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Original Article

Decolourization and Detoxification of Malachite Green by *Enterobacter cloacae* NAM-9415 Isolated from Flower Vase Water

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Abstract

Introduction: The appalling environmental hazards associated with the use of a triphenylmethane dye i.e., Malachite Green (MG) was unveiled by the National Institute of Health in 2004. However, in spite of the successful ban of MG in the US, UK, and several European countries, it continues to be the most commonly used dye in microbiological laboratories and a few textile industries. In the present study, the bio-remediation potential of a bacterium isolated from a flower vase filled with traces of MG dye solution was investigated.

Materials and Methods: The physicochemical parameter for degradation of MG was optimized. Also, considering the fact that the dyes are complex molecules and their breakdown products may be unsafe for environmental disposal, toxicity tests were carried out using an aquatic invertebrate (*Daphnia magna*) as a model organism.

Results: The bacterium was identified as *Enterobacter cloacae* NAM-9415 by 16S rRNA analysis. It showed 96% decolourization of MG at 500ppm dye concentration when cultured at optimum conditions of incubation i.e., 15 h at 45 °C under shaker (120rpm) conditions using nutrient broth medium (pH 7). In addition, it also showed tolerance to high salt concentrations of up to 6g%. Moreover, the breakdown products supported the growth of daphnids in our study.

Conclusions: The above observations indicate the suitability of *E. cloacae* NAM-9415 for biodecolorization of textile effluents.

Keywords: Decolourization, Detoxification, Malachite Green, *Enterobacter cloacae* NAM-9415, Toxicity Tests

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Introduction

Synthetic dyes are primarily derived from petroleum products containing unsaturated groups of molecules known as chromophores. Statistically, over 70% of all known dyes are categorized under azo-dyes, while over 15% constitute of anthraquinone dyes.[1] These dyes are exclusively used in several large scale industries like textile, cosmetics, printing and fashion, resulting in huge profits triggered by the appealing appearance of finished products. Due to the extensive application of these dyes, they have received meticulous attention by researchers and environmentalists.[2,3] On the other hand, in spite of the common use of triphenyl-methane (TPM) dyes in microbiological and pathological laboratories, relatively few studies have focused on the dangers associated with this group of dyes. [4,5] Few examples of TPM dyes include Malachite Green (MG), basic fuchsin and crystal violet. Other uses of TPM dyes include the production of printing ink, hectograph and dyeing of pure wool or silk.[6]

Although, none of these sectors can compete with textile or cosmetic industries and the production of MG is insignificant in comparison to other dyes, the millions of kilograms of this dye produced annually worldwide substantially contribute to the environmental hazards. In spite of the environmentally unfriendly nature of TPM dyes, not only are they discarded as untreated samples but are also used in several oral and dermatological medicinal preparations in several countries.[7] They are also used in aquaculture for controlling the spread of parasitic infections in fish. However, there have been reports of the reproductive abnormalities, tumor formation and deteriorative action of TPM dyes on the nuclei and mitochondria of edible fish tissues and rabbit.[7,8] In addition, the TPM dyes act as an aromatic xenobiotic compound and also affect the photosynthesis activity in aquatic life because of reduced light penetration at concentrations as low as 10 mg/L.[9,10] Furthermore, the degradation product of MG i.e. Leuco-malachite green and carbinol is recognized to be several times more toxic to humans and animals.[7,11] Even though the above findings were publicly proclaimed by the National Toxicology Program in the form of published study undertaken by the National Institute of Health in 2004,[12] and

successful ban on the use of MG in the US, UK as well as several European countries, unfortunately many developing countries rely on MG and other potentially harmful dyes due to its stability and cost effectiveness.[13]

Commonly, all TPM dyes are re-calcitrant molecules that are regarded as toxic, carcinogen and a mutagen.[14,15] It is for this reason that treatment of these dyes prior to discharge is mandatory by environmental regulations in most countries. Although several physical techniques of effluent treatment such as adsorption, sedimentation, filtration, segregation and chemical techniques such as precipitation, coagulation/ flocculation, ion exchange, membrane filtration, electrolysis, oxidation and reduction etc., have been used to eliminate the dyes from waste waters, they suffer drawbacks like the excess use of chemicals, sludge disposal, labour intensiveness and high expenditure.[16] As a result, the interest is focused on biodegradation protocols using micro-organisms, since most apparently, biological processes of effluent treatment surpass the above mentioned drawbacks and provide challenging alternatives to the existing technologies.

