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Decolorization of Malachite green dye by *Stenotrophomonas maltophilia* a compost bacterium

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Abstract

Background: The release of triphenylmethane dyes, like malachite green (MG) and crystal violet (CV), into the environment is a cause of concern due to its toxicity, mutagenicity and carcinogenicity.

Result: A bacterial strain that is capable of decolorizing both dyes was isolated from the composted neem oil-seed cake. The strain was characterized as *Stenotrophomonas maltophilia* based on the 16S rRNA gene sequence and designated as isolate TPMD-1. The kinetic study of the dye degradation revealed the efficiency of the above isolate to degrade MG. The effect of substrate concentration, pH, temperature, and agitation on the decolorization of MG by the isolate was also studied. The MG degradation rate was slightly more in neutral pH at 28–30 °C and 150 rpm. The UV-spectroscopy, HPLC, and FTIR analyses of the dye sample before and after bacterial treatment revealed the high ability of *S. maltophilia* TPDM-1 in the removal of MG from the media. The genetic snapshot of the isolate by PCR amplification and sequencing showed the presence of genes '*lac*' and '*tmr*' that codes for laccase and triphenylmethane reductase.

Conclusion: This study presented the first report of *Stenotrophomonas maltophilia* in the degradation and detoxification of MG dye by oxidoreduction, which could be used for the bioremediation of aquatic environments contaminated by MG.

Keywords: Malachite green, Crystal violet, Dye decolorization, Exponential decay, *S. maltophilia*

Background

The triphenylmethane (TPM) dyes are brilliant and intense by colored synthetic dyes with $(C_6H_5)_3CH$ as their backbone that finds application in the textile industry (Shah et al. 2013). This dye accounts for nearly 30–40% of total dye consumption in the dyeing of cotton, wool, and silk fabrics (Carliell et al. 1998). Besides this, these dyes are also used in coloring biological materials, wax, varnish, cosmetics, paper and leather, and plastic industries (Shah et al. 2013). Certain TPM dyes such as crystal violet (CV) and malachite green (MG) are extensively used in the textile industry (Wu et al. 2008) and the colored effluents are let into water bodies. These effluents contain aromatic amines and phenols that are hard to degrade (Robinson et al. 2001) and affect aquatic life by blocking the photosynthetic process (Raghukumar et al. 2008). The untreated dye effluents are also reported to be toxic to humans beings by way of injuring internal organs (Hassaan and Nemr 2016). The CV was reported to induce tumor in fishes, while MG induces hepatic tumor in rodents and causes reproductive abnormalities in rabbits and fishes (Cho et al. 2013). Because of these, the removal of TPM dyestuffs from the contaminated water source is deemed essential.

The conventional wastewater treatment does not degrade all the aromatic ring structures, including

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aromatic amines, phenols, as it is recalcitrant (Du et al. 2011). Hence, a combination of physico-chemical methods such as adsorption, floatation, coagulation, chemical oxidation and the use of membrane bioreactor is employed to degrade dye effluents (Wang et al. 2011). These techniques are expensive and generate vast volumes of sludge and toxic by-products that are the source of secondary pollution (Saratale et al. 2011). However, biological methods are available, which are eco-friendly and completely mineralize organic pollutants (Pandey et al. 2007). Recent developments in the field of bioremediation include the use of dye degrading microorganisms and the potential degrading enzyme system they produce (Ali et al. 2009; Wang et al. 2012; Yang et al. 2014) and metabolic pathways (Jang et al. 2005). This approach is cost-effective and environmentally friendly. Certain fungal and bacterial strains were shown to involve in the degradation of both CV and MG by the mechanism of adsorption and degradation (Ali et al. 2009; Jadhav and Govindwar 2006).

The biochemical studies of the decolorization of TPM dyes indicated that the involvement of laccases and lignin peroxidases produced by fungi and triphenylmethane reductase (TMR) produced by bacteria (Jang et al. 2005; Shin and Kim, 1998; Tekere et al. 2001). Laccases are a part of the sizeable multicopper oxidase reductase family enzymes that catalyze the four-electron reduction to water from a suitable substrate (Reiss et al. 2013). This enzyme is involved in the biodegradation of lignin, which is the noncarbohydrate polyphenolic component in the wood. Additionally, it has broad substrate specificity and is capable of degrading aromatic phenolic and non-phenolic pollutants in wastewater (Prins et al. 2015). Although fungal laccases are more extensively studied, bacterial laccases are an advantage due to their molecular nature (Prins et al. 2015; Narayanan et al. 2015; Kuppusamy et al. 2017). Similarly, triphenylmethane reductase (TMR), a member of the dehydrogenase enzyme has been reported in several bacteria that are involved in the degradation of textile dyes, particularly the MG by NADH-dependent reduction (Wang et al. 2011; Jang et al. 2005).

The present study aims to isolation of bacterial strains from compost samples capable of degrading TPM dyes, particularly the MG. *Stenotrophomonas maltophilia* isolated from the compost was determined for its ability to decolorize the MG and the study of the degradation kinetics. An attempt was also made to isolate and confirm the role of '*lac*' and '*tmr*' genes, for dye degradation in bacteria.

Methods

Isolation and screening of bacterial isolates for TPM dye degradation

The compost of neem oil-seed cake at the thermophilic stage was collected and serially diluted (10^{-6}) and spread on nutrient agar plates and incubated at 37 °C for 24 h. Individual colonies of bacteria on agar plates were identified based on the colony characteristics and pure cultures were obtained and maintained on nutrient agar. The bacterial isolates were cultured separately in mineral salt basal medium (3 g peptone, 10 g glucose, 0.6 g KH_2PO_4, 0.001 g $ZnSO_4$, 0.4 g K_2HPO_4, 0.0005 g $FeSO_4$, 0.05 g $MnSO_4$, 0.5 g $MgSO_4$ and 20 g agar) amended with either malachite green (MG, 100 mg L^{-1}) or crystal violet (CV,100 mg L^{-1}). The colony cultures were observed for the high zone of clearance. In another experiment, the bacterial culture was inoculated to mineral salt broth (50 ml) supplemented with either the MG or CV at the same concentration used above and incubated at 30 °C in a shaking incubator (150 rpm). The culture broth was collected at an interval of 24 h for 96 h and centrifuged (Mini spin, Eppendorf 10,000 rpm (g), 2 min) and the supernatant was collected and the absorbance measured at 618 nm for MG and 590 nm for CV using a UV–Vis spectrophotometer (Thermo Scientific, Biomate 3S). The measurement was taken up to 96 h, and maximum dye degradation (%) at each time interval was calculated as:

$$A_0 - A_t/A_0 * 100 \quad (1)$$

where A_0, initial absorbance of the media, A_t, absorbance of the media at the interval of '*t*' time.

