB supplemented with 0.0025% (w/v) TB under varying conditions. The samples were incubated at different temperatures (27°C, 32°C, 37°C, and 42°C), pH values (pH 5, 6, 7, 8, and 9), with and without agitation (0 and 100 rpm) for 6 d. The decolorization percentages were measured every 2 d, and color intensity changes were recorded using the same spectrophotometric method. The wavelength peak of the solution in the absorption spectrum was used to determine the remaining TB concentration. The effect of pH on decolorization activity was also measured by incubating the samples in NB with various buffer solutions such as 20 mM acetate buffer (pH 5.0), 20 mM citrate buffer (pH 6.0), 20 mM phosphate buffer (pH 7.0), 20 mM Tris-HCl buffer (pH 8.0), and 20 mM glycine-NaOH buffer (pH 9.0) at 37°C without agitation. The results were then analyzed to determine the optimal conditions for decolorization.

Sequencing data analysis

The selected isolates' partial 16S rRNA gene sequences were processed using Sequence Scanner 2 (Applied Biosystems, USA) and BioEdit software. The sequence was deposited in GenBank and can be accessed with accession no: PP346385. The acquired 16S rRNA sequences of the isolates were aligned with the NCBI GenBank database by the BLAST algorithm (http://www.ncbi.nlm.nih.gov/BLAST/Blast.cgi) and compared with previously deposited 16S rRNA sequences. A phylogenetic tree was constructed using MEGA-11 software (Available online at https://www.megasoftware.net/).

RESULTS AND DISCUSSION

Isolation and characterization of textile dye-decolorizing bacteria

A total of 35 isolates were obtained from industrial effluent sewage samples from the administrative village of Jelambar, West Jakarta, Indonesia. Therefore, spot inoculation was performed using NA plates containing 0.0025% (w/v) TB dye, and incubated at 37°C for 6 d to identify the most effective decolorizing agent. Of the isolates, only isolate TB2 was found to be the most effective in terms of its decolorizing ability. Consequently, TB2 was selected for further characterization, beginning with studying its cell and colony morphology.

The morphological characteristics of isolate TB2 were studied by observing its colony and cellular forms. The colony appeared circular, flat, with an entire margin, moderate in size, smooth in texture, glistening, non-pigmented, and translucent. The cellular form was identified as rod-shaped, Gram-negative bacteria (Table 1).

Biochemical tests were performed to identify the characteristics of isolate TB2. The results were catalase positive, indole positive, and Voges-Proskauer negative. Additionally, it showed positive results in citrate utilization and nitrate reduction assays. The complete biochemical profile of isolate TB2 is shown in Table 2, which includes the production of acid and gas by the fermentation of different sugars in the media and glucose, respectively. Based on the characterization results, which refer to Bergey's manual, TB2 belongs to the genus *Aeromonas* (Guerrero 2001).

Table 1. Morphological characteristics of isolate TB2

Morphology	Isolate TB2
Colony morphology	
Form/shape	Circular
Elevation	Flat
Margin	Entire
Size	Moderate
Texture	Smooth
Appearance	Glistening
Pigmentation	Non-pigmented
Optical property	Translucent
Cell morphology	
Cell shape	Bacilli
Gram	Negative

Table 2. Biochemical properties of TB decolorizing bacteria

Biochemical properties	Isolate TB2
Catalase	+
Indole	+
ONPG hydrolysis	-
Arginine Dihydrolase	+
Citrate utilization	+
Voges-Proskauer (VP)	-
Nitrate reduction	+
Acid production from:	
Glucose	+
Arabinose	+
Cellobiose	-
Inositol	-
Mannitol	+
Mannose	-
Raffinose	-
Rhamnose	-
Salicin	+
Sorbitol	-
Sucrose	+
Trehalose	+
Xylose	-
Adonitol	-
Galactose	+
Methyl-D-Mannoside	-
Methyl-D-Glucoside	-
Inulin	-
Melezitose	-
Gas production from glucose fermentation	-

Note: +: positive results, -: negative results

Molecular characterization of bacterial isolates

The 16S rRNA sequencing was conducted using a BLAST homology search to identify the isolated bacterial species. Before the sequencing, the 16S rRNA gene of isolate TB2 was amplified and confirmed as a single DNA band of approximately 1.4 kbp agarose gel electrophoresis. The results indicated that the TB2 isolate had the highest similarity (99.85%) to the *Aeromonas caviae* strain GSH8M-1 (AP019195.1). A second strain, *Aeromonas hydrophila* WS05, also showed 99.78% similarity.

