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Efficiency of Bacterial Strains in Decolorization of Hazardous Dyes under Sustainability Conditions

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ABSTRACT

Synthetic dyes are frequently used as additive compounds in different industries. The dramatic increase of toxic dyes and their strong durability and stability in aquatic environments lead to rising severity of both the environment and human health. The current study shows the efficiency of bacteria as biological agents in decolorizing some hazardous dyes, without energy supply and chemical substance uses. In this study, the potential of two identified proteus strains (P. strain C and P. strain Y) isolated from the main opened wastewater of Soran City-Iraq was assessed for decolorization of Methylene blue (MB) and Methyl orange (MO). The bacterial strains were separately and together as a consortium tested at different concentrations of the selected dyes (10, 30, 50 and 100 mg/l). After 48 hours of incubation, the decolorization efficiency of the two isolates was significantly observed with respect to the concentrations, and this efficiency lowered with increasing concentration. The decolorization efficiency for MB dye ranged from 67% to 75% by P. strain C, and from 73% to 79% by P. strain Y. Whereas, the efficiency of the mixed culture of the two isolated bacteria (consortium) ranged in 79-88%. MO was more easily degradable than MB, and the efficiency was >90% by individual experiment, and >96% by consortium. In conclusion, the two newly isolated proteus strains were confirmed as promising biological agents for degradation of MB and MO. Therefore, the isolates can be proposed as biological agents for bioremediation under environmental sustainability.

1.Introduction

Synthetic dyes are considered as the most abundant group of textile dyes and up to 100,000 dyes have been developed that are commercially used as additive colorants for different purposes, therefore the annually worldwide releasing amount of dyes range from 7,108–10,000 kilograms (Bouras *et al*., 2021). Sehar *et al*. (2022) indicated that a significant amount of the dyes is released and drained into the immediate environment, particularly wastewater streams. Synthetic dyes are consumed and frequently contribute to freshwater reduction via different methods, including using synthetic dyes for industrial purposes (Jiang, 2023). The discharge of untreated wastewater including dyes directly into water bodies threatens aquatic creatures and finally disturbs aesthetic nature and surrounding society (Telke *et al*., 2015; Almroth *et al*., 2021). On the other hand, using untreated municipal wastewater for irrigation purposes is a health concern due to the toxicity of products by different types of chemical contaminants including heavy metals (Ahmad *et al*., 2024).

Dyes can be classified as either synthetic or natural chemical compounds that can be used to impart color to material, leather and wooden surfaces, the absorption and permanent bonding of the dyes are due to the complexity of the structure which leads to withstand harsh conditions (Khan *et al*., 2013). Different types of dyes are categorized based on their origin, structure and application (Akpomie and Conradie, 2023). Among them, synthetic dyes are deteriorating aquatic environment, their durability and stability lead to persistently suspend and persist in effluents (Nguyen and Juang, 2013). The durability of dyes resulted from the occurrence of covalent bonding between dye and colorant material (Alegbe and Uthman, 2024). The opening of synthetic dyes into the water body leads to increasing turbidity, BOD and COD, also causes a reduction of dissolved oxygen levels due to covering the surface of the water, photosynthetic activity, and water quality (Khan *et al*., 2013; Patel *et al*., 2021). Therefore, bioremediation and removing dyes must proceed before release into aquatic environments (Hassan *et al*., 2021). The degradation of azo dye is triggered by involving a catalytic mechanism to breakdown the azo bond (–N=N), the degradation is supported by contributing azo-reductases, laccase and peroxidase enzymes (Jasińska *et al*., 2024). The aromatic amines as the residue of the decolorization process in water is considered as the main contributor to threatening living cells and carcinogenic (Zhang *et al*., 2023). However, the biodegradation of dyes can be performed aerobically and anaerobically, but some dyes eg: methylene blue (MB) as a toxic and carcinogenic dye easily released to natural water sources, it is difficult to degrade with severity for humans, organisms and the environment (Khan *et al*., 2022). Methyl orange is another recalcitrant, toxic and mutagenic synthetic dye, and known as organic, heterocyclic and sulfonated with high water solubility (Haque *et al*., 2020).