Kinetics of dye decolorization

The rate of decolorization of dyes was studied at a different dye concentration of 100, 200, 300, 400 and 500 mg L^{-1}. The bacterial isolate was inoculated to the dye mineral salt basal liquid medium supplemented with either of the dyes (pH 6.5 at 28 °C). The sampling of the culture broth was done for 96 h at an interval of 24 h, and the extent of degradation was determined by using the formula (1). Further, the rate of degradation of selected dyes was determined by the first-order exponential decay model:

$$C_t = C_0 e^{-\lambda t}, \quad (2)$$

where λ = decolorization rate (h^{-1}), C_0 and C_t (mg L^{-1}) concentration of dye at the zero time and at a time '*t*' (h) (Mutafov et al. 2006) using the Sigma Plot (ver. 14.0). The half-life of each dye at different concentrations was determined by

$$t_{1/2} = 0.693/\lambda \quad (3)$$

where $t_{1/2}$ is the half-life of the decay quantity.

Optimization parameters

The effect of variation in pH, temperature, and rate of agitation on MG dye discoloring ability of bacterial isolate was studied at 100 mg L^{-1} MG dye concentration. The 24 h old bacterial culture (100 µl) was inoculated to mineral salt liquid media (50 ml) contained in a 100 ml conical flask. The pH of the broth was adjusted to 6, 7, or 8 (using 0.1 N NaOH or HCl) and incubated at 28, 30 or 35 °C in a shaking incubator at static (0 rpm),100 or150 rpm for 48 h. The experiment was arranged in a completely randomized design (CRD) with three replicates. The extent of decolorization of dye in each set was determined by the measuring absorbance at 618 nm, as described previously.

Biodegradation analysis

The discolored bacterial supernatant product (without bacterial cells) of MG dye was extracted with an equal volume of ethyl acetate at 0 h (control) and 24 h (after decolorization of the medium). The extracts were evaporated to dryness over anhydrous sodium sulfate (Na_2SO_4) and crystals obtained were dissolved in a small volume of HPLC grade methanol, and the same sample was used for HPLC and FTIR analysis (Jadhav and Govidwar, 2006). The HPLC (Shimadzu, Japan) analysis was carried out on a dual λUV-VIS detector and a C18 column with methanol and water (0.1% formic acid) as the mobile phase, methanol (40%) for 2 min. and 95% methanol for 8 min. with a flow rate of 0.5 ml min^{-1} at 40 °C and the compound (MG) detected at 265 nm.

Fourier Transform Infrared Spectroscopy (FTIR) analysis of the degraded product of MG at 0 and 24 h was carried out using the Thermo Fisher Scientific instrument (FTIR Nicolet 6700FT-IR). The FTIR analysis was done in the mid-IR region of 400–4000 cm^{-1} at16 scan speed. The samples were mixed with spectroscopically pure potassium bromide (KBr) in the ratio of 5:95, pellets were fixed in the sample holder, and the analysis was carried out.

Phytotoxicity

The effect of toxicity of MG before and after degradation was determined by seed germination test using sorghum (*Sorghum vulgare* Pers.) and finger millet (*Eleusine coracana* (L.) Gaertn) and by measuring radicle and plumule length. Seeds were first surface disinfected with sodium hypochlorite (0.01%, 1–2 min) and rinsed for 2–3 times with distilled water and placed in Petri dishes (20 seeds per plate) containing blotting paper discs previously soaked with MG solution (100 mg L^{-1}), before and after treatment with bacterial culture. The Petri dishes were incubated in darkness in the laboratory conditions for 24 to 48 h. The seed germination (%) was calculated.

Identification of dye transforming bacterial isolate by 16S rRNA sequence

Its 16S rRNA gene sequence identified the MG dye discoloring bacterial isolate TPMD-1. The bacterial culture was grown in the LB broth for 24 h and the culture was centrifuged at 10,000 rpm for 2 min. to obtain the pellet of cells that were used for the extraction of genomic DNA. The genomic DNA of the bacterial isolate was extracted by a modified alkaline lysis method (Sambrook et al. 1989). The purified RNA-free genomic DNA was subjected to PCR amplification by using universal primers specific for 16S rRNA gene forward primer (5′ GTT AGATCTTGGCTCAGGACGAACGC3′) and 16S gene reverse primer (5′ GATCCAGCCGCACCTTCCGAT ACG 3′). The amplification was performed using a thermal cycler (Eppendorf Master cycler, USA) and the reaction involved an initial denaturation at 94 °C for 1 min followed by 35 cycles of 94 °C for 1 min, 55 °C for 30 s annealing and elongation at 72 °C for 1 min. and the final extension was set at 72 °C for 12 min. The amplified DNA was sequenced and analyzed using the online NCBI BLAST tool program (http://www.ncbi.nib.gov/blast). The phylogenetic analysis was carried out to study comparative genomics to derive the evolutionary relationship of the bacterial species by using the character-based neighbor-joining analysis method (MEGA ver.7.0), as explained in the Tamura-Nei model (Tamura and Nei 1993).

Molecular snapshot of bacterial isolate for gene '*lac*' and '*tmr*'

The genomic DNA of the MG degrading bacterial isolate was evaluated for the presence of laccase gene (*lac*) and triphenylmethane reductase gene (*tmr*). Both genes were identified by PCR amplification using a specific pair of

Table 1 Primers used in the amplification of *lac* and *tmr* genes from *Stenotrophomonas maltophilia* isolate TPMD-1

Primers	Sequences	References
Lac F Lac R	5′ATGTCCTTTACCCGTCGA CAAATGC 3′ 5′TCAGACCACCGCAAT CGCGGCCATC 3′	Verma (2017) Designed for isolation of laccase gene from *Pseudomonas putida*
Tmr F Tmr R	5′GATAGGAGGCATTCA CCTTG 3′ 5′AGACTCTATGGATGC GCGCG3′	Jang et al (2005) Designed for isolation of tmr gene from *Citrobacter* sp. KCTC 186,611

primer sets under specific reaction conditions (Table 1). The amplicons obtained were run on 1.2% agarose gel, also loaded with a 100 bp DNA ladder. The PCR product that was separated corresponded to primers and eluted from the gel using a gel extraction kit (GeNei TM). All amplicons were sequenced and the sequence confirmation was further compared with those in the Gene Bank (http://www.ncbi.nib.gov/blast). The phylogenetic analysis was done to study comparison based on the character-based maximum likelihood analysis method (MEGA ver. 7.0).

