Degradation mechanism and toxicity reduction of methyl orange dye by a newly isolated bacterium *Pseudomonas aeruginosa* MZ520730

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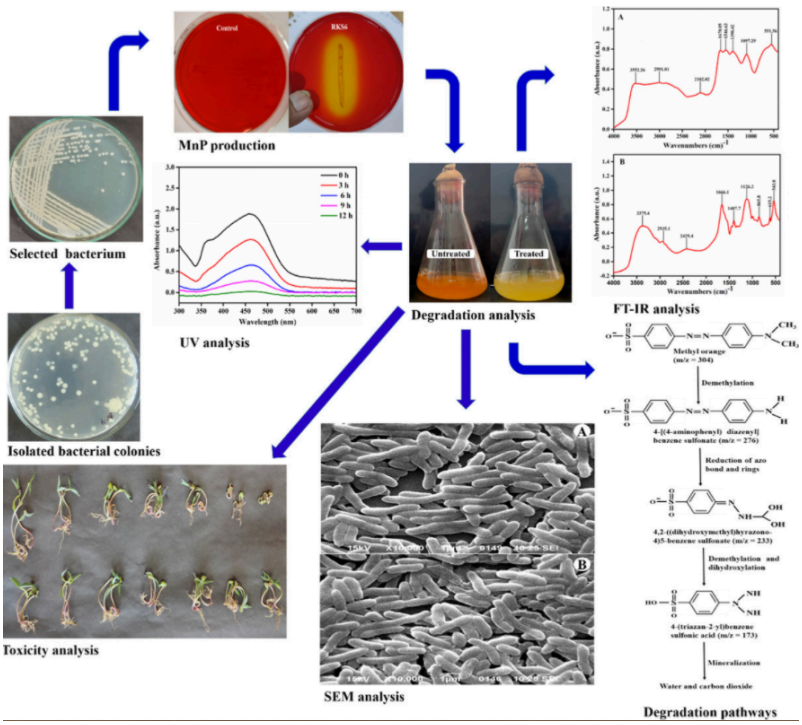
Highlights:

- MO dye is used in textile, tannery, paper, printing, food and medicine industries.
- MO dye toxic to living organisms.
- *Pseudomonas aeruginosa* showed 99% removal of MO dye with 96% reduction of TOC.
- MO dye degradation was confirmed by FT-IR and LC-MS analysis.
- Bacteria treated MO dye was recorded less toxic as compared to untreated dye.

Graphical abstract



Abstract

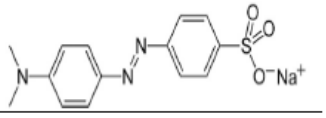
Methyl orange (MO) dye is recalcitrant in nature, hard to degrade and if released into the soil and aquatic resources could cause serious threats on environment and human health. MO is toxic to plant growth. Bacterial treatment may be a sustainable solution for its degradation and decolourization. In this work, a bacterium (RKS6) was isolated from textile industry wastewater and sludge samples and identified as *Pseudomonas aeruginosa* based on the 16S rRNA gene sequencing analysis. RKS6 showed more than 99% decolorization of MO dye (100 mg/l) and 96% reduction of total organic carbon (TOC) within 12 h, at 30 °C, pH 7 at static conditions. RKS6 also produced MnP enzyme of molecular weight ~53 kDa as characterized by the SDS-PAGE analysis. Further, LC-MS analysis showed that MO dye was degraded into 4-[(4-aminophenyl) diazenyl] benzene sulfonate, 4, 2-((dihydroxymethyl) hyrazono-4) 5-benzene sulfonate, 4-(triazan-2-yl) benzene sulfonic, water and carbon dioxide by RKS6. Toxicity assessment showed that the solution treated by the bacterium allowed 90% seed germination indicating that RKS6 was effective in mineralization and detoxification of MO dye and can be effectively used in industrial wastewater treatment.

1. Introduction

Azo dyes are widely used in dyeing, textile, paint, leather, pulp paper, medicine and cosmetic industries [1,2]. Azo dyes have high fastness, low cost, easy to use, and high stability to light, temperature, detergents, chemicals and microbial degradation in comparison to natural dyes [1–3]. Globally, more than thousand tons of different dyes are used in textile industries (TIs) [4,5] and ~28,000 tons of unfixed dyes are released into the environment annually [5,6]. Azo dyes are the largest and most versatile group of synthetic dyes, which consists one/ many azo (-N=N-) bonds and sulfonic (SO^{3-}) bonds, attached to different functional groups such as amino, hydroxyl, methyl, nitro, carboxyl and sulfoxyl [5,7]. Approximately 70% of azo dyes are used in TIs worldwide [5]. Methyl orange (MO) is a synthetic, organic, heterocyclic, sulfonated and high water-soluble anionic dye [3,5,7]. MO dye is widely used in dyeing, leather, textile and pulp paper industries [5,7,8]. It's also used as a pH indicator in many research laboratories [7].

