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ARISTON PUBLICATIONS
ChemSci Advances
ISSN: 2997-9587 www.aristonpubs.com/chemsci-advances https://doi.org/10.69626/csa.2024.0088

RESEARCH ARTICLE

Biodegradation of Malachite Green Dye by Novel Isolated Bacillus Species AAV from Garden Soil

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ABSTRACT: Malachite green (MG) dye commonly use in textile and paper printing industry to a great extent. Textile effluent released from industries including dyes must be treated before release into the environment due to its toxic nature. In present study, newly isolated bacterial species was able to decolorize MG dye (100%) in 1 h at shaking condition and 94% at static condition. 16S rRNA analysis revealed an isolate as Bacillus sp. AAV. Lignin peroxidase, laccase and tyrosinase enzymes from Bacillus sp. AAV showed significant increased activity during decolourisation. There was 138, 369 and 164% trek in activity of LiP, laccase and tyrosinase after MG decolourization, respectively. Bacillus sp. AAV could decolourise MG efficiently at alkaline pH (8-10) and at 40°C temperature. Biodegradation was monitored by UV-VIS spectrophotometer. Gas chromatography-Mass spectroscopy data was used to propose the degradation pathway of MG dye. The newly isolated Bacillus sp. AAV was able to decolourise ten various types of dyes and textile dye effluent. Phytotoxicity test by using seeds of wheat and sorghum was also carried out to check toxicity of MG dye metabolites. The study reveals that there was no toxicity with respect to tested seeds. This study suggests that Bacillus sp. AAV could be a useful microorganism or its enzyme system for textile dyes and effluent treatment.

Keywords: *Bacillus* sp. AAV, 16S rRNA, Malachite green, biodegradation, laccase.

Received: 05 March 2024; Revised: 13 April 2024; Accepted: 25 April 2024

1. INTRODUCTION

With the inception of the industrial revolution, the bi-products formed or the waste generated has always been a cause of concern to the environment and the biodiversity where the waste is released. Similar is the case with the 'Textile Industry'. The textile industry plays an important role in the global economy as well as in human's routine. It is concurrently becoming one of the main sources of environmental pollution in the world in terms of quality and quantity [1]. The dyes or their remnants after the dyeing process are released into the water. There are numerous textile industries in the India, ultimately leading to more water pollution [2, 3]. Dye is an indispensable part used to impart color to materials. The waste generated during the process and operations of the dyes contain inorganic and organic contaminants leading to the hazard to the ecosystem and biodiversity causing an impact on the environment. It is estimated that around 10,000 different dyes and pigments are used in industries and over 7×10^5 tons of these dyes are produced annually worldwide [4]. Approximately 10 to 15% of dyes are lost in the effluent during the dyeing process [5]. Dyes are classified into various classes. One of the major

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classes includes the azo, arithroquinone, and triphenylmethane dyes [6].

Malachite green (MG) dye is widely used in dyeing silk, cotton, leather, wool, etc. It is a triarylmethane dye; cause of concern, due to its reported toxic effects [7]. The toxicity of this dye increases with time, temperature, and concentration [8]. It has been known to cause mutagenesis, carcinogenesis, chromosomal fractures, and respiratory toxicity [9]. Leucomalachite green is a reduced form of MG, used in aquaculture industries [10], and is reported to bio-accumulate in fishes and is a potential human health hazard [11].

There are various techniques in use to treat wastewater such as physical methods (adsorption), chemical methods (reduction), biological methods (stabilization pond), etc. [12]. Most of the techniques used for wastewater treatment require chemicals that are high-priced and produce a large amount of sludge [13]. Decolorization is defined as the removal of color by changing the chromophoric group to non-chromophoric while biodegradation, is the biological breakdown of the substrate (dye) [14].

In 1980, [15] the reported deposition of MG and crystal violet in the water and the sediment of the Buffalo River in the New York (USA). Their findings revealed the presence of aniline dyes in the aquatic environment. Aniline dyes are potential mutagens and carcinogens [16]. Synthetic dyes being intransigent molecules are difficult to degrade. Biodegradation of dyes is gaining popularity as conventional methods of wastewater treatment are ineffective to deal with the recalcitrant xenobiotic compounds. Several microorganisms have been found to decolorize and degrade dyes efficiently [17]. Various bacterial enzymes such as laccase, lignin peroxidase, azoreductase, tyrosinase, etc. are known to aid in dye decolorization [18]. A wide range of aromatic amines are biodegraded rapidly and thus microbiological treatment of textile wastewater is an effective way to deal with toxic dyes [19].

