Biodegrading evaluation of azo dye group – Congo Red, Methyl Blue and Methyl Orange using bacteria isolated from textile wastewater sediment

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Abstract. Textile wastewater is one of the most water polluted sources affecting the environment, ecosystems, and causing human malignancy because it contains azo dye pigment group. The treatment requires various types of physical and chemical methods which are time-consuming and hazardous environmental effects. The biodegradable method using microorganisms for textile effluent has been applied due to its environmental friendliness and affordability. Our study aims to identify the microorganisms that are capable of simultaneous degradation of three monochromatic colors belonging to the azo dying group, including Congo Red, Methyl Blue, Methyl Orange via qualitative experiments using Potato Dextrose Agar (PDA) media plus the three monochromatic colors and quantitative experiments using Pt-Co calibration curve to identify the biodegradable efficiency of each and combined bacterial isolated strains. The results showed that six bacterial strains isolated from textile waste sludge were capable of decolorizing the three azo - monochromatic pigments. Moreover, we found that strain Bacillus Subtilis E1 owning Quorum Sensing mode had the highest decoloration efficiency with color removal performance for Methyl Orange, Methyl Blue, and Congo Red was 83.7%, 30.6%, and 94.4%. In particular, the combinations of two Bacillus Subtilis strains yielded a multi-color decolorizing performance with an efficiency of over 80% for each pair.

1. Introduction
Together with the national industrialization and modernization, the textile – dyeing - clothing industries of Viet Nam have had many changes and occupied an important position in the national economy, leading to an increasing number of factories. This is one of main Vietnam's industries which accounts for the country's economic development [1].

Textile wastewater is generated from plants which have not been treated in both dye and fabric dyeing processes are one of the main routes for toxic dyes to be exposed to the environment [2]. According to worldwide statistics, more than 100,000 dyes are available with an estimated annual output of over 7x10^5 tons used and discharged with wastewater [3]. Mainly dyes containing aromatic ring (anthraquinone and triphenylmethane) or azo group (azobenzene) [4], azo is one of the largest and most versatile groups of
organic dyes [5] which are widely used in textile, printing, cosmetics, pharmaceuticals, food processing industry and laboratories [6]. Therefore, dyed sewage not only contains very high pollution and color, contains many organic compounds with color, durable structure, difficult to decompose but also highly toxic to humans and animals [7]. If untreated wastewater is entire would cause serious environmental pollution, destruction of ecosystems, and reduce the self-cleaning of the receiving water.

The biological approach of textile dyeing wastewater treatment has overcome the limitations of chemical methods and has been developing rapidly in recent years. Previous studies, including Eliana Pereira Chagas et al. (1999) studied the decolorization of azo dyes by two species of fungi, *Phanerochaete chrysosporium*, and *Pleurotus Sajorcaju*. The result showed that the two fungi were able to decomposed Orange G dye, yielding sunset yellow FCF color, and New Coccine dye, yielding ponceau 4R color, in the liquid medium, and the combined of these two strains completely discolored the New Coccine dye. Strain *P. Chrysosporium* completely removed Orange G and 60% of Tartrazine, yielding yellow color, while *P. Sajorcaju* had the weaker decolorized ability with 50% Orange G and a maximum of 20% of Tartrazine decoloration [8]. Pimjai Suwannawong et al (2010) studied the decolorization of Rhodamine B and Congo Red by the laccase enzyme from the *Lentinus polycarpous* strain. The result showed that the pure laccase enzyme from *L. Polychrous Lév*. was able to degrade the Congo Red better than Rhodamine B in the presence or absence of ABTS as an intermediate reodox [9]. Mohamed S. Mohmoud et al. (2016) studied the biological treatment of Red Azo dyes in textile wastewater by the *Aspergillus Niger* strain. The degradation ability of *Aspergillus Niger* was evaluated at a 1,000 mg/L of Red Azo initial concentration, incubated at 250 rpm stirring, 28 ± 1°C, and pH 9.0. The biodegradation efficiency was 58.6% after seven days of incubation [10]. Alicia Paz et al. (2017) have successfully studied *Bacillus Aryabhattai* strain in the decolorization of synthetic dyes such as: Brilliant Blue G-250 (Blue), Carmine Indigo (gray blue), Remazol Brilliant Blue R through experimental model by adding 1ml of *B. aryabhattai* to textile wastewater at concentration of 50 – 180 mg/l in flask 250ml erlen contains 50ml of medium and incubated in the dark to avoid changes in chemical structure under the influence of light. The results of both color reduction and COD reduction open up for the potential application of B. aryabhattai in biological treatment to actual wastes from the textile industry [11]. Wafa I. Abd El-Rahim et al. (2017) isolated 19 strains of fungi capable of eliminating 20 azo dyes, five types of *Aspergillus* and one *Lichtheimia sp*. Among them, 5 species of *Aspergillus* can be decolorized well: *A. niger*, *A. terreus*, *A. oryzaem*, *A. flavus*, *A. fumigatus*, and *A. alabamensis* and a single *L. Corymbiferastrain*. This opens up a new potential for further application of fungi in azo dyes decolorization [12] [13].

