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Evaluation of plasmid-mediated decolourisation of vat dyes by indigenous bacterial isolates from local textile factories in Itoku, Abeokuta

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ABSTRACT
Effective mitigation and control of environmental damage brought on by the improper disposal of textiles industrial effluent is particularly noteworthy. Biodegradation of textile wastewater is emerging as a successful, environmentally friendly, and promising strategy and microbial cells that contain plasmids have specific capabilities. The present study aimed to determine the presence or absence of plasmid-mediated indigenous isolates in the decolourisation of vat dyes. Studies were carried out with four bacterial isolates, namely: *Klebsiella oxytoca, Bacillus firmus, Staphylococcus aureus* as well as *Bacillus macerans* using 100 mg/L of vat dyes (vat red 15, vat brown 1 and vat black 27) in mineral salt medium for 5 days. Bacterial isolates were cured of their plasmid using sodium dodecylsulphate (0.06 g, 0.08 g, 0.11 g, 0.22 g, 5 g and 10 g) and 10 µL of ethidium bromide (EtBr) at 40 ^{O}C and decolourisation was rebated. The results showed that the plasmid weight of the bacterial isolates were 1500 bps and their plasmids were not cured until when sodium dodecylsulphate and ethidium bromide were combined. High decolourisation occurred before plasmid curing with the highest value of 96% by *Bacillus macerans* for vat black 27 dye and vat red dye decolorization activity of *Staphylococcus aureus* was the lowest at 40%. After plasmid curing, the decolourisation activity of the isolate reduced tremendously to 7% by *Klebsiella oxytoca*. The result obtained revealed the decolourisation abilities of selected isolates and also showed that decolourisation of vat dye is plasmid-mediated revealing that the gene encoding for dye decolourisation is harbored in the plasmid.

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INTRODUCTION

The textile industry significantly contributes to global warming with annual emissions of 1.7 million tons of CO2, or 10% of all greenhouse gas emissions worldwide. Clothing is the fourth most ecologically conscious consumer sector in Europe, behind food, housing, and transportation (Leal et al., 2022). Several contaminants, including dispersants, levelling agents, salts, carriers, acids, alkalis, heavy metals and different colours are present in dyeing effluents (Velusamy *et al.,* 2021). Dutta *et al.* (2022) noted that dyes, detergents and other contaminants present in the textile wastewater undergo chemical and biological changes, consume dissolved oxygen, destroy aquatic life and pose a threat to human health as many of these contaminants are highly toxic in nature.

The textile industry, which employs around 35 million people globally, is among the most ancient and most significant production sectors around the globe that has made a significant contribution to the growth of many economies (Desore and Narula 2018; Lellis *et al.,* 2019). However, the textile industry happens to be one of the most harmful despite its undeniable necessity. Preparatory textile finishing processes including desizing, scouring, bleaching, and dyeing are under the purview of the textiles sector's wet processing segment. Large quantities of chemicals, including salts, alkalis, surfactants, pigments, colors, and water are used throughout these operations (Kant 2012). Water is a valuable but limited resource that is necessary for fundamental human survival, yet one in three people globally is believed to lack access to clean water and it has been estimated that one in three people worldwide do not have access to clean water due to industrial pollution (WHO, 2017).