Statistical analysis

All data are expressed as mean values ± standard deviation. Comparisons among multiple groups were made with a one-way analysis of variance (ANOVA) followed by Dunnet *t* test ($P \leq 0.05$) by using the software PAST 4.03.

Results

Isolation and molecular identification of TPM dye discoloring bacteria

Several bacterial isolates were isolated at the thermophilic composting stage of neem oil-seed cake. Among them, six isolates exhibited the ability to decolorize TPM dyes on agar plates, and one of the isolates, strain TPMD-1 was selected for its strong TPM dye decolorizing activity and capable of degrading both the CV and MG dyes. The bacterial isolate was characterized by its non-motile, gram-negative rods and expressing catalase activity. The 16S rRNA sequences were deposited in Gene Bank (accession no. MN830183) and the sequences matched with that of the isolate TPMD-1 cited in the Gene Bank were done. The phylogenetic tree was (Fig. 1) constructed based on the sequence data by the neighbor-joining method, which indicated that the isolate TPMD-1 was closest to *Stenotrophomonas maltophilia* strain QS3 with 96% similarity.

Kinetics of CV and MG dye transformation

The experimental data showed a good fit for the first-order kinetic model suggesting that the decolorization process depended on the concentration of the dye (Table 2). The low dye concentration (100–200 mg L^{-1}) has a short half-life coupled with large decay constants in both dyes. The MG dye decolorization was significant ($P \leq 0.005$) with a shorter half-life than the CV.

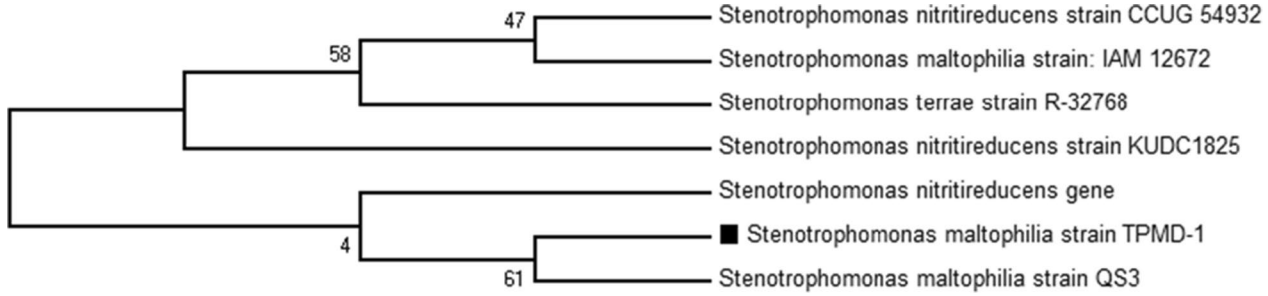

Fig. 1 Phylogenetic distribution of isolate TPMD-1 in comparison to those from NCBI database by neighbor-joining analysis based on their 16S rRNA gene sequences

Table 2 Degree, dye removal rate and half-life period of decolorization of melachite green (MG) and crystal violet (CV) by *Stenotrophomonas maltophilia* isolate TPMD-1

Concentration (mg L^{-1})	MG			CV		
	Degree ofdecolorization (%)	λ (h^{-1})	$t_{½}$ (h)	Degree of decolorization (%)	λ (h^{-1})	$t_{½}$ (h)
100	99.46 ± 0.50^{a}	0.049 ± 0.003^{A}	14.14 ± 0.1^{p}	99.04 ± 0.65^{a}	0.04 ± 0.02^{A}	17.32 ± 0.12^{p}
200	98.24 ± 0.57^{a}	0.031 ± 0.002^{B}	22.35 ± 0.13^{q}	97.50 ± 0.54^{b}	0.023 ± 0.03^{J}	30.13 ± 0.11^{q}
300	85.9 ± 0.53^{b}	0.014 ± 0.004^{C}	49.51 ± 0.09^{r}	81 ± 0.5^{c}	0.010 ± 0.05^{C}	69.3 ± 0.10^{s}
400	79.5 ± 0.55^{d}	0.012 ± 0.003^{D}	57.76 ± 0.1^{r}	75 ± 0.43^{e}	0.006 ± 0.03^{E}	115.5 ± 0.09^{t}
500	67.4 ± 0.51^{f}	0.009 ± 0.004^{F}	77.01 ± 0.13^{u}	54.51 ± 0.47^{g}	0.003 ± 0.03^{G}	231 ± 0.11^{v}

Mean values ± SD followed by different letters in same column indicate significant differences according to DMRT ($P < 0.05$)

'a-g' was used to compare degree of decolorization of both dyes, 'A-G' used to compare rate constants, and p–v to compare half-life periods of two dyes

Effect of physical parameters on MG decolorization

The temperature treatments affected the decolorization activity (non-significant) of *S. maltophilia* TPMD-1 (Table 3). In contrast, pH caused a significant ($P \leq 0.005$) difference in decolorization activities. The decolorizing ability of the isolate was also affected under static (0 rpm) and constant shaking at 100 and 150 rpm. The optimum decolorization activity was reported at pH 6 to 7 at 28–30 °C; under this condition, the shaking did not affect discoloration (either 100 or 150 rpm). An increase in pH to 8 and temperature to 35 °C reduced the decolorization efficiency, but an increase in agitation speed (150 rpm) caused a slight improvement.

