Dye degradation by early colonizing marine bacteria from the Arabian Sea, India

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Abstract

Malachite green (MG) dye belongs to the triphenylmethane group, a common environmental pollutant that threatens non-target organisms. The potential of the early colonizing marine bacterium *Pseudomonas* sp. ESPS40 isolated from the Arabian Sea, India, to decolorize malachite green (MG) was assessed in the present study. The bacterium ESPS40 exhibited a higher ability for MG degradation (86-88%) at varying NaCl concentrations (1-3%). The most increased MG degradation (~88%) was observed at 1% NaCl. The strain ESPS40 showed degradation up to 800 mg MG L^{-1} concentration. Further, enzyme activities such as tyrosinase (63.48-526.52 U L^{-1}) and laccase (3.62-28.20 U L^{-1}) were also analyzed with varying concentrations (100 mg L^{-1}-1000 mg L^{-1}) of MG during the degradation process. The dye degradation was confirmed by Fourier transform infrared spectroscopy (FTIR) and high-performance liquid chromatography (HPLC). The outcome of the present study demonstrated the potential of *Pseudomonas* sp. ESPS40 for efficient degradation of MG at higher concentrations. Thus, the bacterial strain (*Pseudomonas* sp. ESPS40) can be utilized as a potential candidate for the biodegradation of MG in wastewater treatment.

Introduction

Synthetic dyes are extensively used in textile, pharmaceutical, cosmetics, paper making, solar cells and acrylic industries (Chang and Lin 2001; Shindhal et al. 2021). Water pollution by the textile industry alone is one of the major concerning issues in the world (Roy et al. 2020). Annually, one million tons of synthetic dyes are produced globally and around 0.28 million tons are released as textile effluents (Jin et al. 2007; Jadhav et al. 2016; Periyasamy et al. 2019) and partly discarded directly into the aquatic environment which risks the aquatic/marine life (Chen et al. 2003). Dye present in the water bodies reduces the photosynthetic activity and oxygen solubility by inhibiting the light penetration, thus affecting flora and fauna of water bodies (Berradi et al. 2019; Saratale et al. 2009). Given environmental and health risks due to textile effluents, there is a need for safe disposal and removal of dyes from the aquatic environment which has attracted the attention of environmentalists and health professionals (Srivastava et al. 2004; Venil and Lakshmanaperumalsamy 2010; Gopinathan et al. 2015). Previously, different approaches like ozonation, electrocoagulation, reverse osmosis, adsorption, membrane filtration, flocculation, photocatalytic and photo-oxidation are practiced to remove dyes from textile effluents (Daneshvar et al. 2007; Jain et al. 2003; Wu et al. 2018). However, these methods are less efficient, generate secondary waste and expensive (Sghaier et al. 2019). Hence, there is a need for an alternative eco-friendly, efficient and cost-effective approach to degrade such dyes.

Bioremediation is a biological approach with the eco-friendly practice of degrading aromatic dyes into less toxic end-products. There are few reports about aromatic dye degradation by microorganisms like bacteria, fungi and algae (Chen et al. 2003; Chang and Lin 2001; Dave et al. 2015; Jasinska et al. 2015; Phugare et al. 2011; Roy et al. 2018). The biofilm-forming ability favors the bacterial activity for biotransformation of xenobiotic compounds (Shukla et al. 2014; Lee and Yoon 2017). Biofilm-forming bacterial communities have an advantage over planktonic counterparts because they tolerate a harsh

environment. Dye degradation studies using marine microbes could be explored for their potential to degrade different dyes efficiently for a sustainable environmental management (Dash et al. 2013; Xu et al. 2016; Wang et al. 2020).

Malachite green (MG) is a triphenylmethane dye that is commonly used in the textile and dyeing industries (Roy et al. 2020; Song et al. 2020). Additionally, MG is widely used in aquaculture as a fungicide and parasiticide (Srivastava et al. 2004) because of its high efficiency and low cost. The release of MG in the environment is of great concern because of its toxicity, mutagenicity and carcinogenicity; thus its use has been banned by several countries (Li et al. 2009; Qu et al. 2018; Jasinska et al. 2015; Wu et al. 2018). MG is still being used in many countries because of its low cost, ease of availability and high efficacy (Cha et al. 2001; Monga et al. 2021; Qu et al. 2018; Srivastava et al. 2004). Triphenylmethane dyes are stable and difficult to degrade; consequently, their residues enter in to the food chain and cause threats to human health (Panandiker et al. 1993). Some MG decolorizing or degrading bacteria from marine origins, such as *Tenacibaculum* sp. HMG1 (Qu et al. 2018), *Bacillus vietnamensis* sp. MSB17 (Kabeer et al. 2019), *Escherichia coli* (Xu et al. 2020), *Pseudomonas* sp. MSB4 (Raja et al. 2013) etc. had been reported previously and used as a resource for the degradation of MG dye. These isolates cannot degrade MG with higher concentrations; therefore, in the present study, we used the higher concentrations of MG.

The present study aimed to isolate and characterize the MG dye degrading early biofilm-forming bacteria from the Arabian Sea, India. The bacterial strain ESPS40 showed a maximum of 88% degradation of MG at 1% NaCl concentration. The strain was also able to degrade MG at higher NaCl (3.5%) and dye (800 mg L^{-1}) concentrations. The most promising bacterial isolate ESPS40 was identified as *Pseudomonas* sp. strain ESPS40 by 16S rRNA gene sequence analysis. The isolate ESPS40 was further studied for degrading MG dye under various dye concentrations and salinity levels. The degraded products of MG were confirmed by Fourier transform infrared spectroscopy (FTIR) and high-performance liquid chromatography (HPLC).