The present study emphasizes the potential of *Enterobacter cloacae* NAM-9415 for biodegradation of MG. Moreover, considering the possibility of toxic nature of biodegraded components of MG, the toxicological tests of decolourised MG were performed using daphnids as model organisms.

Materials and Methods

Chemicals and Media Used in the Study

Triphenylmethane dye MG (λ_{max} 620nm) used in the current study was manufactured by Lobachemie, Mumbai, India. All other nutrient media were obtained from Hi-media Pvt Ltd., Mumbai, India. Dye stock solutions of 10 mg/ml were prepared in sterile distilled water and used for further experiments.

Sample Collection

A water sample was collected from a flower vase showing decolourization of MG (previously inoculated) and used as a test sample for screening dye decolorizing and degrading bacteria.

Enrichment of Dye Degraders

One ml of the above sample was inoculated into two flasks, each containing 100 ml of sterile Nutrient Broth (NB) and 50 ppm of MG. After inoculation, the flasks were incubated at RT for 24-48 h under shaker (150 rpm) and static conditions respectively, until decolourization was observed. Un-inoculated broth containing 50 ppm MG was used as control and incubated under both static and shaker conditions.

Isolation

Isolation of bacterial species was carried out from the flask showing decolourization, on Nutrient Agar (NA) plate containing 50 ppm MG. The morphologically distinct bacterial colonies were picked up for the dye decolourization study and maintained on NA slants containing 50 ppm of MG. The selected isolates were checked for their maximum degradation capacity using plates containing dye in the range of 100-1200 ppm with an interval of 100 ppm.One isolate showing maximum degradation ability was selected.

Identification of Dye Degrading Bacteria

Primary identification of the isolate was done on the basis of morphological, cultural and biochemical tests, and the strain was confirmed by 16s rRNA gene sequence analysis. PCR based 16S rRNA gene amplification and sequencing of isolated bacterium was carried out using universal primers at Sai Systems Private Limited, Nagpur, India.

Dye Decolourization Assay

For decolourization studies, 50 ml sterile NB containing 500 ppm MG dye was inoculated with 1 ml of 18-24 h old test isolates (0.2 OD_{540nm}) and incubated at RT (~30 °C) under static and shaker conditions until decolourization was observed. Following incubation, the cell-free supernatant was collected by centrifuging small aliquots (10 ml) of the inoculated media at 5000 rpm for 25 min. The decolourization activity was expressed in terms of percentage (%) decolourization using a UV-Vis spectrophotometer and determined by monitoring the decrease in absorbance at the maximum wavelength of dye (i.e., 620 nm).[17] The un-inoculated culture medium containing 500 ppm MG dye was used as an experimental control to check abiotic loss of the dye, and sterile NB without test culture or dye was set up as blank. All experiments were carried out in triplicates and the mean values were represented. The obtained data was checked statistically for standard error and standard deviation. The percentage (%) decolourization was calculated using the following equation:

$$\% \text{ Decolorization} = \frac{A-B}{A} X100$$

Where A is the initial absorbance of control dye and B is the observed absorbance of the decolourized dye.

Dye Degradation Assay

A qualitative degradation assay was carried out with the help of UV-Vis spectrophotometer. The comparison between UV-V is spectra of MG dye and its decolourized metabolites indicated its possible degradation by test organism.

Study of Various Physicochemical Parameters

The biological decolourization and degradation of any pollutant is affected by several environmental factors in any ecosystem. The most important of them are pH, temperature, oxygen, time and substrate concentration etc. For optimization

experiments, firstly, the decolourization was monitored by using 50 ml of various culture media containing 500 ppm MG dye to determine optimum medium for decolourization assay. Different nutrient medium used in our study were glucose yeast extract broth, NB, Luria bertani broth, M9 medium with and without glucose, synthetic medium and mineral salt medium. These media were inoculated with 1 ml of 18-24 h old test isolates (0.2 OD at 540 nm) and incubated at RT under static and shaker conditions until decolourization was observed.