Several chemical, physical and biological techniques have been proposed to remediate water bodies and remove dyes, physicochemical strategies involve chemical additives, energy consumption and high sludge production including toxic by-products (Holkar *et al*., 2016). Due to the high durability, stability and non-decomposable of synthetic dyes, biological treatment is advocated as an alternative strategy to remove/degrade synthetic dyes (Sehar *et al*., 2022; Imran *et al*., 2015). Biological treatments are considered as distinguished and promised strategy for decolorization due to; high efficacy, cost effective, environmentally friendly and sustainability (Sadeghi *et al*., 2019; Tang *et al*., 2022). Moreover, easily applicable, less sludge generation, no additive chemical reagents, energy-saving and the byproducts are generated and decomposed non-toxically (Ali *et al*., 2022). The efficiency of bacteria is estimated up to 100% for the biodegradation of dye, and mixed bacterial strains as a consortium frequently outperform individual bacteria in removal efficiency (Guo *et al*., 2020). For instance, different strains of bacillus species, *Proteus mirabilis* and *Pseudomonas aeruginosa* achieve the degradation of synthetic dyes. And these bacteria were documented as assured degraders for breaking down the presented bonds of azo dyes, adequately (Mohanty and Kumar, 2021).

Nowadays, synthetic dyes are delineated for their high stability and resistance to photocatalyst and chemical degradation. The main reason for conducting this study was to confirm the potential of the isolated bacteria to degrade the selected synthetic dyes. To focus on this issue, the present study designated to isolate some native bacteria from the selected wastewater, which are considered as great degraders and screening their degradation efficiency for candidate dyes. Announcement of the ability of the isolates as a promised strategy among biological treatment techniques, the availability and persistency of those bacteria in their original nature (wastewater) is the main reason for self-purification as a biological aspect in combination with physicochemical aspects for environmental protection and wastewater treatment systems. More importantly, this study was intended to address the issue of using the studied untreated wastewater for irrigation along the sewage canal.

2.Materials and Methods

2.1. Sample collection

The sample of water was collected from one of the main canals of municipal wastewater of Soran City- Erbil, KRI, the adjacent location mainly the main canal of wastewater situated at a latitude of 36.662854˚ and longitude of 44.544966˚. in a 150 ml dark bottle prewashed with wastewater and directly transferred into a laboratory.

2.2. Preparation of the reaction mixture

In this study, three candidate dyes were prepared for the decolorization experiment (Crystal violet, Methylene blue and methyl orange). Stock solution of each dye was prepared, and different concentrations of 10, 30, 50 and 100 mg/l were performed in the experiment by diluting a specific amount of stock solution in nutrient broth media. On the other hand, young cultures of the isolated bacteria were prepared in nutrient broth.

2.3. Isolation and screening of bacteria for decolorization

The collected sample was aseptically cultivated in nutrient broth in a ratio of 10:100 ml, and incubated at 30±2 °C for 24 hours under certain conditions. The proliferated bacteria were spread on nutrient agar plates and incubated for the next 24 hours, single colonies were subcultured to obtain a pure culture (Akrayi, 2011). The individual colonies were cultured separately, and common characteristics were perceived, as shown in Plate 1.

The isolated strains were cultured in nutrient broth separately and mixed as a consortium to screen the ability to degrade the selected synthetic dyes by amending 200mm of CV, MB and MO stock solutions to 5 ml young culture (Mohanty and Kumar, 2021).

2.4. Dye decolorization experiments and quantitative analysis

The experiment was conducted in two sets (one for isolated bacterial strains individually and the second one for a developed consortium of the two isolates). The media of decolorization contained 5ml of each dye concentration and enriched with 0.1ml of proliferated bacteria, and incubated at 30±2 °C for 48 hours. Afterward, to detect the exact quantity of decolorization the samples were centrifuged at 5,000 rpm for 5 minutes and the supernatant part was measured at 597 nm using a UV/Vis spectrophotometer (Model: AE-S60-4UPC), according to Ben *et al*. (2023). The experiment was conducted in replicates of two for each concentration of dyes. The measurement of decolorization efficiency was calculated as:

$$\% \text{ Decolorization} = \frac{A0-A1}{A0} * 100 \qquad (1)$$

where *A0* is the absorbance of the media before treating, and *A1* is the absorbance of the media treated with the bacteria at the end of the experiment.