Materials And Methods

Dyes and chemicals

Malachite green (MG), congo red (CR), methyl orange (MO), methylene blue (MB), indigo carmine (IC) and reactive red (RR) were purchased from Sigma-Aldrich Pvt. Ltd., India. All the dyes used in the present study were of reagent grade. Zobell marine agar (ZMA) and Bushnell Haas (BH) broth media were purchased from Himedia.

Bacterial isolation

The bacterial isolates used in the present study were previously isolated from glass and plastic surfaces from three different sites, i.e., Alang, Diu and Sikka (Gujarat, India) (Kumar et al. 2022). The surfaces were submerged in the seawater for 24 h to allow early biofilm-forming bacteria to colonize the surfaces. The

surfaces were carefully transferred to filter-sterilized autoclaved seawater and brought to the laboratory in sample boxes within 12 h of collection. The surfaces were washed gently with sterile seawater to remove loosely attached planktonic cells. Biofilms from the surfaces were scraped with the help of a sterilized scalpel and dissolved in 10 mL sterile seawater. Serial dilutions were prepared and spread on Zobell Marine Agar (ZMA) media and incubated at 30º C for bacterial isolation. Morphologically distinct bacterial strains were picked out and streak plated on separate ZMA plates. The bacterial colonies were identified and axenic cultures were achieved by sub-culturing through the streak plate method.

Screening of dye degrading bacteria

A total of 43 bacterial colonies from the axenic culture plates were screened for dye degradation ability on ZMA supplemented with 50 µg mL^{-1} of each dye (MG, CR, MO, MB, IC and RR) separately. The inoculated plates were incubated at 30º C for 24 to 36 h and observed the zones of degradation around the colonies (Fig. S1). The isolate ESPS40 showed a maximum degradation zone for MG and was selected for further experiment.

Identification of dye-decolorizing bacteria

The bacterial genomic DNA from the selected isolate (ESPS40) was extracted using MP-Biomedical Kit for DNA according to the manufacturer's protocol. The 16S rRNA gene was amplified through polymerase chain reaction (PCR) from genomic DNA using primer pair 27F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492R (5'-TAC GGY TAC CTT GTT ACG ACT-3') (Lane 1991). The PCR reaction mixture contained 0.5 µM each primer, 250 µM dNTPs, 1.25 U Taq polymerase, 2.5 µL 10X buffer supplemented with $MgCl_2$, 25 ng DNA template and the final volume was adjusted to 25 µL with DNase-free water (Kumar et al. 2022). The reaction conditions were initial denaturation at 95 ºC for 5 min, 35 cycles of 95 ºC 30 s, 56 ºC 30 s, 72 ºC 60 s and a final extension at 72 ºC for 7 min. The purified PCR product was sequenced (Macrogen Inc., South Korea).

Scanning electron microscope (SEM) analysis

Overnight grown bacterial culture of strain ESPS40 in ZMB was centrifuged (3000 rpm, 5 min) and the cell pellet was incubated in 0.1M phosphate buffer (pH 7.5) containing 3% glutaraldehyde (v/v) for 1 h. After incubation, pellet was washed thrice in phosphate buffer saline (PBS) and centrifuged (3000 rpm, 5 min). The cell pellet was dehydrated by washing with increasing concentrations of (30, 50, 70, 90 and 100%) ethanol for 5 min. A thin smear of the pellet was prepared on a coverslip using an inoculation loop. The coverslip was allowed to dry in a vacuum then coated with a gold stud and scanned using a field-emission scanning electron microscope (JSM-7100F, Jeol Ltd., USA).

Effects of salt concentration on dye degradation

The varying range of NaCl concentrations (0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0%) were optimized for MG (100 mg L^{-1}) degradation in Bushnell Haas (BH) broth media. The bacterial strain ESPS40 was

inoculated and incubated at 30 ºC under shaking conditions (150 rpm). At different time intervals (4, 8, 12, 16, 20, 28, 36, 48, and 60 h), 1 mL sample was withdrawn and centrifuged (8000 rpm for 10 min) to remove bacterial biomass and absorbance was measured at 620 nm. All essays were performed in triplicate. The decolorization percentage of dye was calculated by the following equation (Dhanve et al. 2008; Daneshvar et al. 2007; Parshetti et al. 2006):

$$\text{Decolorization } \% = \frac{\text{Initial absorbance(0h)} - \text{Final absorbance (h)}}{\text{Initial absorbance(0h)}} \times 100$$

Effect of dye concentrations on dye degradation

The effect of varying concentrations of MG dye (200, 400, 600, 800 and 1000 mg L^{-1}) on degradation by bacterial strain ESPS40 was studied in BH broth medium. The strain ESPS40 was inoculated in a BH broth medium containing varying dye concentrations and incubated under shaking conditions (150 rpm) at 30º C. At different time intervals (12, 24, 36, 48, 60 and 72 h), 1 mL sample was withdrawn and centrifuged (8000 rpm for 10 min) to remove bacterial biomass and absorbance was measured at 620 nm and decolorization percentage of dye was calculated as described in the previous section.