The research and application of microorganisms capable of removing dye color is very interested in the world and is always a hot topic because the current textile wastewater has a very large load of 16 - 900 m³/tons of products [14]. In general, most of the studies using microorganisms applied to decolorization showed very positive results, but most of them were applied in single colors. Textile wastewater is composed of a complex of many single colors, which form a color combination. Therefore, good single color treatment will not solve the problem of color removal in textile wastewater. Moreover, they often focus more on fungi instead of bacteria, so this is the basic premise for this topic. The main objective of the study is isolation and selection of strains that able Methyl orange, Methyl Blue, and Congo Red dyes decolorization in typical textile dyeing wastewater of Vietnam. Moreover, the study aim to biological treatment with synthetic combined strains capable of handling many colors, especially in the azo dyes group. The test results can be used as a premise for textile wastewater treatment methods using more eco-friendly and economical biological processes for the dyeing industry in Vietnam.

2. Materials and methods

2.1. Research materials

The untreated textile wastewater and activated sludge were collected at ULHWA Textile and Dyeing Company, located in Loteco Industrial Zone (Long Binh Ward, Bien Hoa City, Dong Nai Province, Vietnam). The samples were collected in sterile plastic bottles, stored at 4 °C for transporting to the laboratory, and preserved in the refrigerator at the same temperature. All chemicals (with the highest
purity available) and culture media were purchased from Merck (Germany). The tested microorganism was grown on Potato Dextrose Agar (PDA) medium

2.2. Methodological approach and experimental design

E1: Isolation, screening, and identification of dye degrading bacteria Methyl Orange, Methyl Blue, Congo Red

First, collected wastewater and activated sludge samples used to isolate dye decolorizing bacteria were maintained in potato dextrose medium (PDA), with the following steps: (i) 10 ml of each sample was added in 90 ml of potato dextrose; incubated in 20 mins, with continuously shaking at 20rpm, all maintained at room temperature (ii) then, 1 ml of enrichment samples were added in 9ml NaCl 0.9% into different concentration 10-1, 10-2, 10-3, 10-4, 10-5 (iii) 0.1 ml of diluted samples were streak on PDA (potato dextrose agar) 2% for the enrichment containing 100 mg/l of Congo Red, Methyl Blue, Methyl Orange dye and incubated within 48h at 370C and triplicate for each concentration. The microorganisms will be selected using Methyl Orange first, then resumed from selected strains capable of decolorizing Methyl Orange and continue to evaluate strains that were capable of decolorizing Methyl Blue and Congo Red. Qualitative assessment by assessing the resolution diameter on agar plates with added dye. Measure 100 mL of PDA medium and 100 mL of Methyl Orange, Methyl Blue, Congo Red (different experiments) 200 mg/L in an erlenmeyer of 250ml. After that, adding 50µl/strains proliferation of isolated strains. Quantitative evaluation of color resolution by using the spectrometers (UV-VIS Thermo scientific- Evolution 60S) with a wavelength of 455nm and calculate the concentration based on the Pt-Co calibration curve. The control sample doing the same added material but not added any microbial strain. The experiment repeated triple times.