2.5. Identification of bacteria

The isolated bacterial strains were identified by the 16S rRNA technique. The genomic DNA was obtained from an overnight culture (Kishor *et al*., 2021). The 16S rRNA genes were amplified with forward and reverse universal primers i.e 27F (5’-TCAAAKGAATTGACGGGGGC - 3’) and 1492R (5’- AGGCCCGGGAACGTATTCAC - 3’). Moreover, the sequences were submitted to NCBI nucleotide blast for detection of the percentage identity of the submitted sequence to existing sequences of NCBI. MEGA 11 software was conducted to join the isolated strains with their relative strains and build the phylogenetic tree based on identity %. Furthermore, the isolated strains were successfully deposited to GenBank-NCBI with provided accession numbers; PQ133629 and PQ133630 for Ww P. strain C and Ww P. strain Y, respectively.

2.6. Experimental design and statistical analysis

GraphPad Prism 10 software was used to statistically analyze the obtained results. One-way ANOVA and Tukey post-hoc was performed as suitable to explore differences between and within various treatments and the significance with a 95% confidence level.

3.Results

3.1. Isolation, screening and identification of bacteria

Two strains of bacteria were isolated from the collected wastewater and microscopically characterized as rod-shaped and gram-negative bacteria. Firstly, their potential for decolorization of the selected dyes were screened and both isolates exhibited the ability to decolorize MB and MO, but decolorization of CV was not observed. The two bacterial strains were identified as Ww P. strain C and Ww P. strain Y by 16S rRNA molecular technique. As a result of nucleotide BLAST, the two strains compared to the database of NCBI with identity 98.2% to other Proteus strains, and the product of MEGA 11 Software shown in the following phylogenetic relationship and homology to few strains of *P. vulgaris*, *P. hauseri* and *P. terrae*, Figure 1 and 2.

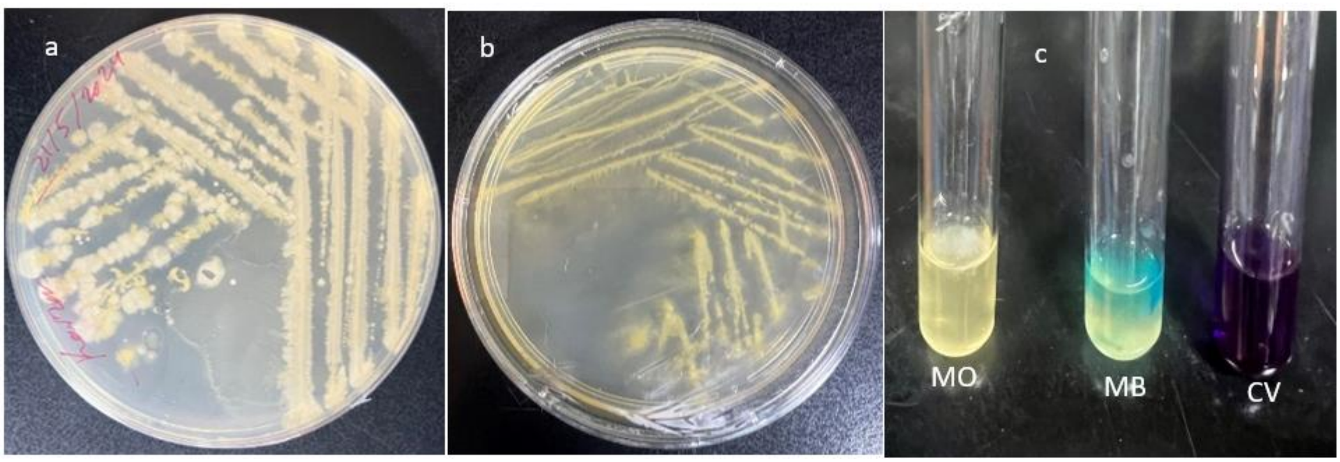

Plate 1: Isolation of bacteria from wastewater, a: mixed culture containing the two strains of bacteria, b: pure culture of Ww. P. strains Y, c: screening of the candidate dyes.