If MO dye was discharged without adequate treatment into rivers, wetlands and agriculture fields, it could cause serious effects in the biota and water/soil ecologies [3,7]. For example, even at very low concentration in water OM causes detrimental effects on water quality due to coloration of water that reduces sunlight penetration, decreases photosynthetic activity, dissolved oxygen content, gas solubility and thus increased BOD, COD, TOC and TDS level [3,5,7]. MO is a recalcitrant dye and causes hypersensitivity, allergies, dermatitis and intestinal cancer in living beings [3,5]. MO dye is well reported to causes high toxicity, carcinogenic, mutagenic and teratogenic effects in humans and animals [3,7,8]. It also reduces soil fertility, crop yield and biodiversity by increasing salinity in soil [5,7]. Therefore, the MO dye should be adequately treated before its final release into the environment.

Table 1. The chemical properties of methyl orange dye.

Name	Synonyms	Chemical class	Consistence	Molecular weight	λmax	Chemical formula	Chemical structure
Methyl Orange	Sodium 4-[(4dimethylamino) phenylazo]benzenesulfonate	Azo dye	Orange powder	327.33 g/ mol	464 nm	$C_{14}H_{14}N_3NaO_3S$	

Many treatment approaches like physico-chemical, advanced oxidation processes (AOPs) and biological methods have been reported for the decolorization of MO dye [5,9–11]. The physico-chemical methods (adsorption and coagulation/flocculation, sedimentation and coagulation) are efficient only for decolorization, but not for the degradation of pollutants [3,5,9,10]. AOPs (including photocatalytic, photochemical and electrochemical oxidation methods) are fast, effective and efficient, but these methods are high in cost, use complicated methods, and may result in incomplete degradation that generate toxic sludge generation as secondary pollutants [9–11].

In contrast, biological treatment is a cost effective and eco-friendly method use metabolic abilities of bacteria, fungi, archaea, algae, yeast and plants to degrade pollutants [3,5,8]. Out of these, bacteria have a high efficiency to decolorize and mineralize a variety of textile pollutants [12]. Also, bacteria are easy to culture, fast-growing, can grow on different substances and adapt to different environmental conditions in comparison to other microbes [2,5]. Bacteria can degrade or mineralize organic wastewater pollutants into H_2O and CO_2 [5,8,11]. Several bacterial strains *Bacillus* sp., *Thermus* sp., *Aeromonas* sp., *Pseudomonas* sp., *Serratia* sp., and *Shewanella* sp. are well reported for the effective degradation of textile wastewater pollutants [4,5,7,9,13,14]. These bacteria possess different degradative enzymes that perform major role in decolorization and mineralization of pollutants [5,9]. Many studies are reported on the decolorization and degradation of MO dye by bacteria [5,16]. For example, Navas et al. [13], reported (33%) decolorization of MO dye by *Thermus* sp. 2.9 within 24 h. *Bacillus* sp. strain (AK1) was decolorized (93%) of MO dye within 18 h [14]. Ayed et al. [15], also recorded 92% removal of MO dye by bacteria within 48 h. But, the isolated bacterium strain (RKS6) showed more effective decolorization (99%) and mineralization (96%) of MO dye within short time in comparison to related published works [13–15].

Thus, MO dye was selected as a model compound for this study due to its wide applicability in different industries and potential in causing serious effects in environment and living beings. A potential ligninolytic (MnP) enzyme producing bacterium was isolated from TIWW and sludge sample and used in degradation and mineralization of MO dye at different

optimized conditions (pH, temperature, dye concentration and static and shaking). The MnP enzyme and degradation of MO dye was confirmed by SDS-PAGE and UV–vis spectroscopy, FT-IR and LC-MS analysis, respectively. The degradation mechanism of MO dye was also discussed. Further, the untreated and treated MO dye was evaluated for its toxicity by a seed germination test using *Phaseolus mungo* L. seeds.