Validation of dye degradation using FTIR and HPLC

The bacterial isolate ESPS40 was inoculated in BH broth supplemented with 1% (v/v) NaCl and 100 mg L^{-1} MG dye and incubated at 30º C (shaking 150 rpm) for 36 h. The bacterial biomass was removed by centrifugation and the discolored supernatant was extracted with an equal volume of ethyl-acetate: methanol (1:5). The extracts were evaporated to dryness in a rotary evaporator and the dried product was dissolved in 400 µL of methanol. Further, the sample was filtered through a syringe filter (0.22 µ). The structural composition of the extracted product was analysed through Fourier transformation infrared (FTIR) (Spectrum EX, PerkinElmer, USA) and high-pressure liquid chromatography (HPLC) (Shimadzu, Japan) analysis.

For the FTIR analysis, samples were mixed with ultrapure potassium bromide (KBr) for pellet formation and the pellet was fixed on the sample holder for absorbance. The transmittance was recorded in the mid-IR range (400-4000 cm^{-1}). The degraded product was also analysed using HPLC by injecting 20 µL of filtered samples with a flow rate of 1 mL min^{-1} in a C18 reverse-phase column with methanol and water (9:1) as the mobile phase (HPLC). The samples (0 h) without bacterial treatment were also used as a control.

Enzyme assay

The enzyme activities were measured after 24 h of bacterial treatment in BH broth medium containing 100 - 1000 mg L^{-1} malachite green. After incubation, bacterial culture was centrifuged at 8,000 rpm for 10 min and supernatant was further used for the enzyme assays. Tyrosinase activity was determined in

0.01% catechol and 20 µL enzyme solution in 100 mM phosphate buffer (pH 7.4) at 410 nm (Du et al. 2011). Laccase activity was measured with 2,20 -azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS). The reaction mixture comprised of 5 mM ABTS, 100 mM acetate buffer (pH 4.5) and 20 µL enzyme then measured at 410 nm (Babu et al. 2015). Blank was prepared with sterilized Milli-Q instead of an enzyme sample. One unit of tyrosinase and laccase activities was measured as a U L^{-1} using the formula given below (Holme and Peck 1996):

$$\text{Enzyme activity (U } L^{-1}) = \frac{(\Delta A \times \text{Vt} \times \text{Df} \times 10^6)}{(t \times \varepsilon \times d \times \text{Vs})}$$

Where, *U* enzyme activity µmol min^{-1} L^{-1} , *ΔA* final absorbance − initial absorbance, *Vt* total reaction volume (mL), Df dilution factor, t time, ε molar extinction co-efficient (M^{-1} cm^{-1} or L mol^{-1} cm^{-1}), d optical path (cm), Vs sample volume (mL).

Statistical analysis

All the experiments performed in this study were in triplicate and the results were reported as mean ± standard deviation (SD).

Results And Discussion

Isolation and screening of Bacteria

The bacteria used in the present study were previously isolated on glass and plastic surfaces (early-biofilm forming) from three coastal sites (Alang, Diu and Sikka) (Kumar et al. 2022). The different isolates were selected based on colony morphology (43 isolates) for the screening of different dye degradations which showed varying clear zone formation after 24 to 36 h of incubation (Fig. S1). Based on dye degradation efficiency, the bacterial strain which showed maximum clear zone formation in plate assay was selected. Isolate ESPS40 was chosen for further study because it showed dye degradation for all the six dyes used (Fig. S1). We also observed that the ESPS40 strain degrades dyes with a maximum clearance zone (12 mm) with MG (Fig. S2). Many dye degrading bacteria have been isolated from the Arabian sea (Kabeer et al. 2019).

Molecular identification of dye degrading bacteria

16S rRNA gene sequencing is an effective method for bacterial identification and phylogenetic analysis (Patel 2001). The comparison of the 16S rRNA gene sequence of bacterial strain ESPS40 with available sequences in the EZBioCloud database (https://www.ezbiocloud.net) showed the highest similarity with the genus *Pseudomonas* (Fig. 1). A phylogenetic tree based on known representatives of *Pseudomonas* species is presented in Figure 1. The highest homology (98.5%) for ESPS40 was with *Pseudomonas khazarica* TBZ2, a marine bacteria known to degrade polycyclic aromatic hydrocarbon compounds (Tarhriz et al. 2020). Based on phylogenetic affiliation to the closest match, the strain ESPS40 was

named *Pseudomonas* sp. ESPS40. The appearance and colony morphology varies from species to species and the colony features serve as an important indicator for bacterial identification (Engelkirk and Duben-Engelkirk, 2008). The strain ESPS40 was found to have an unusual rod-shaped image under scanning electron microscopy (Fig. S3).

Effects of salt concentration on dye degradation

The effect of varying salt concentrations on the MG dye decolorization by bacterial strain ESPS40 was observed (Fig. 2). The dye decolorization percentage ranged from 2.4 (4% NaCl) to 36.1% (0% NaCl) and 38.87 (4% NaCl) to 87.76% (1% NaCl) in 4 and 60 h, respectively. The decolorization of MG was observed to be maximum at 0% NaCl upto 12h but in a later period, it was maximum at 1% NaCl. However, the higher concentration of NaCl substantially inhibited the decolorization activity which may be because of the negative effect of high salt concentrations on strain ESPS40. Shang et al. (2019) also observed reduced decolorization of MG at higher NaCl concentrations by marine bacteria (*Klebsiella aerogenes* S27).