In the present study, we look over the suitability of *Bacillus sp.* AAV for the degradation of the triarylmethane dye, Malachite green. This work revealed the potential of *Bacillus sp.* AAV is to be implemented to treat textile dyes and industrial effluent degradation.

2. EXPERIMENTAL DETAILS

2.1. Chemicals

2,2′-Azinobis (3-ethylbezthiazoline-6-sulfonate) (ABTS) was purchased from SRL, India. Tartaric acid, *n*-propanol, and catechol were purchased from Sisco Research Laboratories, India. All chemicals used were of the highest purity and analytical grade.

2.2. Selection and screening of micro-organisms

Soil samples were collected from MITCON's Garden situated in the agriculture college campus, Shivajinagar, Pune, India. The samples were collected in sterile bottles and transferred to the laboratory for analysis. A serial dilution of water samples was done and the dilutions of 10^{-4}, 10^{-5}, and 10^{-6} were spread plated on the nutrient agar media (g l^{-1}: peptone 5, beef extract 1.5, yeast extract 1.5, NaCl 5, agar 15) supplemented with 50 mg l^{-1} MG dye. The plates were incubated at 37° C for 24 h. The desired colonies were selected based on their ability to form clear zones on these plates and pure culture of the colony with maximum diameter was prepared.

2.3. 16S rRNA sequencing

16S rRNA sequencing of an isolated strain of bacteria was carried out at NCIM, CSIR-NCL, Pune, India. The nucleotide sequence analysis was performed at the BLASTN site at the NCBI server (http://www.ncbi.nlm.nih.gov/BLAST) [20]. The alignment of the sequences was performed by using CLUSTALW program V1.82 at European bioinformatics site (http://www.ebi.ac.uk/clustalw) [21, 22]. The sequence was submitted to the NCBI website for publically available after critical check (https://submit.ncbi.nlm.nih.gov/subs/?search=SUB13605261). The branching diagram or phylogenetic tree was prepared to see the closely related species against the isolated bacterial strain.

2.4. Study of physicochemical parameters

The effect of static and shaking (120 rev min^{-1}) conditions on the decolourization of MG dye (50 mg l^{-1}) was studied. *Bacillus* sp. AAV strain was grown for 24 h at 35°C in 250 ml Erlenmeyer flasks containing 100 ml nutrient broth and MG dye was added in both flasks. The sample was collected at different time intervals and centrifuged at 8000 g for 20 min. The absorbance of supernatant for each time interval was recorded at 600 nm.

2.5. Decolorization measurement

Decolorization was determined by measuring the change in absorbance of culture supernatants at λ_{max} of the MG dye. The percentage decolorization [23] and average decolorization rate (ADR) [24] were measured at different time intervals. All the decolorization experiments were conducted in triplicate with standard conditions. Abiotic controls were always taken. The ADR was calculated as follows:

$$\textbf{Average decolorization rate} = \frac{\mathbf{C} \times \%\mathbf{D} \times \mathbf{1000}}{\mathbf{100}\boldsymbol{t}}$$

Where, C = initial concentrations of dye (mg l^{-1}), %D = dye decolorization (%) after time *t*.

2.6. Optimization of decolorization ability for the selected isolate

Decolorization was optimized with respect to the effect of

different carbon sources, pH, metal ions sources, and temperature. The incubation was done under shaking conditions. Bacterial cell-free nutrient broth served as a control.

2.7. Effect of pH, metal ions and various nutrient media on MG dye decolorization

Nutrient broth pH was maintained (5, 7, 9 and 10) by HCl (O.5 N) and NaOH (0.5 N) to determine the optimum pH of the isolated *Bacillus* sp. AAV. Metal ions *viz*. $CaSO_4$, KCL, $BaCl_2$, $MgSO_4$ and Na_2MoO_4 were added individually in 100 ml nutrient broth and *Bacillus* sp. AAV grew for 24 h at 30 ºC in this medium. 50 mg l^{-1} dye was added to 24 h grew culture medium and percentage decolorization was recorded. Various nutrient media such as glucose, fructose, sucrose, tryptone, yeast and meat extract were made and inoculated with *Bacillus* sp. AAV. In 24 h grew culture, MG dye was added and decolorization was observed to find the preferred media of the isolated strain.