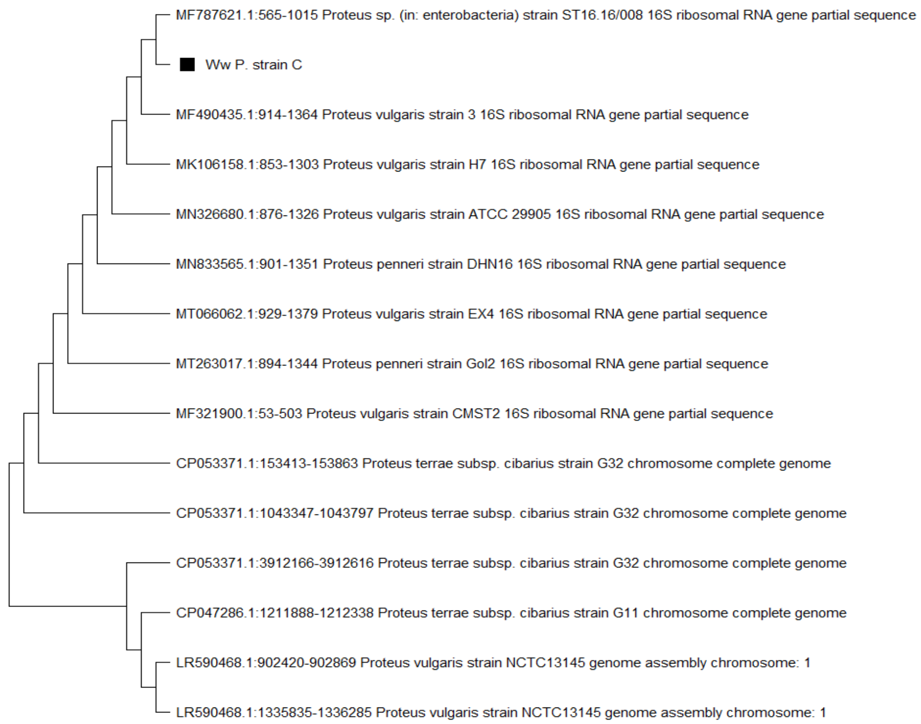

Figure 1: Phylogenetic tree (Neighbor-joining) of the isolated bacteria Ww P. strain C and existing bacterial strains.

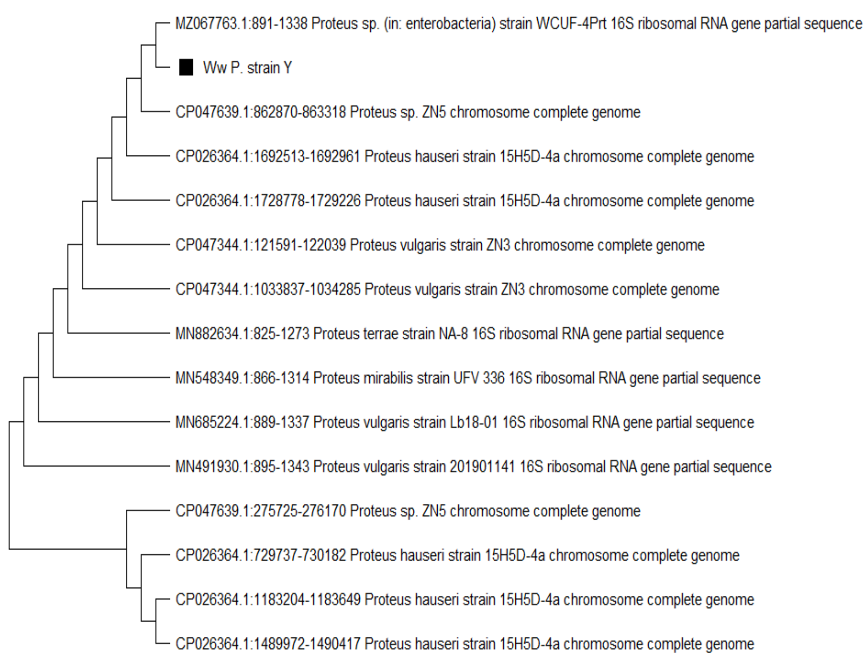

Figure 2: Phylogenetic tree (Neighbor-joining) of the isolated bacteria Ww P. strain Y and existing bacterial strains.

3.2. Biodegradation of synthetic dyes

Decolorization experiments were conducted under certain conditions and the biodegradation of the selected dyes was reported by the isolated bacterial strains after 48 hours of incubation. In general, the results indicated that the two strains (Ww P. strain C and Ww P. strain Y) were evaluated as good degradable bacteria according to the detected absorbances before and after treatment of the different concentrations of dyes. Based on statistical analysis, MB and MO were significantly degraded (P value≤0.05). Moreover, a synergistic relationship of the two isolates was noticed in a mixed culture and their strong degradation ability as a consortium was observed more significantly throughout the study (P value≤0.05), as given in Table 1.