The optimization of physicochemical parameters for biodegradation of MG was investigated by varying one parameter at a time while keeping the others constant. These varying parameters included the pH (4-10), incubation time (24-120 h), temperature (RT, 35 °C, 45 °C, and 55 °C), initial optical density of test isolate (OD_{540nm} 0.2, 0.4, 0.6, 0.8, and 1.0), initial dye concentration (50 ppm to 1200 ppm), aeration i.e., static or shaking (150 rpm), and concentration of NaCl (1-6 %). In addition, a deletion assay was carried out to check the effect of individual components of nutrient medium showing most effective dye decolourization. Finally, an optimized assay was performed by combining the optimized physicochemical parameters.[18,19]

Daphnia Toxicity Test

In order to evaluate the aquatic toxicity of dye effluents, we performed single species laboratory toxicity tests with *Daphnia magna*. The acute toxicity test was conducted by exposing it to various concentrations (1-50 ppm) of MG and its degraded product for 24 h. Distilled water control was used as control. All the toxicity tests were carried out at RT. Test chambers were not aerated during experimentation. The numbers of immobilized daphnids were recorded after 24 h exposure. Daphnids unable to swim for 15 s after gentle stirring were considered as immobile/dead.[20]

Results

Screening of Dye Decolorizing Organism

In our study, seven distinct isolates were obtained on NA plates from the used test samples. These isolates were tested as potential MG decolourizers and capable of growing on NA plates with 500 ppm of MG. The percentage (%) decolourization observed for these isolates are listed in Table 1. The results were recorded considering the maximum decolourization of MG that was observed in the shortest period of time. In this case, isolate seven showed the highest decolourization potential under shaker conditions in 15 h and hence was selected for the present study.

Table 1. Decolourization Potential of Test Isolates

Isolate number	% Decolourization
1.	23.26%
2.	45.92%
3.	33.21%
4.	37.49%
5.	26.69%
6.	39.01%
7.	88.09%

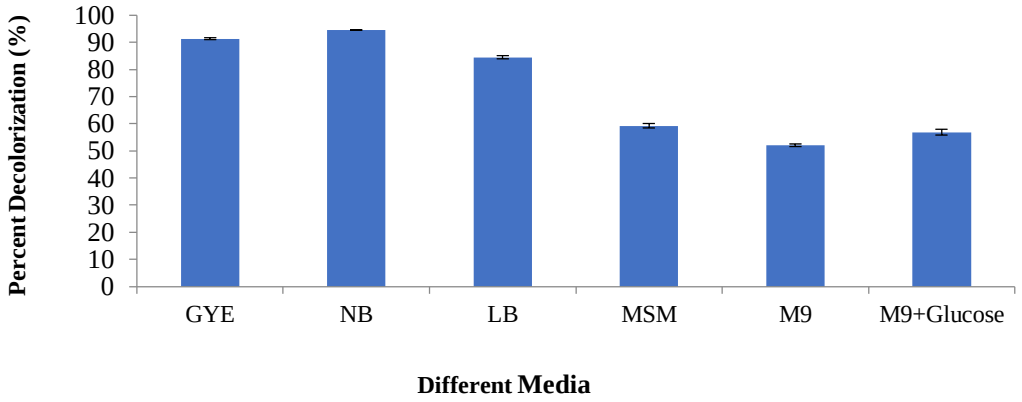

Figure 1. Optimization of Media for Decolorization of Malachite Green Under Shaker Condition by *Enterobacter cloacae* NAM- 9415 in 15h.

Isolation and Identification of MG Degrading Isolate

The preliminary characteristics of isolate seven was determined by morphological, cultural and biochemical tests and was identified as *Enterobacter cloacae*. The colony characteristics of test isolate observed on NA plate are indicated in Supplementary Table 1. The gram nature of the test isolate was confirmed as gram negative by gram staining and KOH string test (Supplementary Figure 1a and b). Upon 16S rRNA analysis, the test isolate showed 99% homology with strains of *Enterobacter cloacae* (Supplementary Figure 2). The isolated pure bacterial species was designated as *Enterobacter cloacae* NAM-9415 and refined sequences were submitted to NCBI with the accession number LC224328.