Biotransformation analysis

The HPLC chromatogram of control (without bacterial treatment-0 h) contained three significant peaks at the retention time of 8.26, 8.812 and 11.85 min. The chromatogram of the bacterial treatment sample had significant peaks at 6.91, 7.31 and 8.92 min (Fig. 2). The FTIR spectrum of control MG (0 h) contains distinct peaks in the fingerprint region (1500–500 cm^{-1}), which corresponded to mono- and para-substituted

Table 3 Effect of pH, temperature and rate of agitation on decolorization (%) of MG by *Stenotrophomonas maltophilia* isolate TPMD-1

PH	Temperature/agitation rate					
	28 °C		30 °C		35 °C	
	100 rpm	150 rpm	100 rpm	150 rpm	100 rpm	150 rpm
6	98.33 ± 0.5^{A}	98.66 ± 0.6^{a}	97.86 ± 0.43^{A}	98.58 ± 0.53^{a}	97.80 ± 0.45^{A}	98.22 ± 0.45^{a}
7	97.39 ± 0.6^{B}	98.21 ± 0.51^{b}	97.16 ± 0.53^{A}	97.64 ± 0.57^{b}	97.12 ± 0.52^{A}	98.01 ± 0.5^{a}
8	90.04 ± 0.62^{C}	96.77 ± 0.62^{c}	89.35 ± 0.3^{B}	91.14 ± 0.42^{c}	84.17 ± 0.6^{B}	89.86 ± 0.67^{b}

The mean ± SD values in a column followed by different letters are significantly different (LSD $P \leq 0.01$)

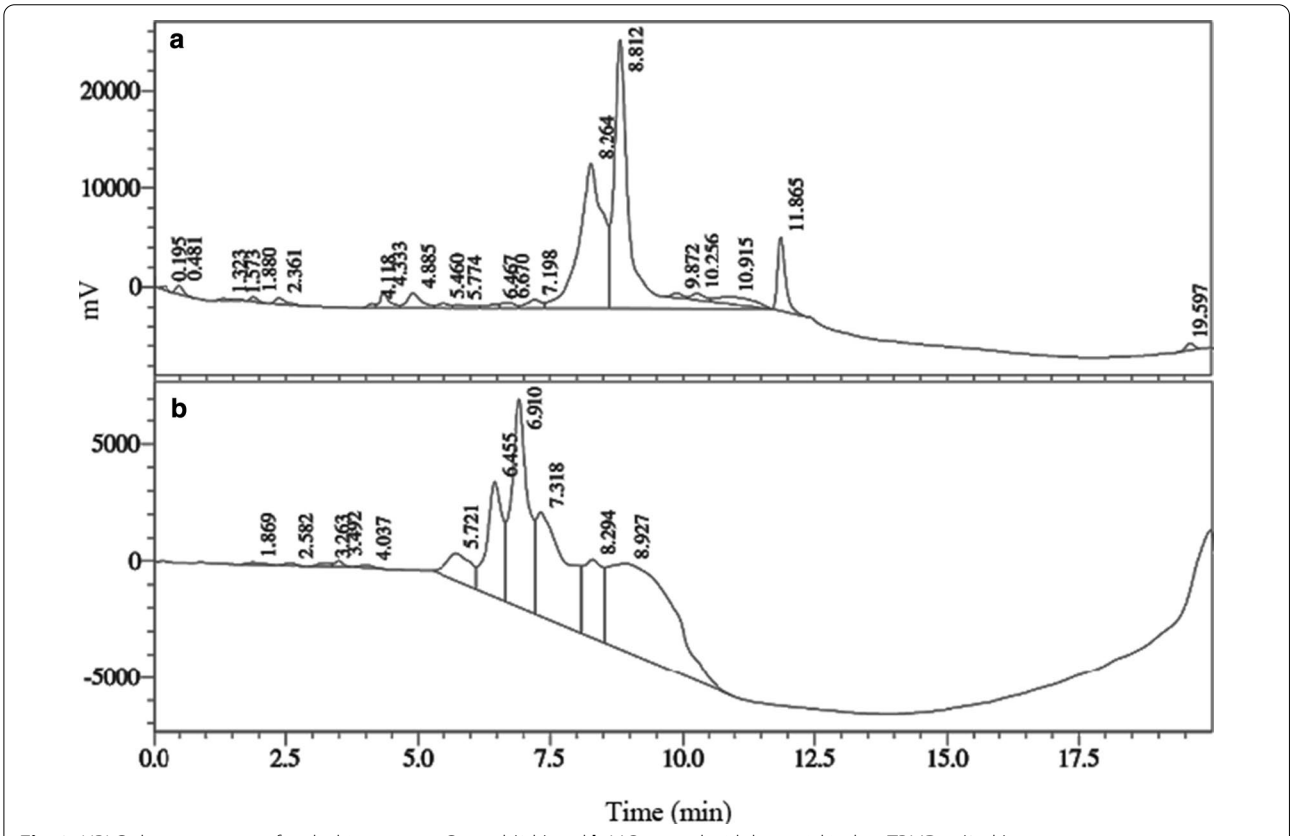

Fig. 2 HPLC chromatogram of malachite green **a** Control (0 h) and **b** MG treated with bacterial isolate TPMD-1 (24 h)

benzene rings that were characteristic of MG. The peaks at 829.76 cm^{-1} for C=C trisubstituted benzene ring, 1503.45 cm^{-1} for N–O stretching nitro compounds, 1768 cm^{-1} for C-H bending aromatic ring, 3027.58 cm^{-1} and 3645.06 cm^{-1} for O–H stretch that are typical peaks of MG were also observed in this study (Fig. 3). The broad peak observed in the region of 3010 to 3850 cm^{-1} for control which corresponded with N–H stretching vibration of primary amines, was absent in the spectrum of the bacterial treated product (Fig. 3). The peaks at 3027.58 indicated the C-H stretching vibrations, and its corresponding bending with aromatic ring vibration occurred at 1768.36 cm^{-1}. The shifting of the peak from 1643.14 to 1768.36 cm^{-1} showed changes in the aromatic ring structure by bacteria treated sample.