In the first experiment, the two isolated bacterial strains (Ww P. strain C and Y) were separately subjected, and the decolorization efficiency for MB dye was ranged from 67 to 79%. Whilst it was increased to the highest value of 88% by mixed culture of consortium (second experiment), as depicted in Figure 4. The two isolates were applied to the experiment separately, the potential of Ww P. strain Y was higher than that obtained by Ww P. strain C, and their ability gradually decreased from the lower to higher concentrations; 10–100 mg/l.

The percent of decolorization among the two isolated strains was non-significant (P value≥0.05). The efficiency of Ww P. strain C for decolorization of MB in different concentrations of 10, 30, 50 and 100 mg/l were in order to: 73, 75, 72 and 67%. Whereas, the values were much higher by Ww P. strain Y (79, 74, 75 and 71%), respectively.

On one hand, the percentage of decolorization between the consortium and each of Ww P. strain C and Y was significantly different (P value≤0.05). Regarding removal efficiency of the consortium for the selected dyes, the maximum rate of degradation efficiency was 88% for the lowest concentration (10 mg/l), and the minimum rate was 79% for the highest concentration (100 mg/l). In the present study, the percentage of decolorization for MO dye was significantly higher than the value recorded in MB decolorization, and it was not less than 90. The efficiency of mixed isolates as a consortium was higher than that displayed individually, but the difference was non-significant (P value≥0.05). The maximum value obtained was 99% for the lowest concentration of 10mg/l, this value was lowered to the lowest of 96% in 50mg/l, as shown in Figure 4. The potential of singular bacterial strains nearly lowered from low to higher concentrations, but the differences were non-significant (P value≥0.05). The decolorization ability of Ww P. strain C and Ww P. strain Y ranged from 90 to 96% and 94 to 97%, respectively.

Table 1: Initial and final absorbances (**λ**) of the studied dyes at different concentrations in mg/l.

Dye	Conc. mg/l	Initial Abs. λ	Final Abs. λ		
			P. strain C±SD	P. strain Y±SD	Consortia
MB	10	0.59±0.0^{a}	0.16±0.03^{b}	0.125±0.025^{b}	0.07^{c}
	30	1.42±0.002^{a}	0.355±0.005^{b}	0.365±0.015^{b}	0.22^{c}
	50	2.27±0.005^{a}	0.63±0.02^{b}	0.575±0.015^{bc}	0.4^{c}
	100	2.9±0.001^{a}	0.96±0.023^{b}	0.855±0.005^{b}	0.6^{c}
MO	10	0.3±0.001^{a}	0.013±0.003^{b}	0.01±0.0^{b}	0.004^{b}
	30	1.02±0.001^{a}	0.045±0.035^{b}	0.045±0.036^{b}	0.03^{b}
	50	1.85±0.004^{a}	0.125±0.015^{b}	0.055±0.045^{c}	0.07^{bc}
	100	2.63±0.002^{a}	0.25±0.02^{b}	0.16±0.04^{c}	0.04^{d}

Note: values in each row with different letters are significantly different at P≤0.05

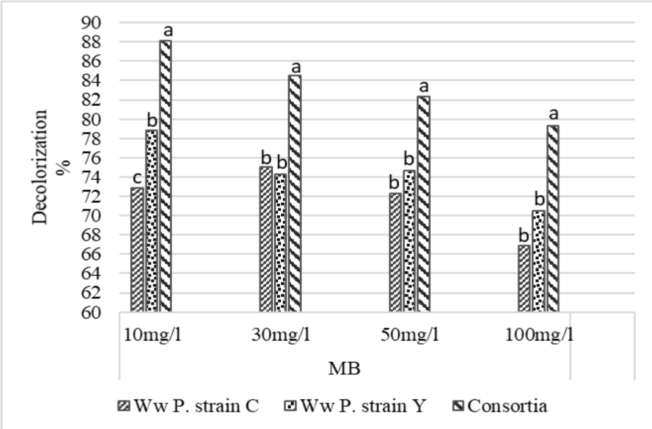

Figure 3: Efficiency of Ww P. strain C and WW P. strain Y compared to mixed culture of Ww P. strains C and Y (consortium) to decolorize MB at different concentrations.