Optimization of Physicochemical Parameters for Dye Decolourization

The NB was found to be the most effective nutrient source for achieving optimum decolourization (Figure 1). The maximum

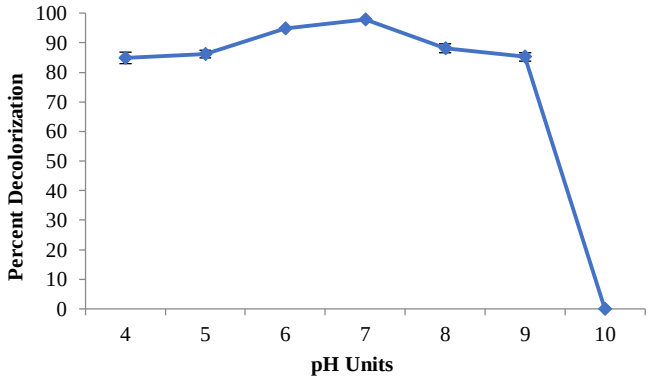

Figure 2. Optimization of pH for Decolourization of MG Dye by *Enterobacter cloacae* NAM-9415 in 15h.

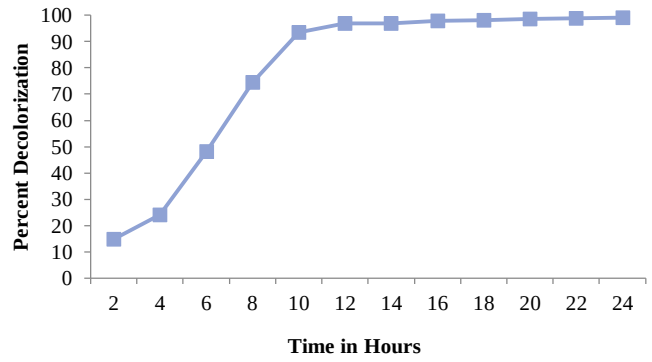

Figure 3. Optimization of Incubation Time for Decolourization of MG Dye by *Enterobacter cloacae* NAM-9415.

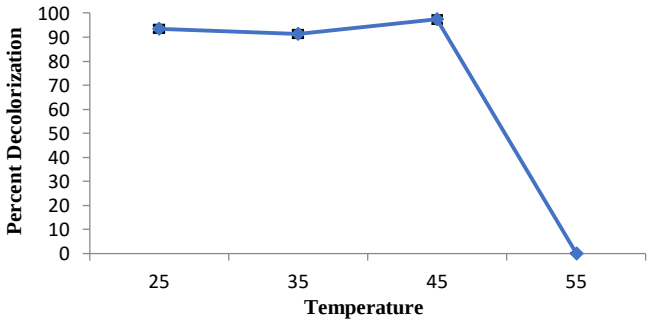

Figure 4. Optimization of Temperature for Decolourization of MG dye by *Enterobacter cloacae* NAM-9415 in 15h.

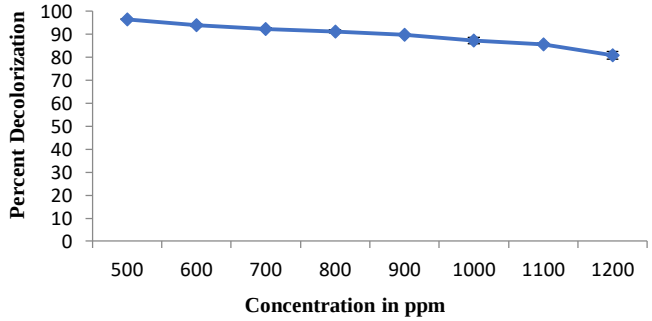

Figure 5. Optimization of Initial Dye Concentration for Decolourization of MG Dye by *Enterobacter cloacae* NAM-9415 in 15h.