According to Panday *et al.* (2007), approximately there are currently 2000 distinct structural dyes being used, and a standard textile factory that produces around 8,000 kilograms of textile each day uses roughly 1.6 million litres of water, with 24% of the total applied into the printing and dyeing divisions (Kant 2012). Almost 80% of all dyestuffs are consumed by the textile industry, and millions of liters of effluents containing massive amounts of grease, dirt, nutrients from dye baths, dye additives, residual dyes, etc. are discharged directly into public drains, where they eventually find their way into water bodies (Elbanna *et al.,* 2010). Textile industries produce wastewater with a variety of characteristics, and the characteristics of the effluent depend on the procedure being used (Verman and Mishra, 2005). Moreover, many dyes are carcinogenic, and their presence in water can induce skin ulceration, severe inflammation of the respiratory system, rupture of the septum of the nostrils, skin irritation, and destruction of the mucous membranes, vomiting, discomfort, haemorrhage including strong diarrhoea when consumed (Ponraj *et al.,* 2011). According to Muthu (2017), the textile sector has a long list of negative effects on the environment and contributes to air pollution by releasing pollutants like dust, particulate matter, sulphur, nitrogen oxides, and volatile organic compounds. The majority of solid waste is made up of wasted packaging, leftover textile fabrics, yarns, and other materials (Lellis *et al.,* 2019).

Unsaturated fatty acids containing the carbon double ion configurations -C=C, -N=N, and -C=N with varied organic molecules are present in synthetic dyes. These characteristics of dyes enable them to adhere securely to fibers, to be exceptionally stable to light, including washing and to be stable in the surrounding for a duration of time (Moyo *et al.,* 2022). They remain for an extremely lengthy period in environment and obstruct the entrance of light and photosynthesis because of their resistant character (Pinheiro *et al.,* 2022). Okereke *et al.* (2016) documented that the indiscriminate release of industrial effluent into our natural ecosystem and life forms has caused major problems for the survival of the flora and fauna, while Bankole *et al.* (2018) and Dafale *et al.* (2010) have noted that the discharge of textile effluents into surface water causes aesthetic issues, impedes oxygen from entering aquatic bodies and light from reaching them and negatively impacts aquatic life.

Vat dye is one of the common dyes used in the dyeing of silk and cotton, especially in local dyeing houses, and their intricate structures account for their capacity to resist deterioration in soil as well as water (Rane *et al.,* 2014). The degradation of fabric wastewater has been the subject of numerous investigations using both physical and chemical approaches. Kim *et al.* (2004) opined that the synthetic nature of dyes and their aromatic molecular structures render them steady and challenging to breakdown. According to Aktar *et al.* (2019), there are benefits and drawbacks to both the chemical and physical approaches of detoxifying textile wastewater in terms of effectiveness,

affordability and byproduct generation. This may help to explain why a variety of techniques are typically employed in order to treat textile dye wastewater as effectively as possible.

Dos *et al.* (2005) and Asgher *et al.* (2013) submitted that these procedures are labor-intensive, uneconomical, rife with methodological difficulties, produce a significant amount of sludge, and are occasionally ineffective. Recently, there has been a lot of interest in using microbial agents to treat dye effluents in an effort to develop more economical and ecologically acceptable strategy techniques (Pinheiro *et al.,* 2022; Samsami *et al.,* 2020*).* In the same vein, Iqbal and Asgher (2013) documented that the bulk of industrial effluents can be effectively treated using microbiological techniques, and Blumel *et al.* (2002) reported that microbial treatment methods are cheaper, more environmentally friendly, and more effective. Namrata and Hitesh (2011) concluded that a vast variety of microorganisms are used in the very broad field of biodegradation to disrupt chemical bonds, and key biological decolorization processes include adsorption, enzymatic release, and breakdown by the biomass.

Plasmids can be found in terrestrial and aquatic habitats and may transfer various kinds of bacteria in nature (Inoue *et al.,* 2007). Plasmids carry genes that give bacteria a variety of benefits. They provide resistance and allow the host bacterium to convert various contaminants into less harmful forms (Carattoli, 2003; Kroll *et al.,* 2010). It is essential to identify novel microbial strains for better textile effluent breakdown due to the variety and complexity of dye structures (Oros *et al.,* 2004). Due to the dearth of information on the decolorization of vat dyes (Jorgewad, 2019). This study thus aimed to investigate the biodecolourisation of vat dyes using native bacteria and to ascertain how plasmids affect the breakdown of vat dyes both prior to or following plasmid curing.