A phylogenetic tree was constructed using the 16S rRNA sequences from several other species within the genus *Aeromonas* to confirm the isolate's taxonomic position further. This analysis showed that the TB2 isolate was clustered with *A. caviae* GSH8M-1, the top result of the BLAST homology search. This confirmed the previous results indicating that the TB2 isolate was *A. caviae*, as shown in Figure 1.

Dye decolorization activity

The effectiveness of decolorization by microorganisms depends on their capacity to remove a range of dyes. Microbial dye removal or degradation can be achieved through various methods, such as adsorption, enzymatic breakdown, or a combination of both (Ngo and Tischler 2022). Therefore, to evaluate dye decolorization activity, plates were monitored for visual changes in color intensity around colonies daily up to day 6, resulting in changes in both the colonies and the medium (Figure 2).

The *A. caviae* strain TB2 colonies were typically unpigmented, but following incubation in media supplemented with TB, the colonies and the medium displayed signs of color changes. The change in color of the colonies from unpigmented to the color of the dyed medium indicated a possible mechanism employed by *A. caviae* TB2, through absorbing the dye from the medium via ion exchange, using hydroxy and carboxy groups on the microbial cell wall as binding sites for dyes (Torres 2020; Ngo and Tischler 2022). Additionally, enzymatic breakdown may contribute to the TB removal observed in the study, involving the complete degradation of dyes using microbial enzymes. In bacteria, this dye decolorization and degradation mechanism involves enzymes, such as azoreductases, which cleave the N=N bond in azo dyes (Ajaz et al. 2020).

The ability of *A. caviae* TB2 to decolorize the TB dye was consistent in both solid NA and NB (Figure 2). Upon centrifugation, *A. caviae* TB2 cells were packed and appeared thick at the bottom of all the tubes. A colorimetric assay with a TB concentration of 0.0025% revealed that the isolate could decolorize up to 66.9% of the TB dye. These results demonstrate the capacity of *A. caviae* TB2 to reduce the dye concentrations within the treated sample.

A previous study reported that *A. caviae* ES50G isolated from textile dyeing effluent could decolorize up to 99.8% of Direct Red within 72 h. Additionally, other closely related *Aeromonas* species, such as *A. hydrophila*, have been shown to decolorize up to 89.02% and 90.32% of 100 mg/L of Reactive Yellow F3R and Joyfix Red RB, respectively, within 72 h of treatment (Srinivasan and Sadasivam 2021). While reports exist on the decolorization of various azo dyes by identified strains, there is currently no available report on TB decolorization within the *Aeromonas* genus in published literature up to the current date.

Effect of agitation on decolorization percentage

To investigate the influence of agitation on the decolorization process, *A. caviae* TB2 was incubated under both static (0 rpm) and shaking (100 rpm) conditions. As shown in Figure 3, agitation decreased the decolorization activity of *A. caviae* TB2, which may be attributed to the inhibition of the decolorization reaction caused by aeration. The biodegradation capability of *Aeromonas* species is primarily associated with the extracellular activity of azoreductase enzymes, which frequently degrade azo dyes under anaerobic conditions (Thanavel et al. 2019). Under shaking conditions, oxygen prevents azoreductase from obtaining the necessary electrons for azo bond cleavage whereas under static conditions, oxygen serves as the terminal electron acceptor, resulting in better decolorization. This is supported by the findings of Seyedi et al. (2020) and Srivastava et al. (2022), who reported better dye decolorization under static culture than under shaking conditions. Consequently, we decided to conduct further decolorization experiments under static conditions, as reported by Barathi et al. (2020) and Montañez-Barragán et al. (2020).