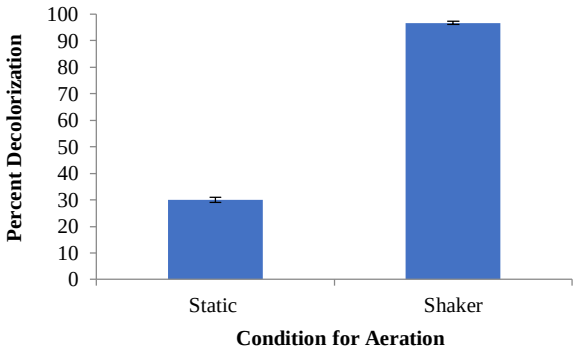

Figure 6. Optimization of Aeration for Decolourization of MG Dye by *Enterobacter cloacae* NAM-9415.

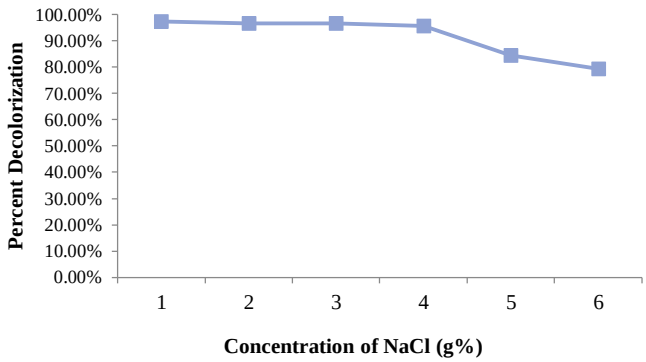

Figure 7. Optimization of NaCl Concentration for Decolourization of MG Dye by *Enterobacter cloacae* NAM-9415 in 15h.

decolourization of MG dye was observed when *E. cloacae* NAM-9415 (1.0 OD_{540nm}) was inoculated in NB (pH 7) containing 500 ppm MG dye and incubated at 45 °C under shaker conditions for 15 h (Figure 3-7). Deletion assay indicated that the removal of the component of NB did not affect the dye decolorization potential of *E. cloacae* NAM-9415 significantly (Figure 8).

Dye Decolourization and Degradation Potential of Enterobacter cloacae NAM-9415 Under Optimized Conditions

An optimized assay was performed by using results of the optimized physicochemical parameters. The maximum decolourization of 98.40% of 500 ppm of MG dye was observed in medium NB with pH 7 at 45 °C and using optical density 1 at 540 nm in 12 h. The confirmation of dye degradation potential of *E. cloacae* NAM-9415 was done based on UV-Visible spectroscopy (Supplementary Figure 3). As observed in Figure, very few peaks were observed in the parent dye molecule. The analysis of metabolites showed presence of new peaks and disappearance of peaks observed in the parent molecule. The new peaks observed in the UV-Vis spectral scan can be attributed to the formation of breakdown products due to metabolic activity of *E. cloacae* NAM-9415.

Daphnia Toxicity Tests

Although several species have been traditionally used for

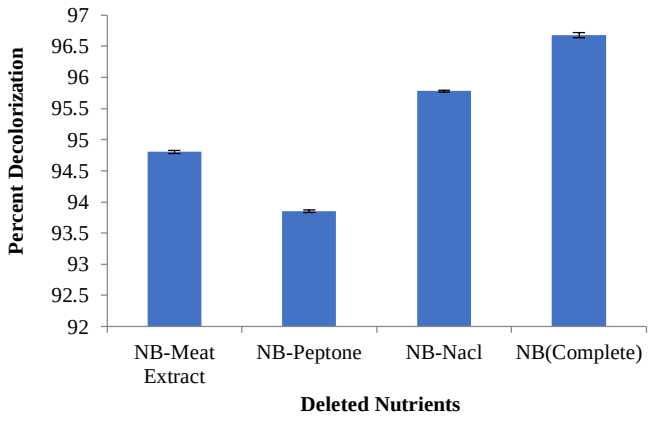

Figure 8. Deletion Assay of Nutrient Broth.