Effects of dye concentrations

The decolorization of dye by strain ESPS40 with varying concentrations of MG (200 mg L^{-1} to1000 mg L^{-1}) was studied at every 12 h of incubation. The decolorization of MG ranged from 17% (1000 mg L^{-1}) to 85% (200 mg L^{-1}) in 72 h of incubation (Fig. 3). The result indicated that the strain ESPS40 could efficiently degrade MG up to 67% and 54% at concentrations of 600 mg L^{-1} and 800 mg L^{-1}, respectively in 72 h. A high concentration of MG (>800 mg L^{-1}) significantly inhibited the decolorization activity. Previous studies showed the bacterial decolorization of MG with varying dye concentrations such as 2.5 mg L^{-1} using *Sphingomonas paucimobilis* (Cheriaa and Bakhrouf 2009), 15 mg L^{-1} using *Enterobacter* sp. (Roy et al. 2020), 20 mg L^{-1} using *Tenacibaculum* sp. (Qu et al. 2018), 50 mg L^{-1} using *Kocuria rosea* (Parshetti et al. 2006), 100 mg L^{-1} using *Brevibacillus laterosporus* (Gomare et al. 2009), 200 mg L^{-1} using *Pseudomonas putida* (Sneha et al. 2014) and 400 mg L^{-1} using *Bacillus vietnamensis* (Kabeer et al. 2019). In the present study, it was observed that the degradation of MG decreased after increasing the dye concentration (>800 mg L^{-1}). Several studies observed similar results where the degradation efficiency decreased with increased concentrations of the dye (Wanyonyi et al. 2017; Song et al. 2020).

Fourier transform infrared spectroscopic analysis

The structural changes of malachite green resulting from bacterial degradation were analyzed using the FTIR spectrum. Considerable variations were observed in the peaks before and after decolorization by bacteria. In non-treated MG FTIR spectra, specific peaks in the range 500-1500 cm^{-1} was observed (mono and para-di substituted benzene rings). The similar pattern have been reported in previous studies (Saravanakumar and Kathiresan 2014; Cheriaa et al. 2012). MG treated with strain ESPS40 showed narrowing of peak at 3366 cm^{-1} and 1651 cm^{-1} refer to O-H, C=C and C-O-C stretching vibrations, respectively (Huang et al. 2014) (Fig. 4). Peaks in the region 2831 cm^{-1} and 2946 cm^{-1} showed C-H

stretching vibrations of aldehyde and alkane, respectively. Slightly smallyer peaks at 2038 cm^{-1} and 2353 cm^{-1} refer to C=C=N and O=C=O stretching, respectively. The absorbance in the region of 1415 to 1455 cm^{-1} in the treated sample was due to C-C stretching vibrations which denotes presence of aromatic rings (Theivandran et al. 2015). A strong peak appearance at 1030 cm^{-1} in bacterial treated dye shows C-N stretch stretching vibration and presence of aliphatic amines (Theivandran et al. 2015). Malachite Green degradation was observed in the solution as evident by the addition of some new peaks and the absence of important peaks. These features were found to be indicative of various types of oxidation events that occurred during the biodegradation due to bacterial activity.

High-performance liquid chromatography analysis

The HPLC of standard malachite green showed peaks at the retention time of 4.22 min and 5.13 min while the treated sample showed peaks at the retention time of 3.97 min and 4.60 min. The decreased concentration of malachite green was confirmed by the peak area. In treated samples, small peaks were also observed which indicated the formation of fragmented products as a result of MG biodegradation by ESPS40 (Fig. 5). New peaks or absence of peaks are generally associated with by-products produced by growing cells after incubation (Dhanve et al. 2009).

Enzyme assay

The degradation of dyes involves various enzymes secreted by bacteria during the biodegradation process. To evaluate the enzymes involved in MG biodegradation, the presence of tyrosinase and laccase enzymes was assayed after 24 h degradation. The activities of enzyme tyrosinase ranged between 63.48 U L^{-1} (at 100 mg L^{-1} MG) to 526.52 U L^{-1} (at 1000 mg L^{-1} MG) and laccase ranged from 3.62 U L^{-1} (at 100 mg L^{-1} MG) to 28.20 U L^{-1} (at 1000 mg L^{-1} MG) (Fig. 6). The result indicated that enzyme activities increased with increasing MG concentrations. Higher MG concentration requires more enzymes for degradation and increased the enzyme activities. In the previous study, laccase purified from *Pseudomonas putida* LUA15.1 showed MG decolorization (Verma et al. 2017). Tyrosinase, MG reductase, lignin peroxidase (LiP), laccase and NADH-DCIP reductase were previously reported to degrade MG by *Aeromonas* sp. (Du et al. 2018). Laccase and NADH-DCIP reductase were also known to degrade MG by *Micrococcus* sp. (Du et al. 2013). The result from the enzyme assay revealed that tyrosinase and laccase took part in MG biodegradation.

Conclusions

The present study showed that *Pseudomonas* sp. ESPS40 can efficiently degrade MG upto 600 mg L^{-1}, but can survive at higher dye concentrations (upto 800 mg L^{-1}). Since, the bacteria used in the present study is originated from marine resources which can withstand a variable range of salinity. The strain ESPS40 showed efficient degradation of MG upto 3.5% NaCl concentration. A higher degradation percentage was observed with 1% NaCl concentration in BH broth media. The FTIR and HPLC data showed the degradation of MG by bacteria. The involvement of enzymes like tyrosinase and laccase

justifies the biodegradation potential of strain ESPS40. Thus, the present study suggests that *Pseudomonas* sp. ESPS40 can be considered a potential candidate for MG degradation and can be used for bioremediation and detoxification of MG in the wastewater. However, further studies need to be conducted before using the strain ESPS40 at the industrial scales.