MATERIALS AND METHODS

Sample collection

Textile dyes

Dyes and effluent were obtained from Itoku, Abeokuta, Nigeria and the dyes were of analytical grade.

Microorganisms

Bacterial isolates (*Klebsiella oxytoca, Bacillus firmus, Staphylococcus aureus* as well as *Bacillus macerans*) have been previously isolated and identified in our previous work (Adebajo *et al.*,2017).

Screening for dye-decolourising bacteria

Mineral base medium comprised in (g/L): Na_2HPO_4 (2.13g), KH_2PO_4 (1.3g), NH_4Cl (0.5g), $MgSO_4$ (0.2g), water up to 1 litre and 1mL of trace element solution per litre and the compositions of the trace compounds (g/L) are as follows: $MnSO_4$.4H2O (0.081), $CuSO_4$.5H2O (0.0782), Na_2MoO_4.2H2O (0.025), $ZnSO_4$.7H2O (0.044), $MgSO_4.7H_2O$ (7.12), $FeSO_4.7H_2O$ (0.498), and $ZnSO_4.7H_2O$ (0.044); $MnSO_4.4H_2O$ (0.081); $CuSO_4.5H_2O$ (0.0782); $Na_2MoO_4.2H_2O$ (0.025); $FeSO_4.7H_2O$ (0.498); Boric acid (0.1) and 0.27 mL of H_2SO_4. One hundred milligrams per liter of the vat dye was incorporated to mineral-salt medium and pure isolates of 0.6 optical density at 600 nm were submerged in normal saline. Mineral salts basal medium (50 mLs) inoculated with 2 mL of culture suspension and 100 mg/L of vat dye and was placed in an incubator for four days at 28°C on a rotating shaker at 110 rpm. Organisms were chosen in accordance with their ability to decolourise the dye. Decolourisation was measured using UV-Visible Spectrophotometer (Jenway, Model 6405, UK) (Bayoumi *et al.,* 2010). The four most potent bacterial isolates having the highest decolourisation potential were selected for further decolourisation experiments.

Decolourisation experiments prior to plasmid curing

In this experiment, 100mg/L of vat red, vat black and vat brown dyes, yeast extract; 0.1g, sucrose; 0.1g were introduced into mineral salt medium (100 mL), pH was adjusted to 7 and was autoclaved at $121^{O}C$ for 15mins. After

cooling, 0.9 optical density (5 mL) inoculum was inoculated and the medium was incubated using a shaker incubator (110 rpm) for five days. Dye medium without the inoculum was used as the control. Following the method of Archana *et al.* (2011) and Nermeen *et al.* (2011), a spectrophotometer at a wavelength of 540 nm was used to determine the decolourisation activity.

$$\% \text{ decolorisation} = \frac{\text{Initial Absorbance} - \text{Final Absorbance}}{\text{Initial Absorbance}} \text{ x } 100$$

Plasmid analysis and plasmid curing experiments

Plasmid DNA of the isolate was extracted from two days broth cultures of the isolates in Tryptic Soy Broth (TSB) using the pure yield plasmid miniprep extraction kit (Promega, USA) according to the instruction of the manufacturer. Agarose (1%) was prepared, it was allowed to cool and 15 µl of ethidium bromide was added to 50 mL agarose. Plasmid DNA (7 µl) was introduced into 3 µl of loading dye to form a sample mixture. The sample mixture and the marker were loaded into the wells. Gels were allowed to run at 100V for 45minutes**,** plasmid DNA bands were then visualized under the UV-transilluminator and the gel photograph was taken.