2.8. Enzyme activities

2.8.1. Preparation of cell-free extract

Cell-free extract (crude enzyme) was made by growing AAV strain in 100 ml of nutrient broth medium at 35°C for 24 h, centrifuged at 8000 g for 15 min. The harvested cells were suspended in 0.1 M phosphate buffer (pH 7.5) for sonication (Sonic-Vibracell ultrasonic processor) keeping sonifier output at 60 amplitude maintaining 4°C temperature and giving 10 strokes, each of 30 s with 2 min intervals. This extract was then centrifuged at 6000 g for 2 min. The supernatant was used as an enzyme source.

2.9. Enzyme assays

2.9.1. Oxidative enzymes during decolorization

Tyrosinase activity was determined in a reaction mixture containing 0.01% catechol in 0.1 M phosphate buffer (pH 7.4). Catechol quinine formed was measured at 410 nm at room temperature by keeping the volume of the reaction mixture at 3.2 ml [25]. Lignin peroxidase activity was determined by monitoring the formation of propanaldehyde at 300 nm. The reaction mixture contained 0.5 ml of 10 Mm *n*-propanol, 2.3 ml of 0.1 M acetate buffer (pH=4.9), and 100 µl 10 mM H_2O_2 [26]. Laccase activity was determined using 2, 2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (10%) as a substrate in 0.1 M acetate buffer (pH=4.9) at room temperature. Oxidized ABTS was measured at 420 nm. One minute incubation period was taken for enzyme assay at room temperature. Blank contained all components except enzymes in all assay procedures. One unit enzyme activity is defined as a change in absorbance units/min/mg of protein.

2.10. Decolorization and biodegradation analysis

Decolorization was monitored using UV-VIS spectroscopic analysis. Identification of metabolites was carried out by GC-MS. For this, 100 ml samples were taken and centrifuged at 10,000 g and extraction of metabolites was carried from the supernatant using an equal volume of ethyl acetate. The extract were dried over anhydrous Na_2SO_4 and evaporated to dryness. GC-MS analysis of metabolites were carried out using Agilent MassHunter MS Engine, equipped with integrated gas chromatograph with a HP1 column (60 m long, 0Æ25 mm i.d., nonpolar). Helium was used as carrier gas at a flow rate of 1 ml min^{-1}. The injector temperature was maintained at 280 °C with oven conditions as: 80 °C kept constant for 2 min increased up to 200 °C with 10 °C min^{-1} raised up to 280 °C with 20 °C min^{-1} rate. The compounds were identified on the basis of mass spectra and using the NIST library.

3. RESULTS AND DISCUSSION

3.1. Results

3.1.1. Isolation and identification of dye-decolorizing bacteria

The bacterial strains were selected on the basis of their ability to form clear zones on nutrient agar plates containing malachite green dye. Among several isolated bacterial strains, one strain was selected on its ability to form a high dye decolourization zone on a nutrient agar plate (data not shown). 16S rRNA sequencing revealed that the isolated strain was identified as Bacillus Sp. AAV (Figure 1). Bacillus sp. AAV decolorized MG dye 100% in 1 h at shaking condition, whereas, decolourization of MG dye was 94% at static condition.

3.1.2. Effect of pH on decolourization

The decolourization of MG dye was studied at different pH (Figure 2(a)). It showed ~99% decolourization at alkaline pH (pH 10) and no decolourization at acidic pH (2-4). *Bacillus* sp. AAV took 15 min for complete decolourization at alkaline pH (Figure 2(a)). The optimum pH of bacterial strain for decolourization was 9-10.

3.1.3. Effect of carbon source and metal ions

The Decolourization of MG was studied among different carbon sources and metal ions. Meat Extract showed a maximum decolourization of 98% in 20 min (Figure 2(b)). Maximum decolourization was achieved with sodium molybdate metal ion, which showed 100% decolourization in 20 min (Figure 3). Noteworthy differences were not observed in the percentage decolourization of dyes in presence of other carbon and metal ion sources.