Declarations

Declaration of conflict of interest

The authors declare no conflict of interest (financial or non-financial).

This MS not included any published work.

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Data Availability Statements

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

Author's contributions

AK, BPVand MK conducted the experiment and analytical work; AK and MK prepared original draft of the manuscript; conceptualization, experiment design, supervised the project and final manuscript preparation by DRC; All authors reviewed the manuscript.

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Figures

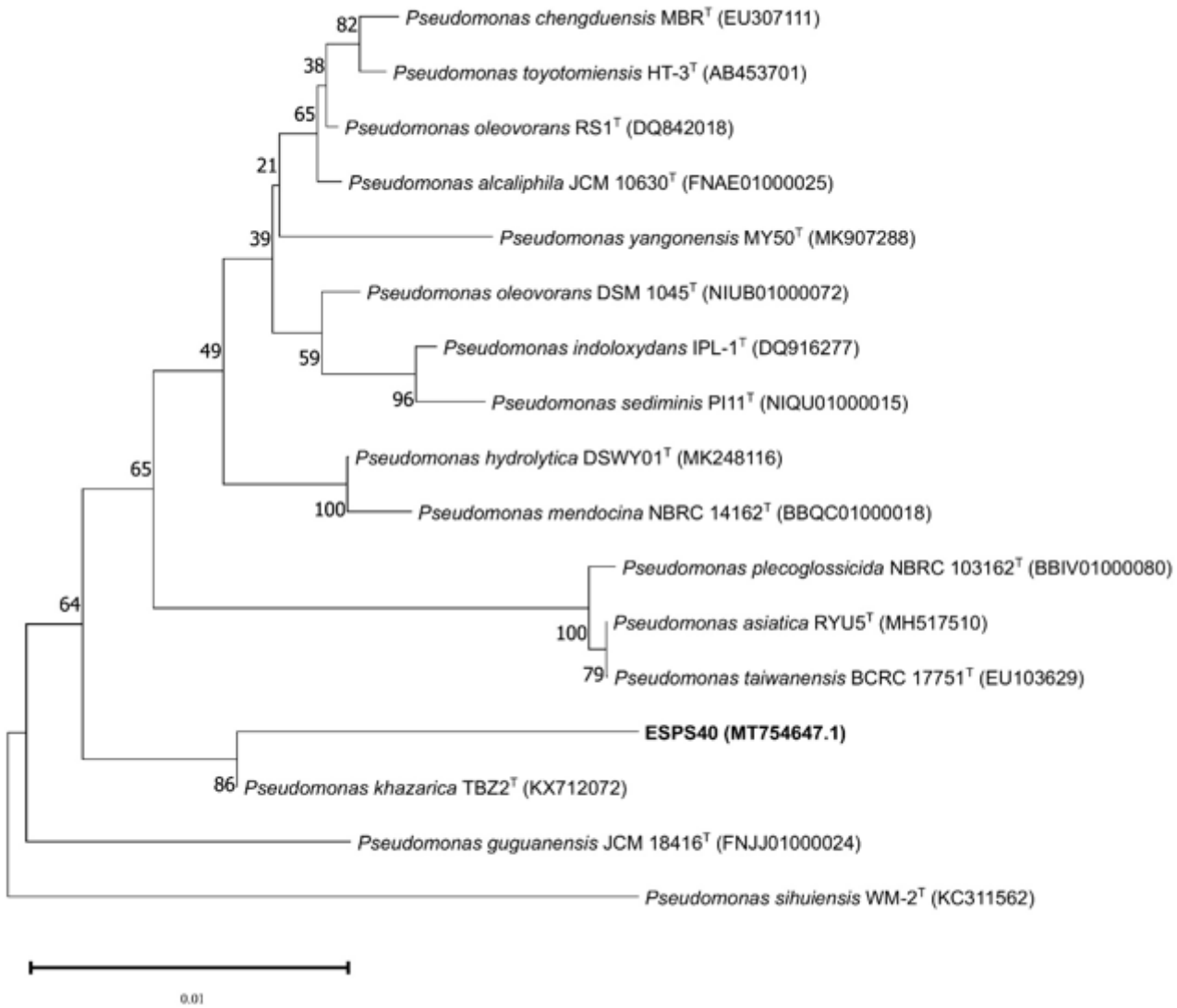

Figure 1

Neighbour joining phylogenetic tree based on a comparison of the 16S rRNA gene sequences of ESPS40 and its closest relatives. The numbers at the nodes indicate the percentages of bootstrap sampling

derived from 1000 replications. GenBank accession numbers are given in parentheses. Bar, 0.01 nucleotide substitution per nucleotide position.