Modified method by Elbanna *et al.*, (2010) and Ojo and Oso (2009) was employed. Different concentrations of curing agents (sodium dodecylsulphate and ethidium bromide) were used. Aliquots (0.06 g, 0.08 g, 0.11 g, 0.22 g, 5 g and 10 g) respectively of Sodium Dodecylsulphate (SDS) were dissolved seperately into 100 mL of tryptic soy broth (TSB broth) to give 0.06%, 0.08%, 0.11%, 0.22%, 5% and 10%. Each of the mixture was boiled and distributed in 15.0 mL portions into MacCartney bottles and then sterilized at 121 ºC for 15 minutes and cooled, 10 µL of Ethidium bromide (10 mg/mL) was added to each of the different concentration in the MacCartney bottles. Each tube containing the curing agent was aseptically inoculated with an overnight culture of a test isolate, incubated for 4 days at 40º C leaving one tube un-inoculated which serves as the control. Plasmid DNA experiment was repeated using Promega plasmid DNA extraction kit as described above after curing.

Decolourisation experiments after plasmid curing

Decolourisation experiments were repeated using the aforementioned procedure after the isolates plasmid had been removed using different concentrations of SDS (0.06 g, 0.08 g, 0.11 g, 0.22 g, 5 g and 10 g) and 10 µL of Ethidium bromide (10 mg/mL). Decolorisation activity was assessed by measuring the drop in absorption on a spectrophotometer at each dye's absorbance optimum (Bayoumi *et al.*, 2010).

RESULTS

Screening Ability to Decolourise Dye

The bacterial species decolorized the dyes in varying degrees. *Klebsiella oxytoca* and *Bacillus macerans* showed the highest level of dye removal of 95.58% and 96.86%, respectively at $P < 0.05$. *Proteus mirabilis* had the least decolourisation of 30% followed by *Pantoea* spp (32.46%). K*lebsiella oxytoca* and *Bacillus firmus* showed dye decolourisation at the second and third ranking of dye decolourisation (Table 1).

Decolourisation of dyes by potential isolates with and without plasmid curing

The following isolates were selected because they had overall best decolourisation ability on vat dye. They include: *Staphylococcus aureus*, *Bacillus macerans*, *Klebsiella oxytoca* and *Bacillus firmus*. Hence, isolates were selected for further decolourisation experiments using vat dyes (vat black 27, vat red 15, vat brown 1).

Bacillus macerans displayed the highest vat black 27 dye decolourisation of 96% after 5 days while *Bacillus firmus* recorded the least amount of decolourisation activity (83%) before curing. After plasmid curing, the

decolourisation activity of the isolate reduced tremendously. *Bacillus macerans* exhibited the least activity of 1% while *Klebsiella oxytoca* showed the highest value of 7% (Figure 1).

Table 1: Screening of Isolates for the Ability to Decolourise Vat Dye at Different Interval

S/N	Sample Sites	Isolates	Time of decolourisation				%
			24h	48h	72h	96h	Decolourisation
1	ITK 1	*Proteus mirabilis*	0.0350 ^{b}	0.0320 ^{ef}	0.0280 ^{f}	0.0245 ^{g}	30
2	ITK 1	*Staphylococcus cohnii spp urealyticum*	0.0370 ^{bc}	0.0250 ^{bcde}	0.0210 ^{cde}	0.0210 ^{e}	43.24
3	ITK 1	*Salmonella salamae*	0.0550 ^{f}	0.0350 ^{f}	0.0300 ^{f}	0.0285 ^{h}	48.18
4	ITK 1	*Staphylococcus aureus*	0.0560 ^{f}	0.0305^{def}	0.0190 ^{bcd}	0.0085 ^{a}	84.82
5	ITK 2	*Pantoea* spp	0.0305 ^{a}	0.0260^{bcde}	0.0182^{bcd}	0.0206 ^{d}	32.46
6	ITK 2	*Klebsiella oxytoca*	0.295 ^{a}	0.0215 ^{bc}	0.0170 ^{b}	0.0090 ^{bc}	95.58
7	ITK 2	*Enterobacter* aerogene	0.0635 ^{g}	0.0320 ^{ef}	0.0230 ^{e}	0.0205 ^{f}	56.70
8	ITK 2	*Bacillus firmus*	0.0360 ^{b}	0.0205 ^{b}	0.0175^{bc}	0.0035 ^{c}	90.28
9	ITK 2	*Bacillus macerans*	0.305 ^{a}	0.0225 ^{bcd}	0.0155 ^{b}	0.0095 ^{a}	96.86
10		Control	0.3250 ^{h}	0.3250 ^{g}	0.3250^{g}	0.3250^{gi}	0

Mean values with the same superscript are not significantly different using Duncan Multiple Range test at ($P < 0.05$).