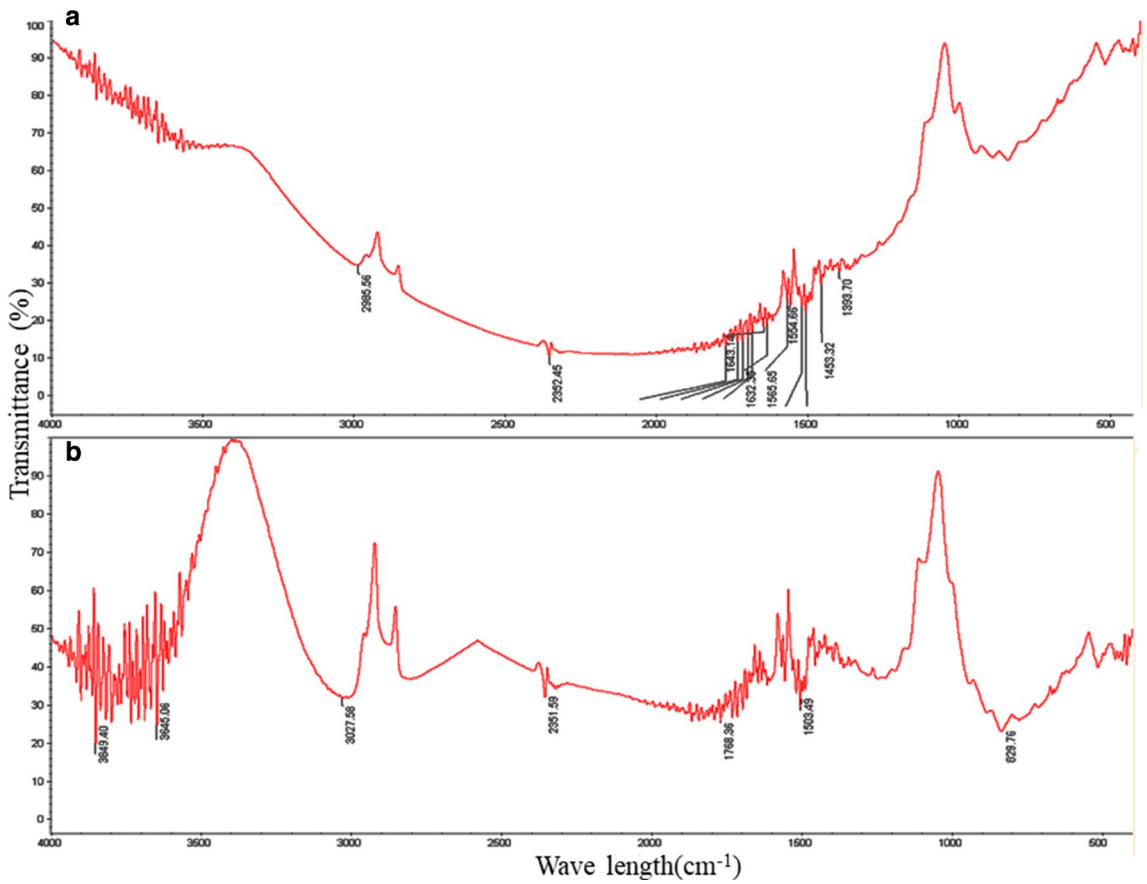

Fig. 3 FTIR spectra of **a** Control (0 h, only basal salt medium + MG) and **b** treatment (24 h, basal salt medium + MG + bacterial isolate TPMD-1)

Table 4 Evaluation of phytotoxicity of MG and degraded product on seed germination and seedling length of *Sorghum vulgare* and *Eleusine coracana*

Treatments	*Sorghum vulgare*			*Eleusine coracana*		
	Germination (%)	Shoot length (mm)	Root length (mm)	Germination (%)	Shoot length (mm)	Root length (mm)
Water	100 ± 0.7^{a}	5.5 ± 0.40^{d}	3.2 ± 0.25^{g}	100 ± 0.6^{A}	5.4 ± 0.4^{D}	3.4 ± 0.3^{G}
Control (MG)	30 ± 0.65^{b}	1.5 ± 0.52^{e}	0.8 ± 0.1^{h}	40 ± 0.7^{B}	1.8 ± 0.45^{E}	0.7 ± 0.15^{H}
Treatment (MG + bacteria)	85 ± 0.58^{c}	4.5 ± 0.43^{f}	2.8 ± 0.25^{i}	90 ± 0.5^{C}	3.8 ± 0.3^{F}	1.5 ± 0.31^{I}

Mean ± SD values followed by different letters in same column insignificantly different according to DMRT ($P \leq 0.05$)

Phytotoxicity

Malachite green is phytotoxic to both seedlings of *Sorghum vulgare* and *Eleusine coracana* when treated to seeds (Table 4). The significantly ($P \leq 0.05$) reduced seed germination and radicle and plumule length. On the other hand, the degraded metabolites of MG did not affect seed germination (Table 4), indicating the reduction in toxicity of metabolites as compared to that of MG in control.

Molecular snapshot of bacterial isolate for '*lac*' and '*tmr*' genes

Analysis of the lac and tmr gene

This gene was identified in *S. maltophilia* TPMD-1 with the aid of the already designed primers specific for the '*lac*' gene (Table 1). The PCR reaction resulted in the amplification of two homologous amplicons of 800 bp (*lac 1*) and 600 bp (*lac 2*) with fragments of the '*lac*' gene (Fig. 4). Further, each band of partial laccase gene was sequenced and analyzed with the corresponding gene sequences documented in the Gene Bank database (http://www.ncbi.nlm.nih.gov/blast). The sequence analysis revealed that the laccase gene of *S. maltophilia* isolates TPMD-1 had 97% homology of large amplicon

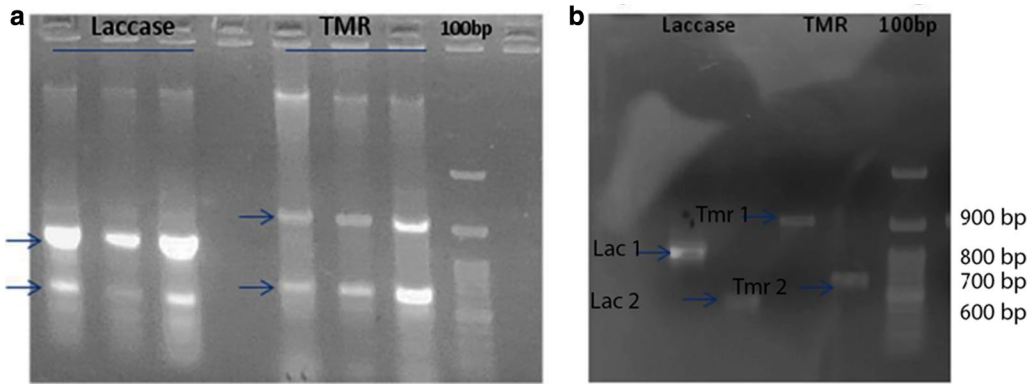

Fig. 4 Agarose gel electrophoresis (1.2% w/v) of laccase (L1 and L2) and TMR (T1 and T2) gene fragments of *Stenotrophomonas matophilia* TPMD-1 amplified by PCR **a** pre-purified and **b** purified laccase and TMR genes (100 bp DNA ladder at the extreme right lane)