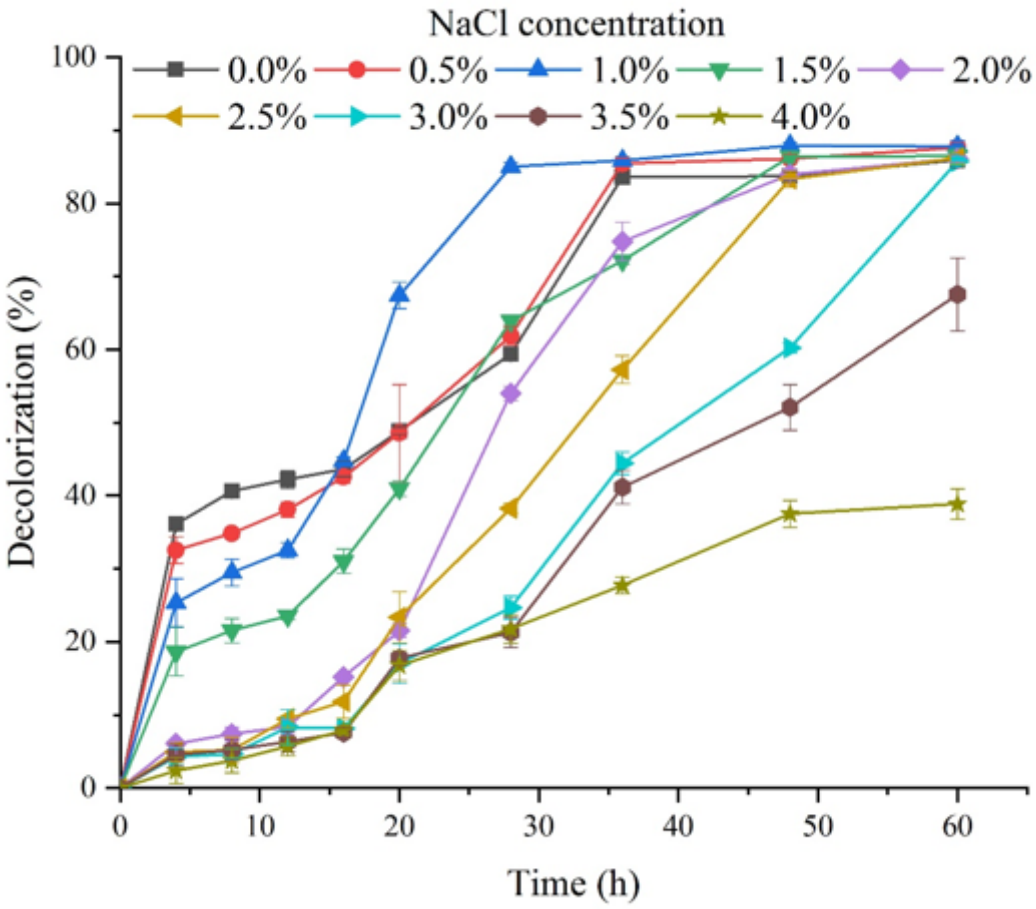

Figure 2

The degradation efficiency of malachite green (100 mg L^{-1}) under different NaCl concentrations (%). The error bars indicate standard deviations (n=3).