E2: Evaluate the multi-color resolution ability of microorganism strains through Pt-Co calibration curve from strains of microorganisms capable of resolving Methyl Orange

Study of Md. Ekramul Karim et al shows that the effectiveness of microbial strains can be improved through the combination of strains (2018) [15]. In addition, due to the fact that wastewater is usually a combination of colors, this experiment wants to evaluate the general color processing capability based on the Pt-Co color scale (according to the criteria for evaluating color in wastewater. of Vietnam). Our experiment used 120 mL of PDA medium supplemented with 120ml (concentration of 200 mg/L for one color) from 40ml Congo Red, 40ml Methyl Blue, and Methyl Orange dyes into a 250ml sterile flask. After that, using a pipette draw 50µl/strains of microorganisms into the flask, each treatment combines 2 strains follow table 1. The experiment was performed with triple replicates for each pair of strains and each type of dye. The control sample doing the same process but not adding any strains of microorganisms. The duration of the experiment was 7 days.

| NUM | CODE | NUM | CODE |
|-----|------|-----|------|
| 1   | O2-4 | 9   | O4-9 |
| 2   | O2-5 | 10  | O5-6 |
| 3   | O2-6 | 11  | O5-7 |
| 4   | O2-7 | 12  | O5-9 |
| 5   | O2-9 | 13  | O6-7 |
| 6   | O4-5 | 14  | O6-9 |
| 7   | O4-6 | 15  | O7-9 |
| 8   | O4-7 |

2.3. Bacterial identification:
The isolated bacterial strains that were capable of decoloration were sent to sequencing for the 16SrDNA (Institute of Tropical Biology, Ho Chi Minh City, Vietnam). Then, the obtained sequencing was BLAST from NCBI using the default mode for the bacterial identification.
3. Results and discussions

3.1. Isolation, screening, and identification of Methyl Orange degrading bacteria

As a result, six isolated colonies showed the good ability to decolor Methyl Orange, especially the O7 and O5 strains with the highest resolution of Methyl Orange. The control sample 28507 Pt-Co concentration, and lightly reduce after 3 and 7 days of incubation, 3.3%, and 6.7% reduction, respectively. After 24h incubation, strains from 6 samples containing microorganisms the Pt-Co value continuously decreased significantly compared to the original control sample. After 72h incubation, the O7 strain showed the best color resolution with the remaining concentration of 6407 Pt-Co (77.52% reduction compared to the original control sample). In contrast, the O4 strain was weakest in resolving Methyl Orange with a concentration of 14973 Pt-Co (47.47% reduction compared to the original sample). Finally, the strain O7 still showed the best color resolution after 7 days incubation with the Pt-Co value 2673 (90.6% reduction compared to the original control sample) and the O5 strain had the second degradation capacity with the remaining concentration of 4640 Pt-Co (83.7% reduction compared to the original control sample), while O2 strain showed the least color resolution (8840 Pt-Co value, 69% color reduction compared to the original control sample).

![Figure 1](image1.png)

**Figure 1.** The average ability graph in Methyl Orange resolution of 6 microbial strains per calibration curve of Pt-Co color after 7 days.

![Figure 2](image2.png)

**Figure 2.** Decolorization of O7 strain on Methyl Orange after 7 days.
3.2. Isolation, screening, and identification of Congo Red degrading bacteria

The isolation result from strains of microorganisms capable of resolving Methyl Orange has obtained 6 capable microorganism strains of resolving Congo Red color, the average resolution diameter ranges from 12 mm to 70 mm. Details of strains are described in the following table. As the results, all isolated microorganism strains were able to decolor Congo Red on PDA culture medium, with the average decolorized diameter ranges 18 - 23 mm, in which R5 has the lowest resolution (18 mm) and the R2, R4 has the highest resolution (23 mm).

The control sample at the initial time had 4483 Pt-Co and decreased 1.8% and 3.85% after 72 hours and 7 days incubation, respectively. For 6 strains or colonies isolated from 6 samples were able to decolorize Congo Red with the capacity compared to the Pt-Co value of the control sample (4483 Pt-Co) after 24 h. After 72 days, Congo Red dye concentration significantly decreased, with Pt-Co value dropped from 31.8% to 74.95% compared to the original value, with R5 strain has the highest capacity decolonization (74,95%). After 7 days, the concentration of Congo Red dye in the samples decreased to 225 Pt-Co value, with the R5 strain showed the best decolorization (94.4% reduction compared to the original control sample).
3.3. Isolation, screening, and identification of Methyl Blue degrading bacteria

The isolation result from strains of microorganisms capable of resolving Methyl Orange have obtained 6 capable microorganism strains of resolving Methyl Blue color, the average resolution diameter ranges from 18 mm to 23 mm. From the results of color resolution analysis on PDA culture medium, all isolated microorganism strains have the ability to resolve Methyl Blue color, the average resolution diameter ranges from 12 mm to 70 mm in which B5, B6 and B7 strain has the lowest resolution with a resolution diameter of 12 mm and the B9 has the best resolution with a resolution diameter of 70mm.