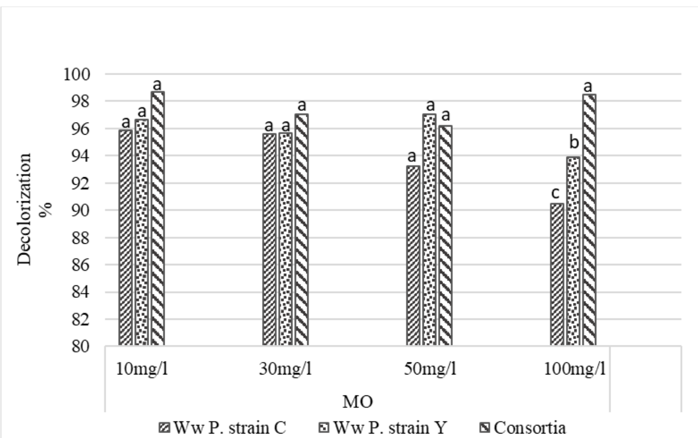

Figure 4: Efficiency of Ww P. strain C and WW P. strain Y compared to mixed culture of Ww P. strains C and Y (consortium) to decolorize MO at different concentrations, the decolorization % is homologous.

4. Discussion

The two isolated bacteria from the studied municipal wastewater were identified and confirmed as proteus spp. and labelled by Ww P. strain C and Ww P. strain Y which are standing for Wastewater Proteus strain C and Wastewater Proteus strain Y. The presence and survival ability of these two strains within studied wastewater and cultivation may be related to the activity of some indigenous genes allowing bacteria to adapt to different conditions, including heavy metal concentrations and the availability of xenobiotics as pollutants. The ability of *Proteus* spp. to tolerate and overcome chemical pollutants not only to utilize pollutants, but also considered as an enhancer to promote plant growth and trigger other microorganisms to provide bioremediation and environmental protection (Drzewiecka, 2016). Due to the viability of these two isolated strains in the studied wastewater, they may have feasibility to be used in municipal wastewater treatment plants and perform wastewater purification. The decolorization process by certain bacteria leads to reduced toxicity against bacterial and fungal strains, this is due to the adverse effects of dyes in disruption of microenvironment and ecosystem (Agrawal *et al*., 2017). Dye is documented as one of the pollutant sources in the main wastewater canal of Erbil City-KRI, and adversely affects the environment and human health (Shekha, 2011). This study focused on degradation of MB and MO dyes, because of their highly toxicity, mutagenic and carcinogenic properties, and their commercially availability in various sectors (Khan *et al*., 2022; Haque *et al*., 2020).

Saeed *et al.* (2022), proposed bacteria to mitigate aquatic pollution specifically for the decolorization process as a promised technique. This study revealed the high potential of the two isolated *Proteus* spp. strains (Ww P. strain C and Ww P. strain Y) to degrade MB and MO dyes at 48 hours, and their ability for decolorization was comparable with other bacteria and dyes that were noticed in other studies. Fareed *et al*. (2022), isolated *Bacillus cereus* strain ROC from wastewater and concluded great potential for degradation of two selected azo dyes “Reactive orange 16 and Reactive black 5”. Moreover, *Salinivibrio kushneri* HTSP as a gram-negative bacterium was considered a potent decolorizer of CBB G-250 and Congo red dyes with an efficiency of more than 80% (John *et al*., 2020). The results of this study showed a higher decolorization rate, more than 80% on average when the bacteria adjusted to the experiment individually, while it was more than 95% for the consortium experiment.

The results indicated that the bio-decolorization efficiency of the two isolates were significantly higher in the consortium experiment in comparison to the individual experiment. This is due to the occurrence synergistic relationship between the proteus spp. strains and their metabolism and similarity in enzymatic reactions lead to enrol as cofactor and helper for degradation of the selected dyes (Drzewiecka, 2016).