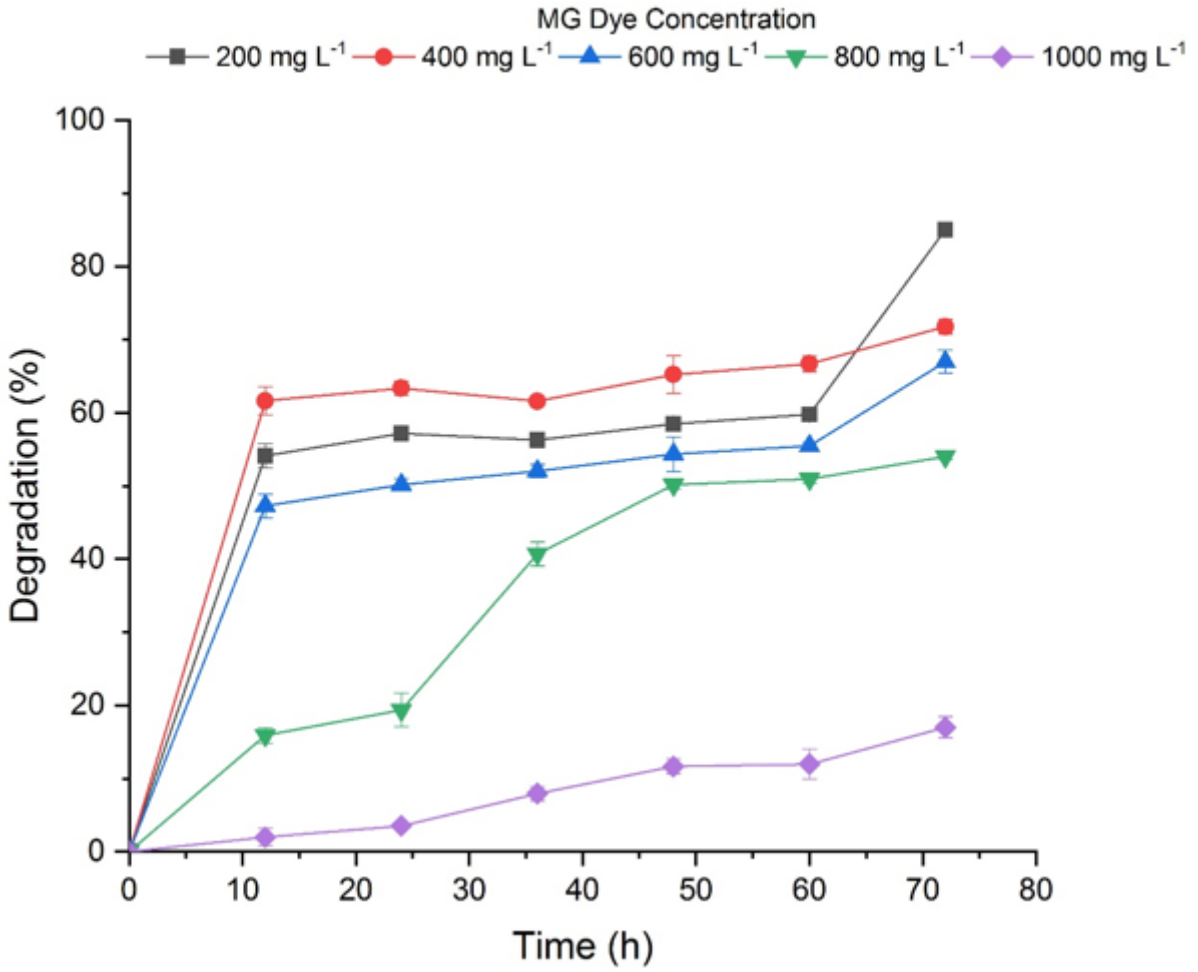

Figure 3

The degradation efficiency of malachite green under different dye concentrations (mg L^{-1}). The error bars indicate standard deviations (n=3).

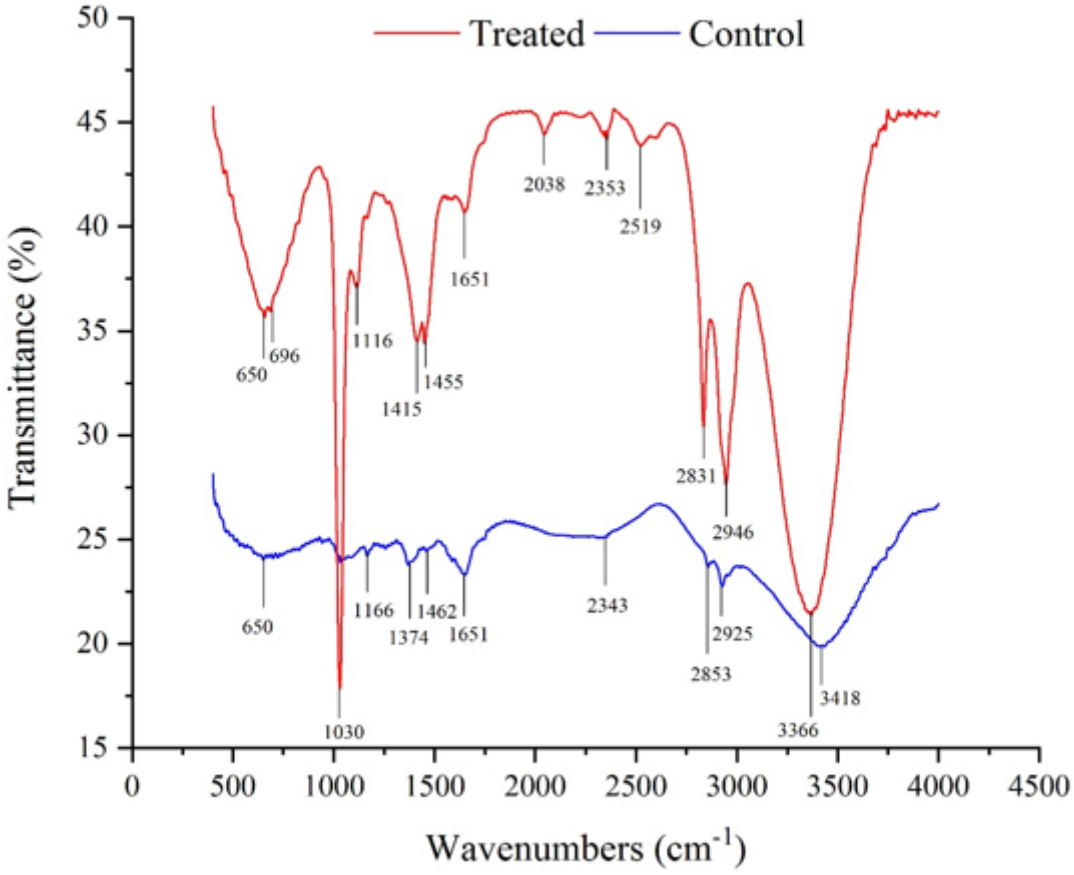

Figure 4

FT-IR spectra of malachite green (A) Control (0h) and (B) malachite green treated with bacterial isolate ESPS40 (36h).