Staphylococcus aureus exhibited the least vat red 15 dye decolorization activity of 40%, compared to a decolorization rate of 94% by *Bacillus firmus* before plasmid curing. Decolourisation activity after plasmid curing for vat red 15 dye also reduced. *Klebsiella oxytoca* (4%) recorded the highest and the lowest by *Bacillus firmus* of 1% (Figure 2).

Decolourisation of vat brown 1 was depicted in Figure 3. Before plasmid curing, *Bacillus firmus* exhibited the highest decolourisation of 84% while *Staphylococcus aureus* recorded the minimum decolourisation of 72%.

Following plasmid curing, highest and lowest decolourisation of vat brown 1 dye were: 4% and 2% by *Klebsiella oxytoca* and *Bacillus macerans*, respectively.

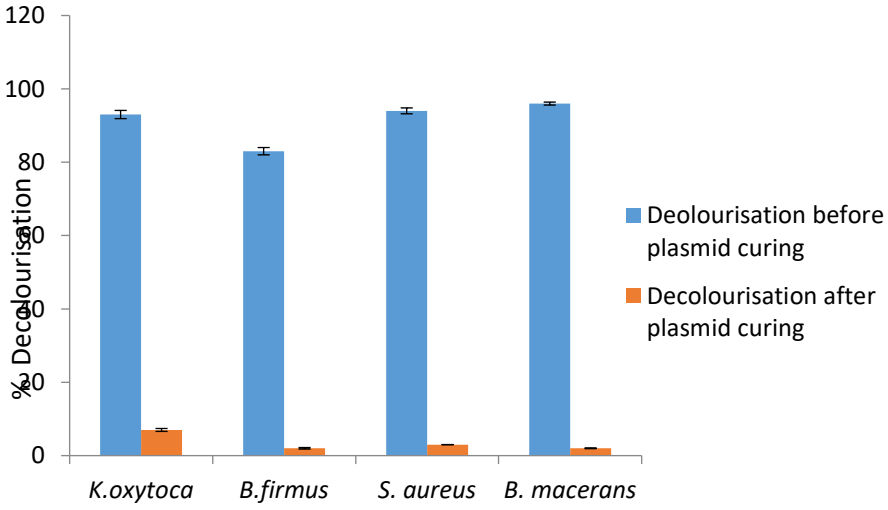

Figure 1: Decolourisation percentage of vat black 27 dye before and after plasmid curing by selected bacteria isolates after 5 days