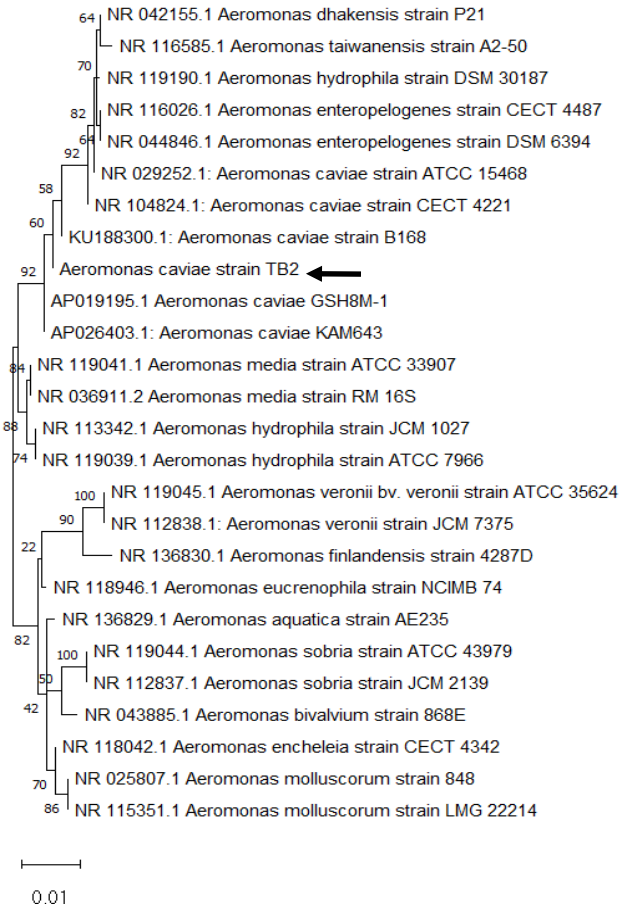

Figure 1. Phylogenetic tree of isolate TB2. The 16S rRNA sequences maximum likelihood tree was constructed using MEGA-11 software with a bootstrap value 100. The black arrow indicates the position of isolate TB2 within the tree

Effect of pH on decolorization percentage

The pH is an influential factor in numerous microbial activities. The effect of pH on the decolorization ability of the isolate was assessed across a range of pH values 5.0-9.0 (Figure 4). The level of decolorization varied depending on the pH during the 6-day incubation period. An increase in pH from 5.0 to 7.0 resulted in a higher decolorization percentage. Peak decolorization activity was observed at pH 7.0 (56.89%). However, the activity was quickly reduced under acidic conditions at pH 5.0 (2.59%) and pH 6.0 (7.93%) and alkaline conditions at pH 8.0 (37.39%) and pH 9.0 (33.28%), as shown in Figure 4. This indicates that the optimal pH for the growth of TB2 has a significant impact on its dye decolorization efficiency.

Previous research has indicated that acidic and alkaline conditions are detrimental to the biodegradation of azo dyes, as the protonation and deprotonation of these dyes at lower and higher pH levels lead to changes in their chemical structure, restricting the adapted microbes from decolorizing them (Masarbo and Karegoudar 2022). Studies have shown that at pH 7, the metabolic activity of the enzyme is most active, allowing it to easily bind to the active site of the dye surface, resulting in increased dye decolorization activity. However, a negative decolorization performance was observed as the pH increased from 7 to 9 (Hashem et al. 2018). The electrostatic force of attraction between the negatively charged surface of the biomass and the positively charged dye cations is strongest at high pH values, making dye molecule transport across the cell membrane a rate-limiting phase in decolorization (Srivastava et al. 2022). Based on these findings, further experiments for dye decolorization were conducted at pH 7.

Effect of temperature on decolorization percentage

Temperature is critical in microbial decolorization because microorganisms exhibit optimal activity within specific temperatures. As shown in Figures 5 and 6, the extent of dye decolorization varied in response to different incubation temperatures, including 27°C, 32°C, 37°C, and 42°C. The results indicated that for temperatures of 27°C, 32°C, and 37°C, decolorization increased steadily from day 2 to day 6, reaching a peak on day 6. However, for the incubation temperature of 42°C, decolorization only increased from 10.51% on day 2 to 12.24% on day 6, without following the same pattern as the other temperatures. The highest levels of decolorization were observed at 27°C, with the isolate decolorizing 77.10% of TB. This was followed by the isolates which were incubated at 32°C and 37°C, decolorizing 63.14% and 59.14%, respectively, after 6 d.

As the temperature increased, the decolorization process improved until it reached the optimal limit, after which a decline in the decolorization percentage was observed. This decline might be due to the loss of cell viability at higher temperatures, which could lead to the denaturation of the azoreductase enzyme (Ajaz et al. 2020; Mishra et al. 2022). Therefore, at the higher temperature of 42°C, the significant decrease in decolorization may have been primarily caused by cell viability loss.