In the control sample at the initial time, the control sample had a concentration of 533 Pt-Co. After 3 days of culture, the control sample decreased to 513 Pt-Co (3.7% reduction compared to the original control sample) and the following days the control sample continued to decrease slightly. By 7 days, the concentration remaining had 503 Pt-Co (5.6% reduction compared to the original control sample).
In the remaining samples at the beginning, 6 samples containing microorganisms had an initial concentration of 533 Pt-Co. After 1 day, the strains concentration increased compared to the original control sample because due to the color capacitor. However, only the B5 strain showed signs of decrease to 517 Pt-Co (3% reduction compared to the original control sample). After 3 days, the strains concentration continuously decreased compared to day 1, the B5 strain continued to reduce the dye concentration to 417 Pt-Co (21.76% reduction compared to the original control sample). By 5 days, the strains had a color uptake phenomenon that increased the concentration, only the B5 and B9 strains continued to reduce the dye concentration to 377 Pt-Co and 397 Pt-Co. By 7 days, the phenomenon of color uptake increases the concentration that occurs in strains B7, B2, and B9. The B5 strain reduced best with the remaining concentration of 370 Pt-Co (30.6% reduction compared to the original control sample).

Figure 7. The average ability graph in Methyl Blue resolution of 6 microbial strains per calibration curve of Pt-Co color after 7 days

Figure 8. Decolorization of B5 strain on Methyl Blue after 7 days

3.4. Evaluate the multi-color resolution ability of microorganism strains through Pt-Co calibration curve from strains of microorganisms capable of resolving Methyl Orange
The control sample had a concentration of 8057 Pt-Co concentration, decreased slightly after 3 days of culture, the Control sample decreased to 7853 Pt-Co (2.5% reduction compared to the original Control) and the following days the Control sample continued to decrease slightly. After 7 days of culture, the residual concentration was 7663 Pt-Co (a slight decrease of 4.88% compared to the original).

In other states at the initial time, 15 samples containing the pair of microorganisms were at an initial concentration of 8057 Pt-Co. After 3 days of culture, multi-color dye concentration decreased significantly, in which the O5-6 strain pair decolored best with the residual concentration of 1923 Pt-Co (76.12% reduction from the original sample), in this pair of strains, there is strong absorption by bacteria biomass, whereas, in 5 pairs of symbiotic strains for high decolorization effect, pairs of O2-6 have the lowest decolorization efficiency with the concentration of 3190 Pt-Co (60.41% reduction from the original sample). In the following days, multi-color dye concentrations continued to decrease, however, on day 5, pair O4-7 had a color uptake phenomenon that increased the concentration. On day 7, multi-color dye concentrations in the samples ranged from 1517 to 2750 Pt-Co, in which O2-9 and O5-6 pairs had the best result with the remaining concentration of 1517 Pt-Co for the O2-9 pair (81.18% reduction compared to the original sample) and 1573 Pt-Co for the O5-6 pair (80.47% reduction compared to the original sample). The pair of R4-7 strain is still the lowest decolorization pair with the concentration of 2750 (65.87% reduction compared to the original sample) because color uptake phenomenon that increased the concentration.

Thus, all 15 pairs of symbiotic microorganism strains were able to decolor of multi-color dye good, especially the pair of O2-9 and O5-6 pairs had the best result with the remaining concentration of 1517 Pt-Co for the O2-9 pair (81.18% reduction compared to the original sample) and 1573 Pt-Co for the O5-6 pair (80.47% reduction compared to the original sample). Therefore, the low decolorization strains combined with other strains with the effect of symbiotic stimulation and support leading to an increase in the ability of decolorization. This is consistent with the hypothesis of K.M. Oliver [15], the author argues that symbiosis originates from the proper interaction in the suitable strains or species will bring different benefits depending on the mechanism of the resonant pairs. Experimental results of Md. Ekramul Karim [16] also showed that the isolated microorganisms strains from the culture containing Novacron Super Black-G colors have the decolorization effect from 40 to 42% but when combined, the decolorization effect increases up to 90%.
3.5. **Morphology of the strains was selected from the combination of strains capable of multi-color resolution.**