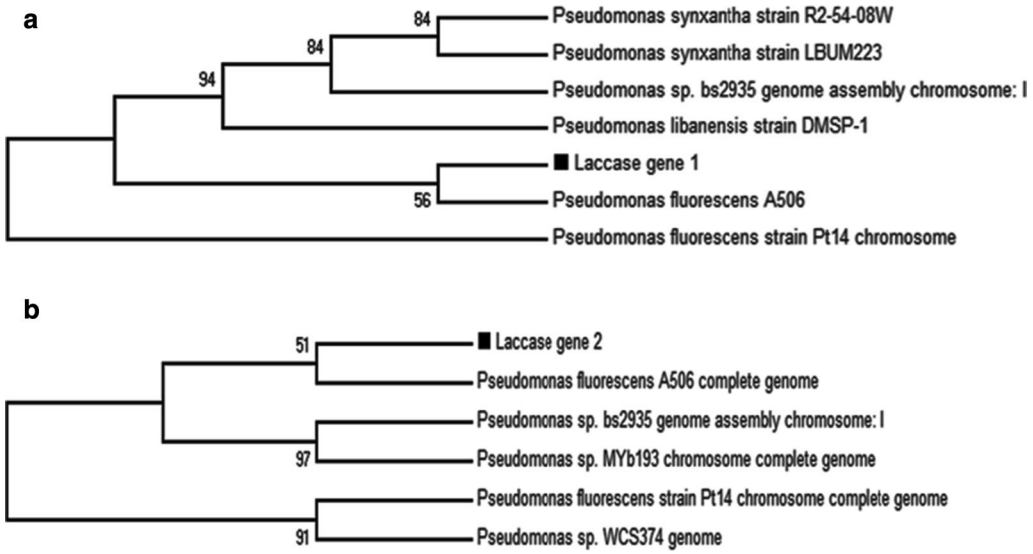

Fig. 5 Phylogenetic representation of **a** Laccase 1 and **b** Laccase 2, amplicons of *S.maltophilia* TPMD-1 in comparison to those from NCBI database by maximum likelihood analysis based on laccase specific primers

(800 bp) and 98% of small amplicon (600 bp) with *Pseudomonas fluorescence* A506 complete chromosome (Fig. 5).

The PCR was performed to determine the presence of gene *tmr* in the genomic DNA of MG dye discoloring *Stenotrophomonas* sp. In this study, two homologous amplicons of 900 bp (*Tmr 1*) and 700 bp (*Tmr 2*), obtained from *S. maltophilia* isolate TPMD-1 (Fig. 4), were sequenced and compared with those of the related genes available in Gene Bank (NCBI BLAST tool). The large amplified gene '*tmr*' sequence with 900 bp showed 97.28% identity with *Pseudomonas* sp., while a small one 700 bp with 83% identity showed similarity with *Stenotrophomonas* sp. Based on this, the phylogenetic tree was constructed (Fig. 6).

Discussion

Literature survey indicated that several bacterial strains had been involved in the decolorization of MG and CV (Sneha et al. 2014; Chaturvedi and Verma 2015; Wanyonyi et al. 2017). In the previous study, Kim et al. (2008) isolated the *S. maltophilia* LK-24, which was found to decolor crystal violet, a TPM dye. An isolate of *S. maltophilia* capable of discoloring both CV and MG dyes is not documented in the literature. Hence this is the first report of the strain of *S. maltophilia* TPMD-1 that degrades both the MG and CV dyes. In other studies, *S. maltophilia* was used in the bioremediation of hydrocarbons (Liu et al. 2007; Urszula et al. 2009; Gao et al. 2013) and chlorinated pesticides (Dubey and Fulekar 2012) and the decolorization of certain synthetic dyes such as methylene blue, toluidine blue, methyl green, indigo blue, neutral red, congo red, methyl orange, and reactive pink, golden yellow MR (Galai et al. 2009; Rajeswari et al. 2015). In addition to the above, strains of *Stenotrophomonas* sp, particularly *S. maltophilia* that are also reported to be resistant to heavy metals (Zhang et al. 2001; Crossman et al. 2008) are shown to produce biofilm with a great level of exopolysaccharide and protein that enabled the bacteria to grow in the presence of toxic heavy metals (Crossman et al. 2008; Ryan et al. 2009). Some strains also adapted to grow in the industrial effluents containing both dyes and heavy metals. Furthermore, in vitro shaking condition induced *S. maltophilia* to produce laccase in the presence of copper and decolorize methyl blue, toluidine blue, methyl green, methyl orange, congo red, and pink (Galai et al. 2009, 2012).

Several authors documented that the decolorization of synthetic dyes follows the first-order decay model (Mutafov et al. 2006; Van der Zee et al. 2001; Selvakumar et al. 2013). The present study adopted this model to explain the rate of decolorization of CV and MG by *S. maltophilia* TPMD-1. This model simplifies the information of the physiology of the dye decolorization process and helps in interpreting the experimental results based on the comparison of numerical values. The comparison of coefficients of the model obtained for the bacterial isolate revealed a significant difference between the decolorization of MG and CV. The factors of the study included an initial concentration of CV and MG and an incubation period in the liquid medium. This difference in decolorization efficiencies of two compounds could be assigned