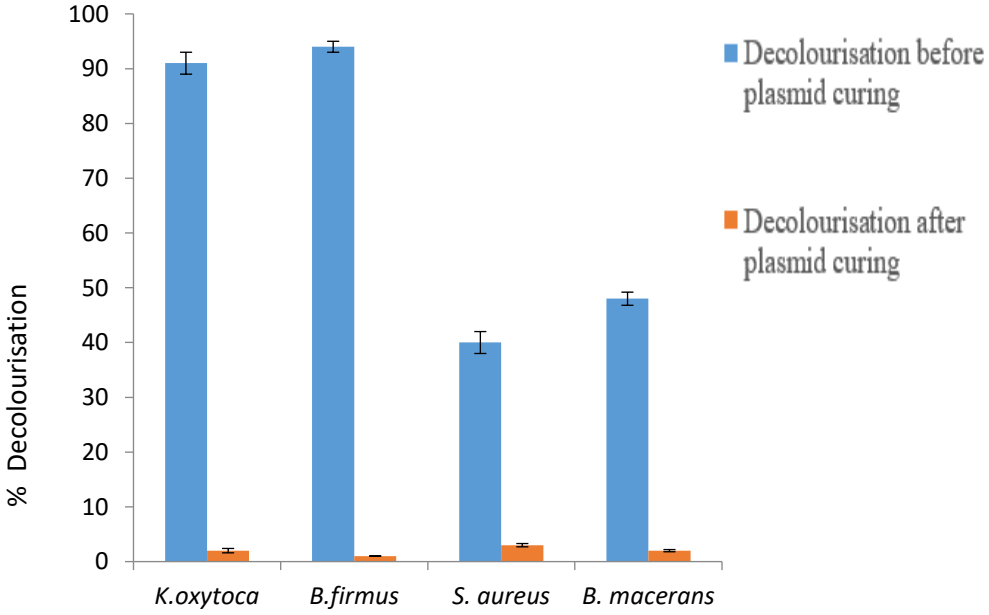

Figure 2: Decolourisation percentage of vat red 15 dye before and after plasmid curing by selected bacteria isolates after 5 days.

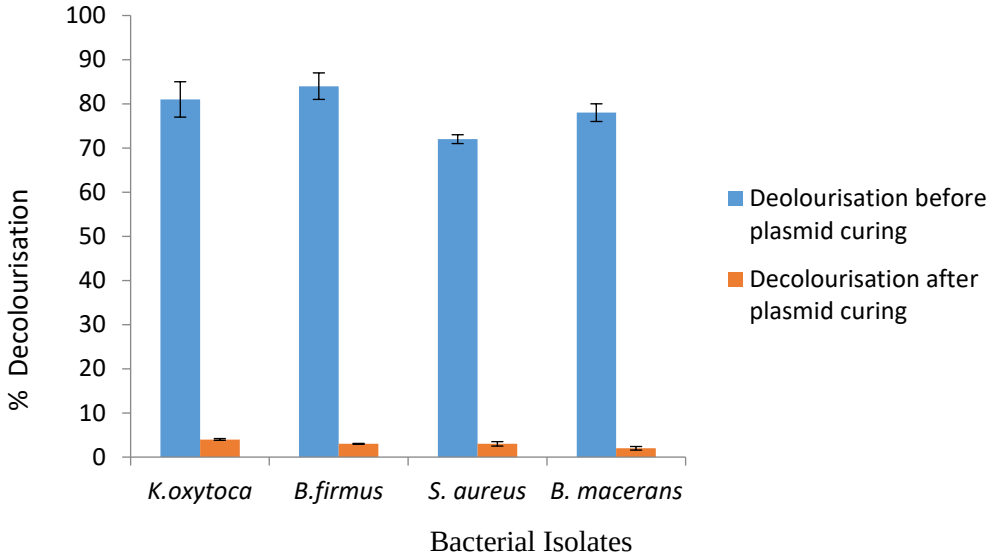

Figure 3: Decolourisation percentage of vat brown 1 dye before and after plasmid curing by selected bacteria isolates after 5 days

Plasmid Analysis

The result obtained showed that all the isolates had a single plasmid with a band size of 1.5 kbp. The plasmid profile of isolates is presented in Plate 1single plasmid with a band size of 1.5 kbp. The plasmid profile of isolates is presented in Plate 1. *Staphylococcus aureus, Klebsiella oxytoca*, *Bacillus macerans* and *Bacillus firmus* were designated as 2, 3, 4, and 5, respectively, while M shows the different weight of the ladder used and 6 serves as the positive control. Sodium dodecylsulfate and ethidium bromide (EtBr) were used for curing experiments at different concentrations. Isolates were cured of their plasmids when 10 microliters of EtBr and 10% sodium dodecylsulfate were used. After curing, decolorization was repeated and the findings demonstrated that all isolates were incapable to carry out decolorization.