Regarding the degradability of MB and MO, MO was more degraded in comparison to MB. However, Crystal violet was screened, but neither Ww. P. strain C nor Ww. P. strain Y achieved bio-decolorization. Because CV is qualified as an antimicrobial, antifungal, and antiparasitic dye (Sehmi *et al*., 2016), and the studied strains were suppressed, and unable to proceed with degradation on CV dye in this study. Overall, the isolated Proteus strains C and Y were considered as non-degraders for CV. Whereas, reported as promised degradation agent of MO and MB with an average of 94 and 72%, respectively. This is due to the chemical structure of the studied dyes and their natural bonding. Generally, dye molecule consists of chromophores as the main efficient group, auxochromes responsible for imparting dyes on the surface and the remained atoms are known as a matrix of the dye (Rane and Joshi, 2021). Methylene blue is a positively charged complex consisting of a polymethine dye and one unit of amino-autochrome (Anushree and Philip, 2019). MB consists of chromophores containing N–S groups and auxochrome containing N- groups on the benzene ring (Yang *et al*., 2017).

It is worth noting that, the bio-decolorization process of MB was limited and not completely removed, but the efficiency of the two isolated bacteria in the current study was substantial, because MB is known for it is toughness to be degraded, due to it is high stability and durability (Liu *et al*., 2020). Methyl orange (MO) was degraded in advance by a consortium of the two isolated bacterial strains, which is considered as a promised value among bio-decolorization processes and the removal efficiency was not less than 90% to 99%. Similarly, previous studies indicated the decolorization rate of MO by *Enterococcus faecium* is 99% (Bera and Tank, 2021), and also *Franconibacter* sp.1MS achieved 96% (Karnwal, 2019). Also, Muksy *et al*. (2024) revealed the efficacy of isolated *Aspergillus terreus* from soil to remove MO was 98%. Regarding the contributed mechanisms of decolorization, Purnomo *et al*. (2019) concluded that demethylation and hydroxylation processes are involved in MO degradation. The degradation occurs step by step: firstly, MO will demethylate via breaking bonds and cleavage occurs in the azo group and aromatic rings, then demethylation and dihydroxylation occur, hereafter MO mineralized into simpler molecules (Bahrudin and Nawi, 2019). This is also true for the biodegradation of MB dye. The study of Pandey *et al*. (2007) revealed that the degradation of aromatic amines depends on chemical structure and surrounding environmental conditions. The degradation of azo dyes occurs via enzymatic reactions through a mechanism involving the destruction of azo bond –N=N (Jasińska *et al*., 2024).

Similarly, to the current study, several studies revealed that mixed culture of bacteria (consortium) achieved complete decolorization, e.g., *Bacillus* sp., *Staphylococcus* sp., *Escherichia* sp., *Enterococcus* sp., and *Pseudomonas* sp. used in a mixed culture as consortium for decolorization of Remazol Brilliant Violet 5R with 100% bio-decolorization (Shah *et al*., 2016). However, in other instances, the consortium did not seem to achieve complete decolorization, e.g., 80% decolorization of Reactive Orange 16 by mixing *Acinetobacter* and *Klebsiella* spp. (Meerbergen *et al*., 2018).

Moreover, the decolorization efficiency of a fungus (*Trichoderma* sp.) for 0.05mg/l CV was 96%, whereas Congo red was not removed under the same condition (Ali *et al*., 2023). This indicates that the degradation of dyes varies according to the composition of the dye, experimental condition and the applied agents. Moreover, the decolorization process is affected by many abiotic and biotic factors, including environmental conditions and the ability of biological agents and participating enzymes (Khan *et al*., 2013).

5.Conclusion

The two newly isolated bacterial strains from the wastewater were confirmed as promised biological agents for degradation of the studied dyes via enzymatic reactions and breakdown of aromatic bonds. A higher percentage of decolorization efficiency was achieved by Ww P. strain Y than Ww P. strain C, and statistically, their efficiency was significant and acceptable for the decolorization of contaminated water by toxic dyes. In individual experiments, the decolorization percentage was between 67% to 97% which was achieved by Ww P. strain C for MB and Ww P. strain Y for MO, respectively. The decolorization rate in the individual experiment was lower than that observed in the consortium experiment. The mixed culture of consortium showed the decolorization percentage in a range of 79 to 99% for MB and MO, respectively. The synergistic relationship between the identified bacterial strains accelerated the process of decolorization in consortium experiment assay. It is concluded that, Ww P. strain Y and Ww P. strain C either individually or together as a consortium can be considered as corresponding biological agents in water purification and sustainability solutions. The studied strains can be recommended to be used by industries and factories in constructed bioreactors for decolorization and degradation of the wasting colours before opening into the municipal wastewater canals.

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Conflict of interest

There is no conflict of interest

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