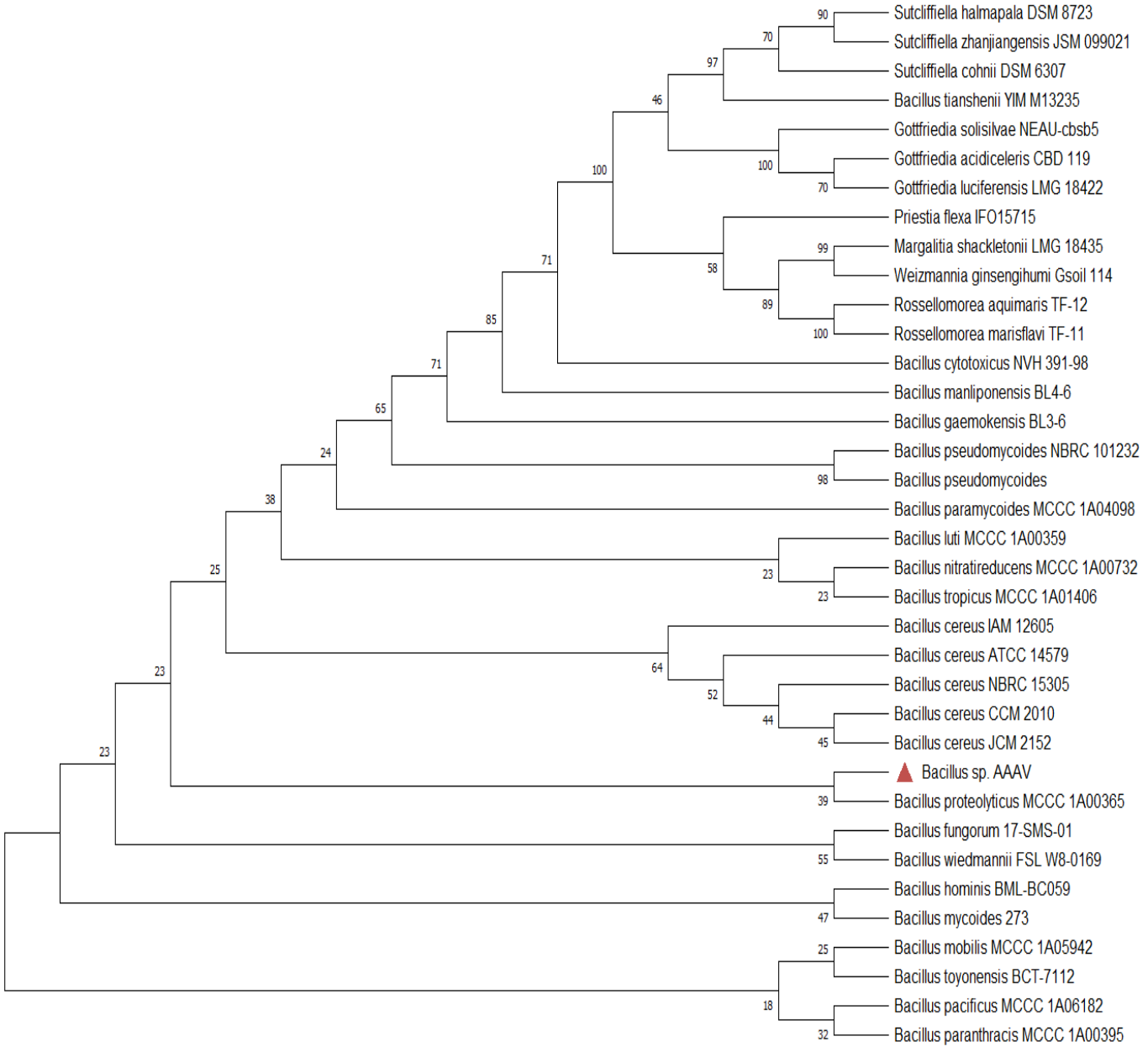

Fig. 1. Phylogenetic tree of the *Bacillus* sp. AAV and related organisms were aligned based on 16S rDNA sequences (neighbour-joining tree). Scale bar: number of nucleotide changes per sequence position. The numbers at nodes show the bootstrap values obtained with 1000 resampling analyses.