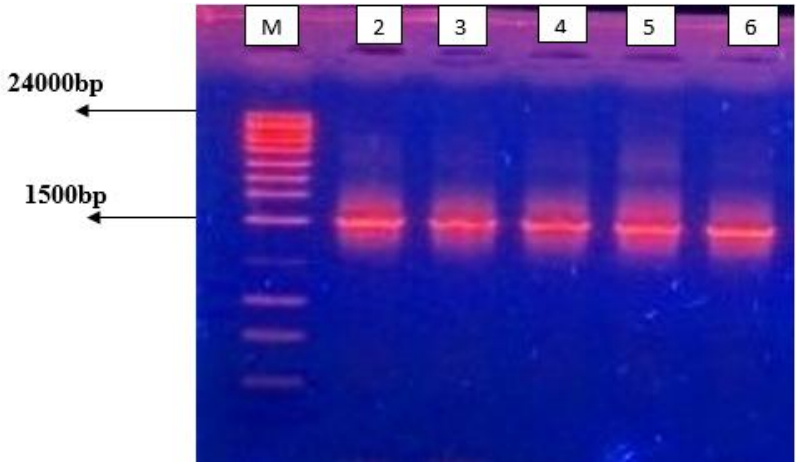

Plate 1: Gel electrophoresis of plasmid DNA profiles of selected isolates. M: UltraRanger DNA ladder; Lane 2: *K. oxytoca*; Lane 3: *B. firmus;* Lane 4: *S. aureus*; Lane 5: *B.macerans*; Lane 6: Positive control

DISCUSSION

A significant issue currently is the pollution brought on by the discharge of a variety of industrial wastewaters in the environment (Vijaykumar *et al., 2007*; Mahmood *et al.,* 2011). Dye waste frequently ends up in water bodies as a result of the coloring process and pollutes the water. In view of the dangers that these dye pollutants pose to both individuals and life in the water, the discharge of untreated wastewater from textile factories has consequently become a significant issue in several nations (Lambert and Davy, 2011). It has been shown that bacterial agents isolated from various sources, including dye-contaminated soil and textile wastewater, have the potential to effectively decolorize and break down dye pollutants, thereby improving the quality of the water (Moyo *et al., 2022*). Many industries have different levels of contaminants in their waste water, and it has been discovered that the presence of pathogenic bacteria can cause epidemics of different water-borne diseases (Okereke *et al., 2016*). The adaptability and activity of particular microbes determine the decolorization efficiency of microbial isolates (Saratale *et al., 2009*). Pereira and Alves (2012) reported that the chromophore link in dyes is destroyed when decolorization takes place.

In this study, it was discovered that four bacterial strains from textile effluent: *Klebsiella oxytoca, Bacillus firmus, Staphylococcus aureus,* and *Bacillus macerans* had the ability to decolorize the textile dye through catabolism and utilization by the isolates. This is in line with the report of Mahood *et al.* (2015), who stated that native isolates could break down the dye under natural circumstances without the need for extra resources for growth. In addition, the bacterial species employed in this work have been linked to dye effluent by Khalid *et al.* (2008), Olukanni *et al.* (2009), Chaube *et al.* (2010), and Gurav *et al.* (2011). The shaking flask experiment was considered because agitation results in a high rate of dye decolorization, which was also confirmed by Bayoumi *et al.* (2010). The decolorization of dye by prospective microbes is a cellular reduction process that necessitates various electron transport channels that are made up of cytoplasmic, periplasmic and exterior membrane molecules (Brige *et al.,* 2008).