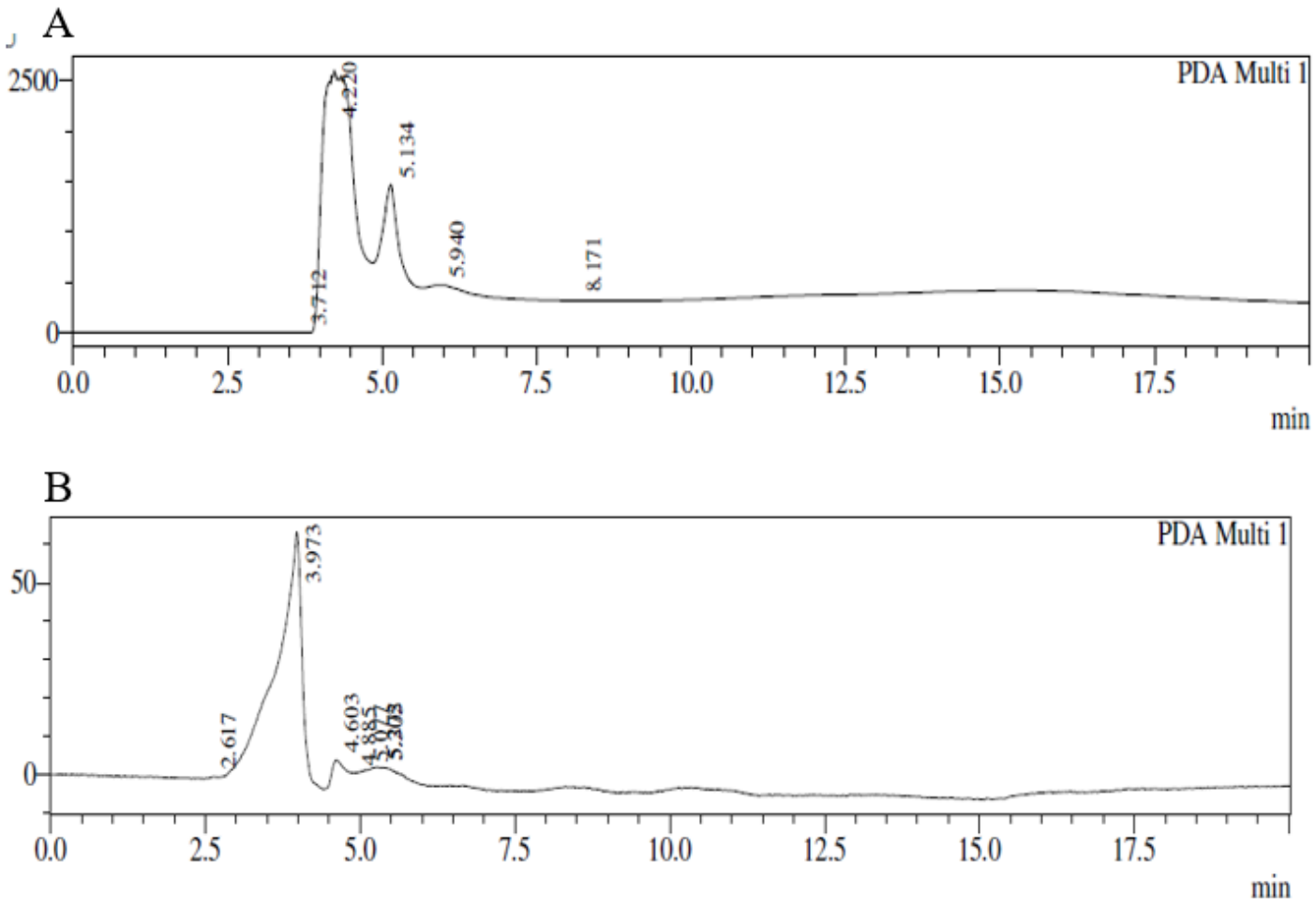

Figure 5

HPLC chromatogram of malachite green (A) Control (0h) and (B) malachite green treated with bacterial isolate ESPS40 (24h).

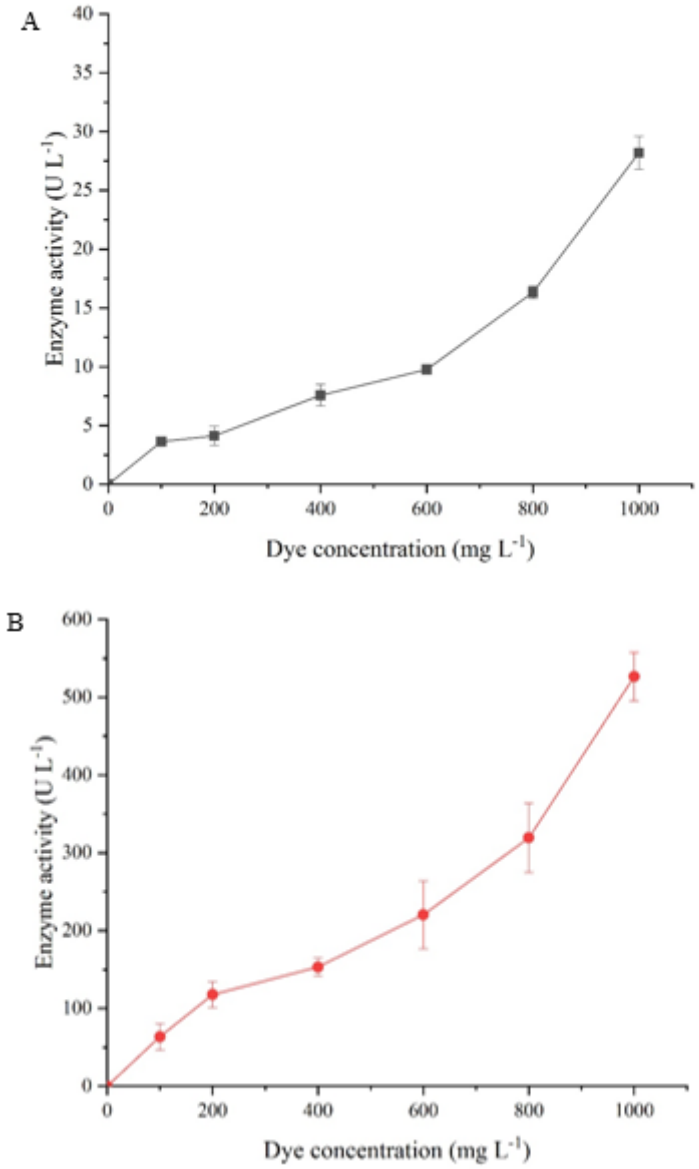

Figure 6

Enzymatic activity with different concentrations of malachite green (treated with isolate ESPS40). The error bars indicate standard deviations (n=3).

Supplementary Files

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- GA.png
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