3.1.4. Enzyme activities while decolourization in batch culture

Decolourization of MG in batch culture is observed and at the same time enzyme activities while also checked. LiP, tyrosinase, and laccase show the activity status of degrading enzymes during the decolourization of MG dye (Table 1). Induction in the intracellular LiP (138%), laccase (369%), and tyrosinase (164%) activity were recorded up to complete decolourization (1h incubation). All enzyme activities' significant induction was observed in the intracellular assay. In the culture supernatant, no significant induction was observed for all the activity assays (data not shown).

3.1.5. Biodegradation analysis

MG dye was scanned to monitor dye degradation by UV-Vis spectrophotometer (400–800 nm) to see the decolourization of dye at various time intervals. Peak observed at 620 nm (0h) was shifted in λmax up to 100% biodegradation of dye (1h). Gas chromatography-mass spectrometry analysis was conceded to examine the metabolites synthesized throughout the biodegradation process.

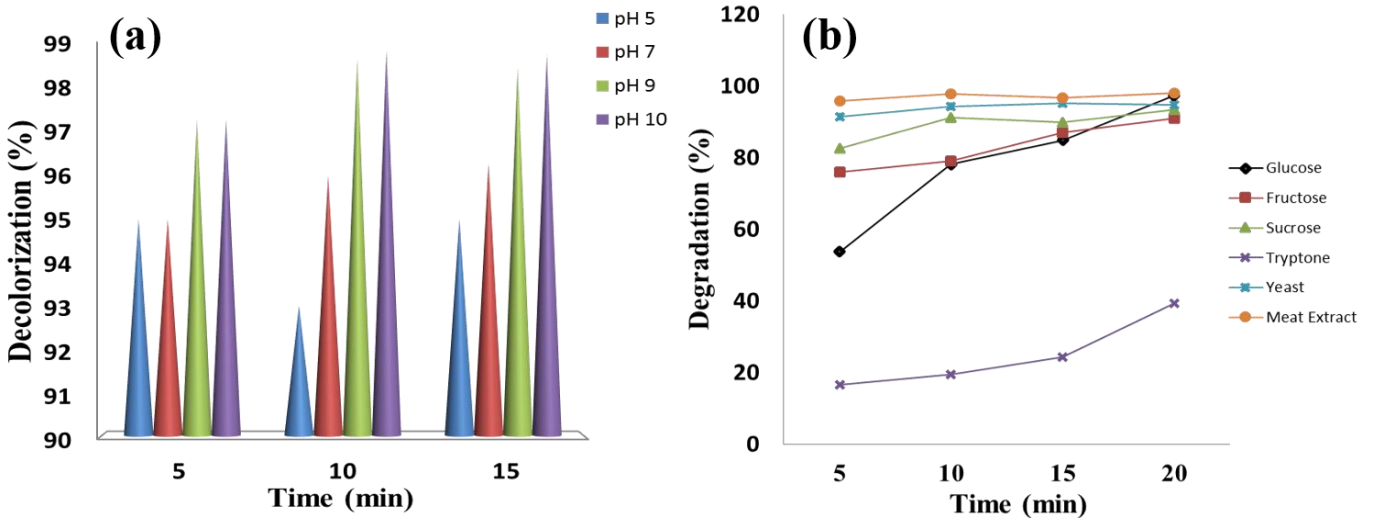

Fig. 2. (a) Effect of pH, and (b) Effect of different nutrient source on the decolorization of Malachite green dye.