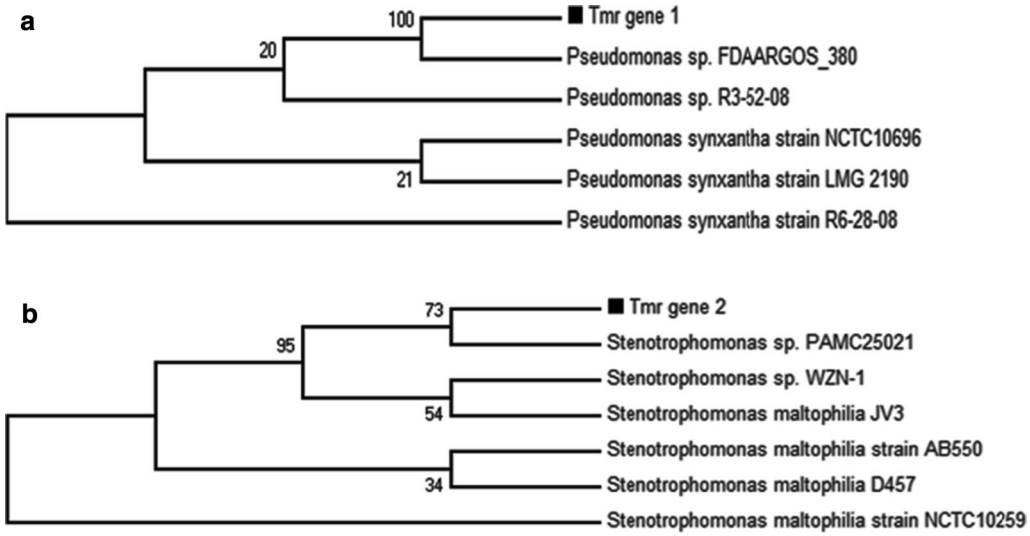

Fig. 6 Phylogenetic representation of **a** TMR 1 and **b** TMR 2, amplicons of *S.maltophilia* TPMD-1 in comparison to those from NCBI database by maximum likelihood analysis based on TMR specific primers

to different chemical structures, wherein MG consists of two dimethyl groups in two side chains while CV consists of three dimethyl groups in three side chains (Rajesh and Uttam, 1999). Similar observations were made, Wang et al. (2011) in the decolorization experiment on MG and CV by *Achromobacter xylosoxidans* MG1. Hence, MG with a short half-life was selected for further investigations. The process of dye degradation is shown to depend on the physicochemical status of the decolorization environment. The MG dye decolorization efficiency was very low in a static condition at different temperature and pH range. This is evident for the oxygen requirement of the organism to decolorize the dye since isolate is catalase-positive and aerobic (Assih et al. 2002). The previous study also reported the importance of shaking or oxygen in the bacterial discoloration of different dyes, including methyl orange, congo red and malachite green (Pradhan et al. 2012). The anaerobic and aerobic conditions influence enzyme-related dye degradation (Khan et al. 2012) and hence, the agitation could be playing an essential role in increasing the availability of oxygen in the liquid medium, thus stimulating the discoloration ability, particularly at alkaline pH (Table 3). However, the requirement of static condition for MG decolorization is also documented (Chaturvedi and Verma 2015) suggesting the ambiguity in the understanding of the decolorization mechanism. The present study indicated that the acidic pH and low temperature could influence the bacterial isolate TPMD-1 as compared to the alkaline pH and temperature > 35 °C. In this context, the acidic textile industrial effluents (Zouch et al. 2018) could be supporting the adaptation of the bacteria resulting in dye decolorization.

In the biological system, decolorization of MG occurs by two processes—absorption or adsorption of the dye by bacterial/fungal cells and degradation. During this process, the intensity of the dye in the medium decreases over time. Hence, HPLC could be a valuable technique to differentiate between these two processes. The disappearance of specific peaks with the concomitant appearance of individual new peaks was observed. This peak corresponded to various metabolites that are produced during the degradation of MG. The characteristic presence of new peaks or the absence of any peak after incubation was attributed to the production of new by-products by growing cells (Chen et al. 2008; Dhanve et al. 2009). One of the limitations of HPLC was the lack of deterministic data to confirm the categories of those substances. The biotransformation of MG dye was confirmed by comparing the FTIR analysis of untreated control with the treated sample. The prominent peak at 829.76 cm^{-1} representing mono or para-substituted benzene ring (C-H bending vibration 850–670 cm^{-1}) (Coates 2000), which is prominent in control, is absent in bacterial treatment. Such observation was also made in *Ochrobactrum pseudogrignonense* strain GGUPV1 and laccase producing *Bacillus thuringensis* RUN1 grown in MG containing media (Khan et al. 2012; Chaturvedi and Verma 2015). The previous reports also documented the presence of benzene ring, C–H and O–H stretching aromatic rings with nitro compounds in the MG controls (Cheriaa et al. 2012; Vijayalakshmidevi and Muthukumar, 2014). Degradation of aromatic structures was supported by the absence of peaks below 1000 cm^{-1} (Vijayalakshmidevi and Muthukumar, 2014). The decreased peak intensity in the fingerprint region could be attributed to the production of a degraded metabolite of MG. Parshetti et al. 2006 opines that the variations in the FTIR spectra of bacterial treatment in comparison to MG (control) indicated biotransformation. The bacterial decolorization of MG involves a series of reactions such as N-demethylation, reduction, benzene removal, and oxidation (Wang et al. 2012). This resulted in the formation of methanone [4- (dimethylamino)], phenyl phenyl (m/z 148) and phenol, 3-(dimethylamino) (m/z 136), phosphinic acid, [p-dimethyl amino)phenyl], methyl ester (m/z318), (E)-2- hydroxy-4 diethylamino-stilbene (m/z239) and Benzylaniline 9 m/z183) (Chaturvedi and Verma 2015; Wanyonyi et al. 2017). The HPLC and FTIR analyses of the present study indicated that the decolorization of MG occurred through degradation and not by cellular adsorption, following changes in the molecular orientation of the pure dye molecule; and the resulted in the formation of fragments as indicated by the formation of new peaks in HPLC and FTIR spectra. Moreover, the colorless microbial biomass after complete decolorization confirmed the dye biodegradation ability of bacterial cells.

The MG and its reduced forms are equally phytotoxic (Culp and Beland, 1996). The toxicity of MG and its degraded products are a primary environmental concern, since they are disposed-of into water bodies without any treatment, and these products find entry to the irrigation system and finally, the cultivation fields and portable water system. The phytotoxicity of MG and its degraded products was determined in sorghum and finger millet seedlings. The findings of this study are consistent with those of the previous reports that MG is toxic to plants, while its degraded products are less toxic (Gomare et al. 2009; Chaturvedi and Verma 2015).