Reductive bond cleavage and the production of colorless solutions are key components of the microbial color degrading process (Mahmood *et al.,* 2015). It is crucial to stress that in order to make the dye harmless to life in all its forms, the selected microorganisms for dye treatment must degrade and remove the dye's color to its basic components. The presence of microbial enzymes in cells gives them the capacity to biodegrade different environmental contaminants (Moyo *et al., 2022),* and research have revealed the existence of broad host-range plasmids, especially in soil, activated sludge, manures, treated effluent, and aquatic environments (Heuer *et al., 2002*; Inoue *et al., 2007*; Moura *et al., 2010*). Moreover, Akiyama *et al.* (2010) found that plasmids had a high potential for mobilization during effluent treatment. The genetic components known as plasmids may contain catabolic genes that break down various waste products and could harbor catabolic genes for the breakdown of different waste products (Carattoli, 2013). The textile dye decolorization ability of the isolates may be attributed to their adaptability to the xenobiotic compounds, and the various strains might target the dye molecule in multiple locations (Elbanna *et al.,* 2010).

Elias et al. (2013) claimed that plasmid curing happens naturally when cells divide or when they are exposed to any chemical or physical factors. Plasmid-curing chemicals were employed in this work to eliminate the bacterial plasmids, and El-Mansia et al. (2000) reported that in order to determine the phenotypic features encoded by a specific plasmid, plasmid DNA must be removed, a process known as curing. When curing agents (SDS and EtBr) were employed separately, it was discovered that plasmids were not removed. This result inspired us to combine various chemical and physical techniques using SDS, EtBr, and a higher temperature of 40°C. SDS acts to dislodge the native plasmid from its site of attachment or cause imperfect replication and unsuccessful plasmid segregation. EtBr is an intercalating substance that prevents the replication of plasmids, while physical agents like elevated growth temperatures (40°C) cause deletions of the plasmid DNA that are whole or partial for the strain (Letchumanan et al., 2015).

The inability to decolorize colors after curing demonstrates that genes needed to decolorize textile dyes were present on the plasmid. For EtBr and SDS, no growth was seen at concentrations greater than 10 µL and 10 g, showing that the quantities are lethal dosages, and the use of higher concentrations of SDS (10g), ethidium bromide (10µL)

and incubation at high temperature during the curing of plasmids suggests that dye-degrading plasmids are highly durable and difficult to cure (Bashar et al., 2010). Elbanna et al. (2010) have also documented that curing isolates with SDS at elevated temperatures was effective in removing plasmids from a wide variety of bacteria. Our findings revealed that decolorization of vat dyes by the bacterial isolates is due to degradation and that potential isolates possess degradative plasmids.

The prospective isolates' capacity to harbor plasmids and neutralize vat pigments is in congruence with the report of Stolz et al. (2012), where crystal violet decolorization by E. coli was reported to be plasmid-mediated, while in the findings of Schluter et al. (2007), most of the Pseudomonas strains that carried degrading plasmids also efficiently degraded dyes. The microorganisms were able to adsorb the dyes into their cytoplasm, secrete enzymes that breakdown the dyes, and utilize the dyes as nutrients, which later brought about the degradation of dyes. Also, it was observed that there was no growth or decolorization in the control tubes, showing that the isolates' metabolic activity was what caused the decolorization.

CONCLUSION

Management of wastewater and water purification is essential to support humanity's swift advancement, mitigation of the environmental damage and health risks. Biological processes that are simple, fast, and economical are used to solve decolorization challenges. This study showed the potential of four unique strains of bacteria in the degradation of textile liquid waste, which will help mitigate the effect of untreated textile effluents upon the ecosystem and living forms. It is fascinating to point out that the strains utilized in the decolorization investigation were all isolated from the dye-industrial effluent. Also, our findings revealed that decolorization is plasmid-mediated, that the gene is located in the plasmids, and that the screened isolates were effective decolorizers of vat dyes. From these findings, it could be inferred that some bacteria can reside in and metabolize dye effluent. These bacteria harbor diverse mobile genetic elements that might be exploitable in biotechnological processes designed for the bioremediation of dye-contaminated industrial wastes.

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