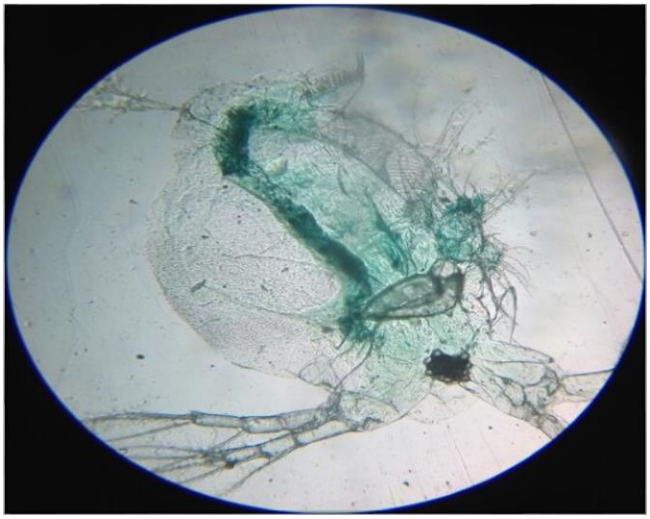

Figure 9. Non-viable Daphnia Exposed to >2 ppm Malachite Green Dye.

evaluating toxicity of dyes, there are no standardized protocols available for testing the safety of degraded metabolic products of dye degradation. However, it is reasonable to study the toxicity of any chemical compounds on fragile organisms like daphnids since they act as sensitive indicators of environmental pollutants. In our study, the daphnids were killed at concentration above 2 ppm of MG (Figure 9). The degraded MG showed presence of active daphnids indicating loss of toxicity. These observations indicated the suitability of treated wastewaters to be discharged in to larger water bodies, or to be used for the purpose of irrigation after analysing its BOD, COD, pH and other similar parameters.

Discussion

Dynamic environments prevail in our ecosystem with variable abiotic conditions like pH, temperature, aeration, availability of nutrition and population density of micro-organisms. Probable changes in any one or more of these parameters significantly affect the growth of living biota in these environment and hence the degradation of dyes, making it extremely important to study these parameters in order to avail maximum output. Moreover, the use of live bacterial cells rather than one or more isolated enzymes presents us with biochemical advantages like naturally occurring buffered environment inside the cell and continuous enzyme production in the correct sequence required for bio-remediation of dyes, cost-effectively. In contrast, the use of purified enzymes poses complications like construction of complex dye degradation pathways and purification of several enzymes. Moreover, buffering the enzymes in-vitro further adds to the practical difficulties and processing cost.[21,22]

Biodegradation of TPM dyes like crystal violet, brilliant green and MG have been carried out by several researchers using pure bacterial cultures of *Pseudomonas aeruginosa*, *Pseudomonas otitidis*, *Agrobacteium radiobacter*, and *Shewanella* sp. NTOU16.[23-26] In addition, few fungal (*Sachharomyces cerivisiae*) and yeast (*Aspergillus flavus*) strains have also been potentially applied as TPM dye degraders.[27,28]

Beginning with the most appropriate growth medium to maximize the decolourization potential of *E. cloacae* NAM-9415 for MG dye, we further optimized the biochemical as well as physical parameters for best results. Complex growth medium like NB readily provides complete nutrition to the growing bacteria and hence prevents stressed environments caused by high levels of dyes in the medium, to some extent. Semi-synthetic medium have also shown to provide good decolourization and degradation of few dyes. A study carried out by Parshetti et al.[29] using semi-synthetic media containing molasses, urea and sucrose, reported 100, 91, 81% decolourization respectively. It has been suggested that pH of the growth medium play an important role in the overall biochemical processes by acting as a rate-limiting factor in decolourization by regulating the transport of dye molecules across the cell membrane.[30] In our study, significant activity was observed in lower pH range (4-6) with an optimum observed at pH 7 (Figure 2). In contrast to our findings, a decrease in decolourization of azo-dyes is observed at lower pH (3-6) by other authors, except that they also reported the optimum pH to be 7.[3,31] This contradiction in our finding is due to the fact that many *Enterobacter* sp. show tolerance to low pH due to various regulatory features like alternative sigma factor (σ^2), 2 component signal transduction system (PhoP/Q; MViA) and the major iron regulatory protein (Fur).[32]