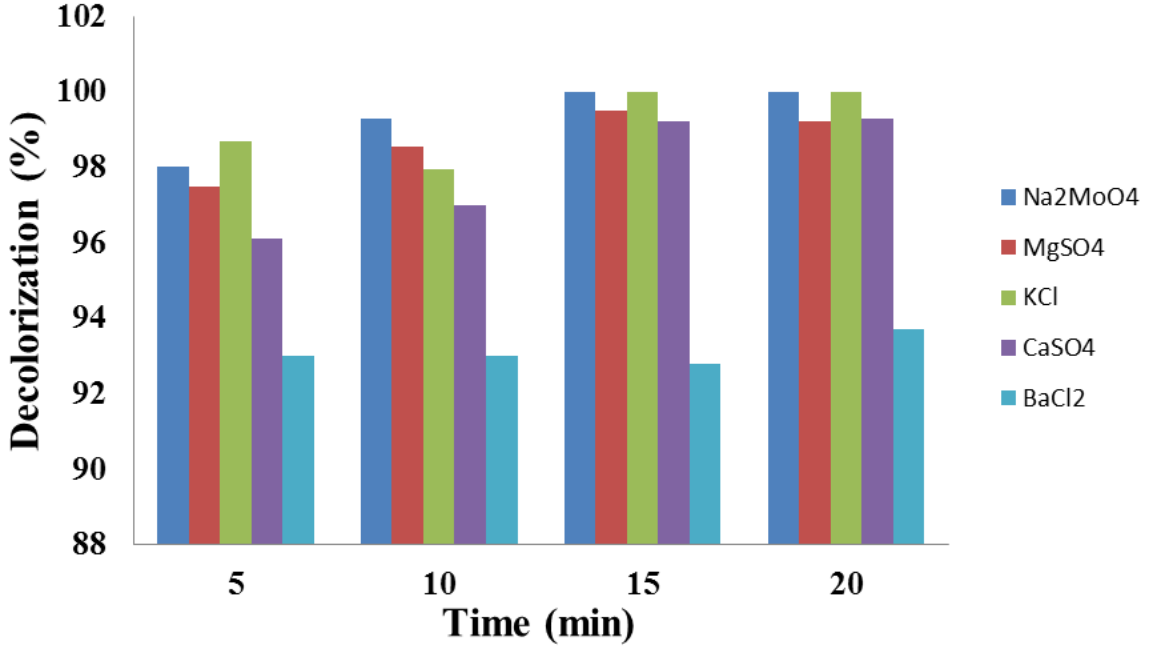

Fig. 3. Effect of metal ions on decolourization of Malachite green dye.

Table 1. Enzyme activity status during decolorization of MG dye by *Bacillus* sp. AAV.

Enzymes	Control (0 h)	Decolorization (100%; 1 h)
LiP	1.523±0.078	2.116±0.07*
Laccase	0.13±0.006	0.48±0.001*
Tyrosinase	1.178±0.003	1.943±0.005*

*Values are mean of three experiments ± SEM.
Significantly different from control (0 h) at $*P < 0.05$.
Activity in units mg^{-1} min^{-1}.

A pathway has been proposed for the biodegradation of MG dye by *Bacillus* sp. AAV at static condition (Figure 4). The first cleavage was probably due to the action of LiP resulting in a benzene ring with a nitrogen molecule. The sequential reactions with symmetric and asymmetric cleavage gave only benzene at the end. The denitration and loss of methyl groups showed the role of various enzymes in the biodegradation of MG dye. All the metabolites are not shown by GC-MS, however, their presence is supposed to propose the degradation pathway. GC-MS spectral image and the proposed pathway are shown in Figure 4.

3.2. Discussion

Bacillus sp. AAV showed excellent decolourization potential in the present work in the broad pH range of (5.0-10.0) and temperature 25 to 40°C which are normal operational parameters for conventional wastewater treatment systems [27, 28]. Some study suggests that bacteria isolated from the actual site of textile effluent are that they are more likely to have the enzymes activated, which facilitate the decomposition of dyes [29, 30]. Contrary to the above study, we found that Bacillus sp. AAV which was isolated from MITCON's garden soil has the excellent ability to decolourize 100% MG dye just in 1 h at shaking conditions and 94% decolourization at static conditions. An increase in the percentage decolourization of MG at shaking conditions because shaking facilitates the transfer and distribution of nutrients and oxygen between the medium and microbial cells [31]. The results obtained in the present study about different pH, it was observed that with the rise in pH, the maximum decolourizing efficiency was reached (98.8%) at pH 10.0. Thereafter, whenever the pH value increases, the decolourization process appeared to decrease [32].