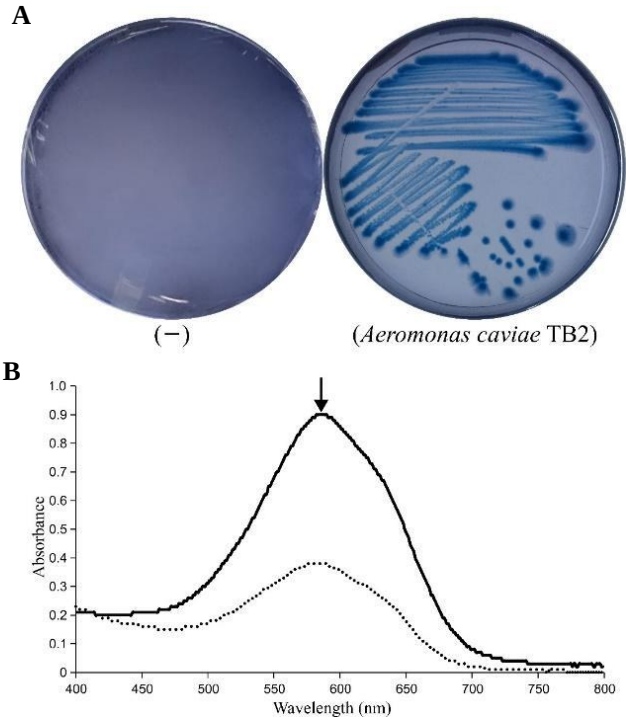

Figure 2. Decolorization activity of *Aeromonas caviae* TB2. A Negative control (uncultured TB-supplemented Nutrient Agar media at 0.0025% (w/v) TB) and the bacteria were grown on TB-supplemented Nutrient Agar media at 0.0025% (w/v) TB and incubated at 37°C for 6 d. B. The bacteria were grown in TB-supplemented Nutrient Broth (NB) at 0.0025% (w/v) TB and incubated at 37°C for 6 d. The profiles were measured using a UV-Vis spectrophotometer. Solid line: NB + 0.0025% (w/v) TB as a control; dotted line: NB + 0.0025% (w/v) TB with *Aeromonas caviae* TB2. The arrow indicates the TB adsorption peak at 590 nm

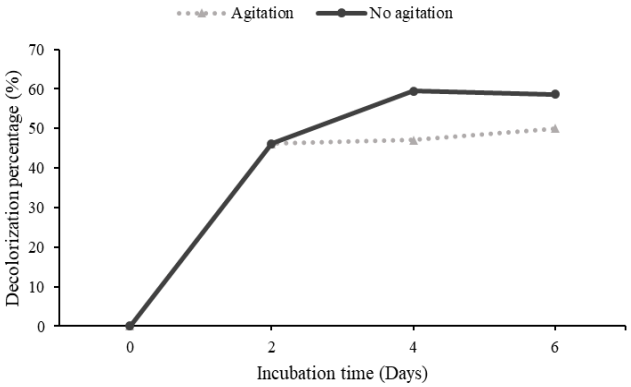

Figure 3. Effect of agitation (100 rpm) on the decolorization percentage of *Aeromonas caviae* TB2. The isolate was incubated in TB-supplemented Nutrient Broth (NB) at 0.0025% (w/v) TB at 37°C for 6 d. Measurements were conducted in triplicate

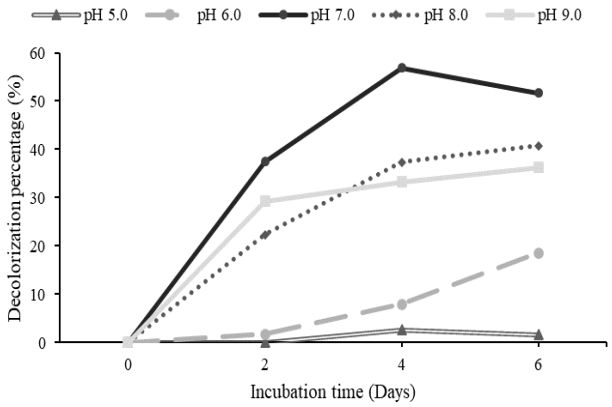

Figure 4. Effect of pH on the decolorization percentage of *Aeromonas caviae* TB2. The bacteria were incubated in TB-supplemented Nutrient Broth (NB) at 0.0025% (w/v) TB at different pH conditions (pH 5.0, with media in 20 mM acetate buffer, pH 6.0 in 20 mM citrate buffer, pH 7.0 in 20 mM phosphate buffer, pH 8.0 in 20 mM Tris-HCl buffer, and pH 9.0 in 20 mM glycine-NaOH buffer), and incubated at 37°C without agitation for 6 d. Measurements were conducted in triplicate