It is observed, in general, that the decolourization process gradually increases after few hours of incubation at a suitable temperature and then reaches a maximum before showing a decline. This is because the bacteria require some time to grow and acclimatize to the environment that aids in the decolourization process. However, at high concentration levels of the dye, it can be toxic to the growing micro-organisms and also increase the competition for the availability

of carbon sources.[33,34] In the present study, maximum decolourization of 500 ppm MG dye was observed after 12 h when incubated at 37 °C (Figure 3 to 5). A recent study reported decolourization of 20 ppm MG dye by unidentified bacterium in a temperature range of 25 °C to 45 °C and pH range of 5-9 in 2 h.[35]

The availability of oxygen also contributes to the decolourization activity. Almost 96% MG was decolourized under shaker conditions in our study, whereas less than 30% decolourization was observed under static conditions (Figure 6). Higher colour removal was observed by shaking because of better oxygen transfer and nutrient distribution as compared to stationary cultures.[36] Hence, aerobic organisms show better activity under shaker conditions whereas micro-aerophilic organisms thrive better under static conditions. Contradicting results are reported by several authors on the effect of shaking/agitation on the microbial decolourization of synthetic dyes depending on the type of micro-organism used in their study.[37,38] In case of our study, where the dye degradation occurs under aerated conditions, the dye may act as the preferred electron acceptor probably due to its lower OR potential than O_2/H_2O couple and higher OR potential than the last electron donor in the respiratory electron transport chain. This can be justified by the fact that the redox reaction takes place only when the OR potential of electron donor is lower than the electron acceptor. Usually O_2/H_2O couple has E° potential value 0.82 V.

Interestingly the decolourization process was not affected significantly by deleting the media composition of NB one by one, in our study (Figure 8). Although the nutrient media lacking NaCl showed negligible decrease in percentage (%) decolourization, it was still used in further experiments considering the fact that high amounts of salt are used in industrial dye baths to ensure fixation of dye to the cellulose fibres. Hence, the effluent normally shows presence of high salt concentration. This fact also increases the necessity to use cultures which can tolerate higher concentration of salts. Salt concentrations up to 15 to 20% have been measured in waste waters from dye stuff industries. Sodium levels are also elevated when sodium hydroxide is used in dye bath to increase the pH. These high concentrations of sodium generally suppress microbial growth at levels above 3 g%. Biodegradation of MG has been studied in MSM medium containing 0.5% NaCl,[37] whereas in our study *E. cloacae* NAM-9415 showed tolerance to 6 g% NaCl; thus proving to be a potential candidate for bioremediation of MG dye due to its salt tolerant ability.

Also, the percentage (%) decolourization in the peptone deleted and meat extract deleted medium was 93% and 94% respectively. This indicates that both components provide important nutrients that enhance the dye decolourization process. Hence, it also indicates that the dye molecule serves as a carbon or nitrogen source, as it showed 94% decolourization in sterile distilled water. While studying the decolourization of TPM, azo and anthraquinone dyes by a newly isolated strain of *Pseudomonas veronii*, another study suggested that crystal violet (50 mg/L) could be used as a sole carbon and energy source for cell growth.[39] Microbial source utilizing the dye as sole source of carbon and energy for their growth are of special interest. Such microbes may be valuable in eliminating the pollutants in nature.

Conclusion

The diversity in the structure of dyes and the composition of dye waste water poses several challenges for its treatment before disposal, using conventional methods. Our study indicated the potential of *E. cloacae* NAM-9415 to degrade MG dye and produce degraded metabolites which are non-toxic to fragile pollution indicators like daphnia. *E. cloacae* NAM-9415 showed tolerance to dye concentration up to 1200 ppm and salinity up to 6 g% NaCl. Hence, in conclusion, the present study reports the suitability of *E. cloacae* NAM-9415 for biodecolorization of textile or laboratory effluents containing TPM dyes.

Authors' Contributions

All the authors made equal contributions to the study design, analysis and literature review. NS carried out the experimental study. MG wrote the first draft of the manuscript and carried out statistical analysis. MG and KA made appropriate changes to finalise the manuscript. All authors approved the final version of the manuscript.

Conflict of Interest Disclosures

The authors declare that they have no conflicts interest.

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