Molecular snapshot of bacterial isolate for '*lac*' and '*tmr*' genes

Laccases (p-benzenediol: oxygen oxidoreductase EC1.10.3.2) are the member of a multicopper protein family, belonging to a group of blue copper protein, and are widely produced in plants, fungi and bacteria (Shraddha et al. 2011). However, bacterial laccase activity has

been more profound than its fungal counterpart (Feng et al. 2015). The bacterial species like *Bacillus halodurans, B. subtilis SF, B. pumilus, Azospirillum lipoferum, Pseudomonas desmolyticum* NCIM 2112*, P. putida* have been reported for laccase production (Narayanan and Murugan, 2014). The gene '*lac*' codes for the laccase, which is previously known as the MG degrader (Yang et al. 2014). The bacterial laccase enzyme is both intra or extracellular (Muthukumarasamy et al. 2015; Dhiman and Shirkot 2015) capable of oxidizing various benzene ring containing compounds (phenols and polyphenols) and plays a vital role in the detoxification of textile effluents and bioremediation process (Couto and Herrera 2006; Chadra and Chowdhary 2015). The oxidation could be attributed to four copper atoms in laccase that, play an essential role in the enzyme catalytic mechanisms (Cha and Cooksey 1991). Various bacterial laccases have been isolated and studied at the molecular level, and laccase production is optimized in bacteria for dye degradation (Couto and Herrera, 2006; Feng et al 2015; Kuppusamy et al. 2017). Unuofin et al. (2019) opine that the exact function of all the bacterial laccase genes discovered so far has not been ascertained; however, they have been linked with resistance to radiation and synthesis of pigments. There is a connection between laccase activity induction and bacterial dye decolorization (Lee et al. 1994; Ayed et al. 2010; Unuofin 2020). The earlier report is available related to the copper depended on an enzyme, multicopper oxidase in *Stenotrophomonas maltophilia* coded by *cop A* gene which has laccase activity, copper resistance and in vitro dye decolorization property (Galai et al. 2011). Similarly, the laccase enzyme from *S. maltophilia* AAP56 is metal-dependent (Cu^{2+}) and copper sulfate stimulates the laccase production and bacteria can decolorize the synthetic dye in shaking conditions (Galai et al. 2009).

A previous study of bacterial biodegradation of MG indicated that the *tmr* gene product was responsible for dye degradation (Wang et al. 2012; Jang et al. 2005). This enzyme can convert the MG into its leuco form (Jang et al. 2005). This is true in *Pseudomonas* sp containing *tmr* 2 gene, which is responsible for discoloration of the TPM dyes including MG, CV and Basic Fuchsin (Li et al. 2009). However, the most efficient TMR substrate appeared to be malachite green because it exhibited favorable structural features when modeled with the ternary complex (Jang et al. 2005). Gene homologs similar to the above were also documented in *Achromobacter xylosoxidans* MG1 and *Exiguobacterium* sp. MG2 and the '*tmr*' gene was amplified in the present study by using similar primers. Similar, observations were also made for *Citrobacter* sp, which is previously known for MG degradation (Wang et al. 2011, 2012). The engineered freshwater cyanobacteria *Synchococcus* containing triphenylmethane reductase gene '*katmr'* was shown to produce a soluble recombinant protein that degraded malachite green into small molecules 4-methylaminobenzoic acid and 4-hydroxyl-aniline and that are nontoxic to wheat seeds (Han et al. 2020). Huan-Mei et al. 2011 opined that '*tmr*' gene could have been horizontally transferred from *Pseudomonas* sp. strain K_9 to another MG degrading *Pseudomonas* sp. strain MDB-1, based on the relatedness of '*tmr*' gene and the associated protein product.

The presence of pair of homologs *lac* and *tmr* genes indicating the gene duplication. This kind of gene duplication and large amplifications that generated several replicons of the same gene within a single bacterium is a common phenomenon. Such gene duplications could have been maintained in the long-term during evolution to increase the number of gene products (Francino 2012). This could be the possible reason behind the presence of multiple laccase genes detected in both fungi and bacteria. The findings of Sakamoto et al. (2015) that distinct multi-copper oxidase genes are present in *Lentinula edodes* supported the above observation. Recently, Unuofin et al. (2019) also identified the five different homologous laccase gene sequences in *Achromobacter xylosoxidans* and *Citrobacter freundii*. This homology suggested that the laccase gene of *S. maltophilia* TPMD-1 performs a similar function.

Conclusion

The TPM dyes like MG and CV are the most commonly used dyes industrially, which is the cause of concern for the pollution of water bodies. A bacterial strain *S. maltopholia* isolate TPMD-1 obtained from the compost of neem oil-cake was efficiently degraded to both MG and CV. The MG degradation rate was slightly more in neutral pH at 28–30 °C. The *Stenotrophomonas maltophilia* TPMD-1 contained TMR and laccase genes that were previously considered as efficient enzymes in the decolorization of MG. Furthermore, the widely distributed bacterium *Stenotrophomonas maltophilia* is saprophytic and has the highest efficiency in the decolorization of MG. This study helps future understanding of the complete biodegradation mechanism of MG or other triphenylmethane dyes with a similar structure.

Abbreviations

MG: Malachite Green; RNA: Ribose nucleic acid; TPMD: Triphenyl methane degrading; TPM: Triphenyl methane; HPLC: High-Performance Liquid Chromatography; FTIR: Fourier Transform Infrared Spectroscopy; TMR: Triphenyl methane reductase; NADH: Nicotinamide adenine dinucleotide; Lac: Laccase; PCR: Polymerase Chain reaction; NCBI: National Center for Biotechnology Information; BLAST: Basic Local Alignment Search Tool; DNA: Deoxyribonucleic Acid; ANOVA: Analysis of variance; KH_2PO_4: Potassium dihydrogen phosphate;

$ZnSO_4$: Zinc Sulfate; K_2HPO_4: Dipotassium phosphate; $FeSO_4$: Ferrous sulfate; $MnSO_4$: Manganese sulfate; $MgSO_4$: Magnesium sulfate; Na_2SO_4: Sodium sulfate; KBr: Potassium bromide.

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Authors' contributions
VUA collection of neem oil-seed cakes, isolate the composting and dye degrading bacterium and conducted the molecular experiments and preparation of manuscript. MBS designed the methodology revision of manuscript. BG, RKK and EN provide laboratory facility and revised the manuscript and All authors have read and approved the manuscript.

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Declarations

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Competing interests
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