From gram stain results show that O2, O6, O9 strains have similar morphological gram stain results, both rod-shaped and gram-positive, which are suitable for biological treatment applications. Strains O5 are the strains with the most different morphology than the other strains. According to the results of gram staining, O5 bacteria are spherical, have a round core inside, stick together in clusters, and have a membrane covering the outer core.

| CODE | DESCRIPTION | MORPHOLOGY |
|------|-------------|------------|
| O2   | Rod-shaped bacteria, gram-positive group | ![Image](image1.png) |
| O5   | Bacteria are spherical, with a round nucleus inside, surrounded by a membrane. Mechanism Quoring Sensing (*) | ![Image](image2.png) |
| O6   | Rod-shaped bacteria, gram-positive group | ![Image](image3.png) |
| O9   | Rod-shaped bacteria, gram-positive group | ![Image](image4.png) |
Most of the O2, O6, O9 strains have uneven resolution performance in monochromatic colors MO, MB, CR in quantitative experiments. If the quantitative experiment in monochrome has 3 colors, the above strains only reduce the concentration in 2 monochromatic colors and the remaining 1 color, the phenomenon of increasing concentration due to staining. The difference is that O5 strains have good monochrome resolution performance in three colors at the same time, there is no accumulation at times for O5 strains.

Bacteria are mainly considered to be separate individuals of unicellular organisms and they do not interact or relate to each other. However, this view has changed when scientists today have discovered that many species of bacteria can communicate and coordinate with each other on a multicellular level rather than just a single cell, through the "Quorum Sensing" mechanism. Miller et al. It has been investigated that bacterial Quorum Sensing is a method that relies on cell density in cell-to-cell communication, regulating gene expression, and when it reaches its maximum. This will create a biofilm connecting the bacterial cells [17]. This phenomenon is called "Quorum sensing". Figure 12 below is the process by which bacteria use the “Quorum Sensing” signal to link, communicate and coordinate with each other at the multicellular level, according to the research of L. Suleman et al [18].

Bacteria bind to each other through "Quorum Sensing", which causes QS to propagate signal molecules, the gene code "invites" bacteria to cooperate, which in turn will increase bacterial density and increase production biomass [19]. In the future, if applied well, it can be confirmed that this will be one of the microorganisms that can estimate the treatment efficiency will increase thanks to the Quorum Sensing mechanism and from there will develop improved treatment Biological wastewater weaving well dyed.

Figure 10. Spore image of O5 strain through a microscope with 100x

Figure 11. An illustration of the bacterial process using “Quorum Sensing” and a spore scan of O5 strain through a 100x microscope.
Through PCR molecular biology method, comparing the 16S rRNA sequence of microorganisms with genetic data banks using BLAST search, the database through BLAST search shows that the O2 and O9 strains have 100% similarity to the Bacillus subtilis strain SR3-30. Based on the results of genetic decoding and comparison with the database through BLAST search, O5 and O6 strains have a 100% similarity with Bacillus subtilis strain E1. These biological substances were created even when Bacillus subtilis was alive or dead. Bacillus subtilis has the ability to consume excess organic matter, reducing the amount of nitric and nitrate [20, reducing the color of azo dyes [21] in wastewater textiles. As for the Quorum Sensing mechanism, B. Subtilis is one of the strong prototypal microorganisms in linking multicellular to forming Biofilm membranes [22]. Thus, Applying the resolution mechanism of Bacillus Subtilis strain and the ability to generate the Quorum Sensing mechanism, in the future, if applied correctly, it is sure that it will improve textile wastewater treatment efficiency.

4. Conclusions
In conclusion, the study isolated and selected 6 strains of Bacteria capable of 3 color resolution of Congo Red, Methyl Blue, Methyl Orange. The O5 strain from the Methyl Orange color resolution strain set is capable of multi-color resolution compared to other strains. Especially, the O5 strain had the name Bacillus Subtilis E1 with a Quorum Sensing mechanism for simultaneous resolution of 3 monochrome colors with color removal performance for Methyl Orange 83.7%, Methyl Blue 30.6%, Congo Red 94.4%. In particular, the two combinations of the two Bacillus Subtilis strains yield a multi-color removal performance with a color removal efficiency of over 80% per pair. Although the research still needs to go through many successive steps, the benefits of dye biodegradation in wastewater and the environment are promising.

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