2. Materials and methods

2.1. Chemicals, textile industry wastewater and sludge collection

MO dye ($C_{14}H_{14}N_3NaO_3S$) was received from Sigma-Aldrich, USA. The potassium bromide, ethyl acetate, methanol, phenol red, mercuric chloride and sodium sulfate were received from SR, Laboratories, India. The culture media (starch, sodium nitrate, urea, maltose, glucose, lactose, peptone, yeast extract, sucrose, agar and peptone) were obtained from Himedia, India. The seeds of *Phaseolus mungo* L. (mung beans) were obtained from Lucknow (local market), U.P., India. All the chemical reagents and solvents used in this work were of the high purify and analytical grade.

Further, TIWW and sludge samples were collected from the Handloom Textile Company, Panki, Kanpur, U.P., India. Kanpur city is one of the largest hubs of TIs, which is located at (80.3500° E, 26.4670° N). Both the samples were kept at 4 °C. The TIWW and sludge was utilized for the isolation of bacteria capable to degrade MO dye.

2.2. Preparation of culture media and methyl orange dye solution

The Minimal Salt Medium (MSM) used in this work was contained (g l^{-1}): Na_2HPO_4 2.4, K_2HPO_4 2, peptone 5, glucose 10, NH_4NO_3 0.1, $CaCl_2$ 0.01, $MgSO_4$ 0.01, and agar 15. All these chemicals were dissolved in distilled water, pH maintain at 7.0 and sterilized at 121 °C for 20 min in autoclave (Labard Instruchuem Pvt. Ltd., India).

To prepare MO dye solution, MO dye powder was dissolved in distilled water at 100–400 mg/l concentration. The sample was sterilized with membrane filter (0.22 μm) (Millipore Ltd.,

Bedford., MA), kept at 4 °C in refrigerator and used in degradation and toxicity analysis. The chemical properties of methyl orange dye are given in Table 1.

2.3. Isolation and screening of methyl orange dye decolorizing bacteria

The MO dye degrading bacterial strains were isolated from the collected TIWW and sludge samples following the methodology of Kishor et al. [12]. Briefly, 8.0 ml of TIWW and 2.0 g of sludge was transferred in an Erlenmeyer flask having 90 ml (v/v) of sterilized MSM broth. To this flask, 150 mg/l of MO dye solution was added and incubated in a BOD incubator shaker (LI-BODS-10, Labard., India) at 30 °C and 100 rpm. After 1 week, 1.0 ml (v/v) aliquot was serially diluted, 25 μl of diluted suspension was spreaded on 100 mg/l of MO dye containing MSM agar plates and incubated in a BOD incubator at 30 °C for 24 h. Afterward, the different morphologically distinct bacterial colonies appeared on plates were selected, picked up and purified by repeating streak plate method.

The isolated bacterial strains were screened based on their MnP enzyme activity and MO dye decolorization efficiency. For MnP enzyme activity, phenol red dye was used as indicator of MnP enzyme activity [16]. Briefly, a loopful culture of all isolated bacterial strains were streaked on MSM agar plates amended with 100 mg/l of phenol red and placed under BOD incubator at 30 °C. After two days, only four bacterial isolates grown and developed a clear decolorization zone around their colonies indicating the MnP enzyme activity.

The isolated bacterial strains that showed MnP enzyme activity were further screened based on their MO dye decolorization efficiency. Briefly, a loopful culture of all isolates were inoculated separately in MSM broth (10 ml) and transferred at 30 °C in a BOD incubator. After 24 h, 1.0 ml (v/v) of culture was inoculated in 100 mg/l of MO dye containing MSM broth (pH 7.0) and incubated at 30 °C. After different time intervals, the sample was taken and centrifugation at 10,000 ×*g*, at 4 °C for 10 min in a refrigerated centrifuge (REMI Instruments Pvt. Ltd., India) to separate the biomass and decolorization was measured at λ_{max} = 464 nm by UV-VIS spectrophotometer (Labtronics, Pvt. Ltd., India). The MSM broth amended with

MO dye without bacterial culture was used as control. The % decolorization was determined following the formula as below:

$$Decolorization\ (\%) = \frac{(R_o - R_t)}{R_o} \times 100$$

where R_o is the initial absorbance and R_t is the final absorbance and the bacteria showing maximum decolorization of MO dye were selected for further study.

2.4. Characterization of methyl orange decolorizing bacteria

2.4.1. Morphological and biochemical characterization

The selected bacterium capable for the degradation of MO dye was morphologically and biochemically characterized according to Whitman et al. [17].