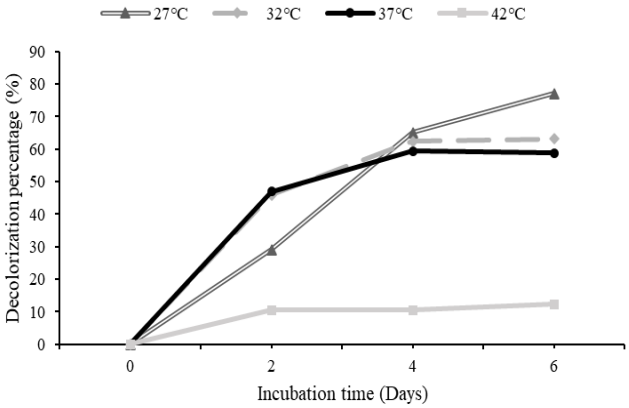

Figure 5. Effect of temperature on the decolorization percentage of *Aeromonas caviae* TB2. The isolate was incubated in TB-supplemented Nutrient Broth (NB) at 0.0025% (w/v), pH 7.0, at different temperatures for 6 d. Measurements were conducted in triplicate

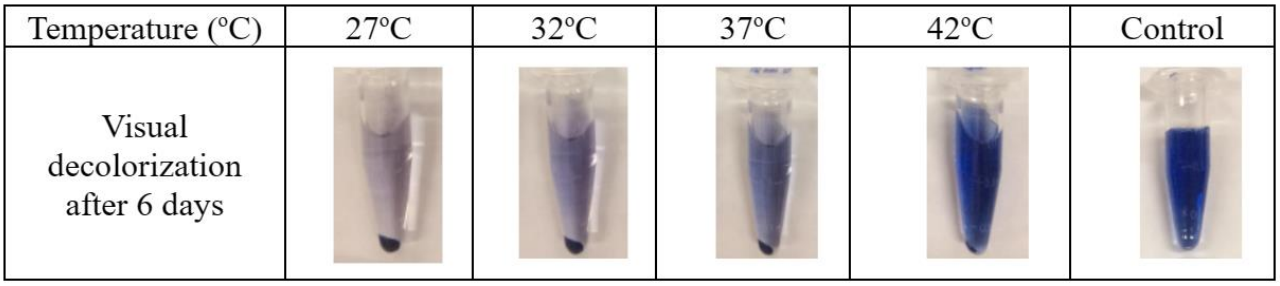

Figure 6. Visual decolorization of *Aeromonas caviae* at different temperatures. The bacteria were incubated in TB-supplemented Nutrient Broth (NB) at 0.0025% (w/v) TB, pH 7.0, at different temperatures for 6 d. The control was Nutrient Broth (NB) containing 0.0025% (w/v) TB and incubated without bacteria

Similar results have been reported by El Bouraie and El Din (2016), who found that the optimum temperature for decolorization by *A. hydrophila* was 30 to 35°C. Numerous studies have indicated that the optimal temperature for favorable biochemical activities, such as bacterial strain growth and enzyme production, falls between 28°C and 35°C (Guo et al. 2020).

In conclusion, the present study successfully isolated, identified, and examined This was accomplished by highlighting the ability of the isolated bacterial culture, *A. caviae* TB2, to remove the textile dye TB with decolorization efficiencies of up to 77.10%. These efficiencies were achieved under static conditions, a neutral pH, and a temperature of 27°C. To our knowledge, this is the first study to report the ability of the *Aeromonas* genus to decolorize TB. These findings imply the potential application of utilizing indigenous bacteria from wastewater for decolorizing dye-bearing industrial effluents, thus offering a potential solution for treating industrial wastewater.

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