Induction in the decolourization percentage was observedwith both metal ions and carbon sources. Sodium molybdate and potassium chloride showed the highest decolourization of 100% in 20 min. Studies suggested that the sodium molybdate significantly enhanced catalytic activity [33, 34] for cationic dye MG degradation [35]. Induction in the enzymatic activity of tyrosinase, laccase, and LiP indicates that the oxidative enzymes are mainly involved in the dye degradation mechanism [13]. Bacterial laccases involve in various processes like the detoxification of industrial effluents mostly textile industry, which degrades various ranges of xenobiotic compounds [36]. Studies reported that the laccase exhibits broad substrate specificity towards aromatic compounds containing hydroxyl and amine groups including diphenyls, polyphenols, diamines, and aromatic amines [37, 38]. Lip and laccase play important role in degradation of MG dye by oxidative and reductive potential. Eventually as reported in many papers, free radicals capable of undergoing further depolymerization, repolymerization, demethylation, or quinone formation [39, 40]. Lip, Laccase and manganese peroxidase are known for lignin degradation [41]. Studies reported that LiP possesses the ability to degrade dyes including Direct Blue-6 and azo dyes [5, 31]. It was reported that the Bacillus cereus DC11 has the ability to decolorize 55 µM of MG (triphenylmethane dye) with 95-98% in 4 h. The bacteria from Enterococcus sp. showed 100% decolourization of MG (0.02 g/L) respectively within 24 h [42]. Others reported that the synthetic CH/ZnO and CH/Ce-ZnO enhance the photocatalytic degradation of MG by 54% and 87%, respectively. The significance of our report is the 100% degradation capability of newly isolated *Bacillus* sp. AAV within 20 min under the aerobic condition with a metal ion source. It breaks all the myths related to the isolation of bacteria from the activated site. Microorganisms from other soil may possess an excellent ability to degrade dyes and are easily available for isolation.

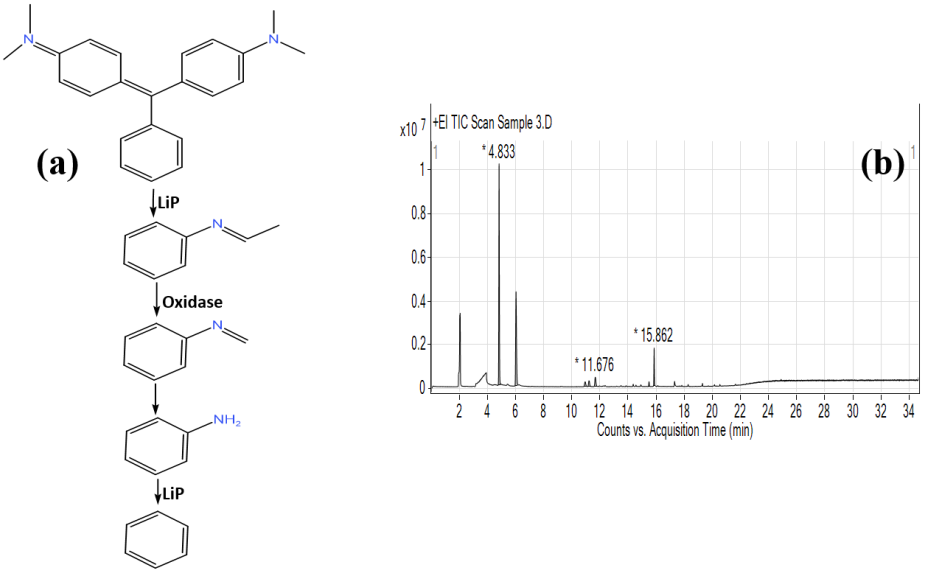

Fig. 4. (a) Proposed pathway for degradation of Malachite green by Bacillus sp. AAV (a). (b) GC-MS spectra of the decolorized MG dye. All the compounds represented have not been found, but their existence is rationalized as necessary intermediates for the final products found. The probable role of the enzymes is hypothesised in the degradation pathway.

4. CONCLUSION

This study reveals that MG dye can be degraded to 100% by isolated novel bacteria. Bacillus sp. AAV bacteria showed the potential to produce various oxidative enzymes to decolorize dye. The outcomes observed during decolourization showed that the activity of different enzymes depends on metabolites produced during decolourization. Hence, the present work demonstrates that Bacillus sp. AAV and/or its enzymes could be the best biological tool for textile effluent treatment. Experiments with refined enzymes can give an idea about the specific role of enzymes that are involved in the decolourization and degradation of MG.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interests.

ACKNOWLEDGEMENTS

We are thankful to the DSIR-SIRO, Govt. of India, New Delhi for recognition and also grateful to the Department of Science and Technology, Govt. of India, New Delhi for recognizing us as Technology Business Incubator in the Life Science area. VVD is thankful to the Mr. Anand Chalwade, MD, MITCON for providing all the facilities. Dr. Sachin Agawane is also thankful to the Director, CSIR- National Chemical Laboratory for providing lab facilities and consumables.

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