2.4.2. Molecular identification

The selected bacterium was identified based on the 16S rRNA gene sequencing analysis. The genomic DNA was prepared form the overnight grown cultures according to method described by [12]. Approx. 5 μL of template DNA was taken for the amplification of 16S rRNA gene and universal primers 27F (5′-AGAGTTTGATCCTGGCTCACG-3′) and 1492R (5′-TACGGTTACCTTGTTACGACTT-3′). 100 ng of DNA template, lX of PCR buffer, 3.0 mM of $MgCl_2$, 200 μM of dNTP each, 25 pmol of primer and 2.5 units of DNA polymerase template was added in reaction sample followed by denaturation, annealing and extension at 94 °C for 1 min, 45 °C and 72 °C (30 cycles), respectively. A gel extraction kit (Merk Biosciences, Bangalore., India) was used to purify the PCR amplified product, which was further sequenced by Biokart India Pvt., Ltd., (India). The 16S rRNA sequences obtained were also submitted in Gene Bank database under accession number MZ520730 and the phylogenetic tree was made by MEGA software version 5.0.

2.5. Effects of different environmental parameter on degradation of methyl orange dye

Different environmental conditions i.e., dye concentration, temperature, pH and shaking rate were optimized because these parameters highly influence the microbial degradation

efficiency [12]. Briefly, a loopful culture of selected bacterium was transferred in sterile MSM broth (10 ml) and incubated in BOD incubator shaker at 30 °C and 100 rpm. After 24 h, 1% (v/v) of culture was inoculated in 50 ml (v/v) sterile MSM broth containing 100 mg/l of MO dye and incubated in a BOD incubator by varying various parameters as pH between 4 and 10, temperature 15–45 °C, dye concentration 50–300 mg/l and static and shaking flask (100 rpm) conditions. The NaOH (1 M) and HCl (1 M) was used to maintain the pH of medium [16]. After 3, 6, 9 and 12 h, 4 ml aliquots were collected and centrifuged at 8000 ×*g* for 15 min at 4 °C to separate biomass. The decolorization was measured at 464 nm by spectrophotometer (Labtronics, LT-2201, India). All tests were carried out in three sets and a flask without bacterial culture was used as control. The % decolorization was measured as per the formula as below:

$$Decolorization\ (\%) = \frac{(R_o - R_t)}{R_o} \times 100$$

where R_o is the initial absorbance and R_t is the final absorbance of MO dye.

2.6. Decolorization and TOC removal of methyl orange dye by isolated bacterium at optimized conditions

The MO dye decolorization and TOC removal was carried out under the optimized conditions. Briefly, a loopful culture of selected bacterium was inoculated 10 ml sterilized MSM broth followed by incubation in an incubator BOD shaker at 30 °C for 24 h. Afterward, 1% of overnight grown culture was inoculated in MSM broth amended with 100 mg/l of MO dye followed by incubation in a BOD incubator at optimized conditions. After 3, 6, 9 and 12 h, a sample of 4 ml was collected and centrifugation at 8000 ×*g* for 15 min at 4 °C to separate biomass. The supernatant obtained was used to monitor the decolorization at 464 nm and the % decolorization was calculated as discussed above.

In addition, the total organic carbon (TOC) in untreated and bacterium treated MO dye samples was measured by Liqui TOC l1 apparatus with an IR detector (Elementar Analysensysteme GmbH Company, Germany) [1]. The conditions for TOC removal were set

as: flow rate 200 ml min^{-1}; temperature 850 °C, irradiation time 1.5–2.5 min and oxygen used as carrier gas (0.95–1.00 bar). The % TOC removal was calculated by following equation:

$$TOC\ removal\ (\%) = \frac{(A_o - A_t)}{A_o} \times 100$$

where A_o is the initial TOC and A_t is the final TOC value.

2.7. MnP activity

Phenol red was used as an indicator for MnP activity [16]. During the decolorization study, 4 ml (v/v) of aliquot was taken from flasks at different time and centrifugation at 8000 ×*g* for 15 min at 4 °C to separate biomass and the supernatant obtained was directly employed to measure MnP enzyme activity.

The MnP enzyme activity was measured as per Kishor et al. [12]. Briefly, 1.0 ml phenol red solution, 1.0 ml phosphate buffer (pH 7.0), 0.5 ml $MnSO_4$ and 1.0 ml supernatant were added to reaction sample. The sample was collected at every 4 min and activity was recorded at 610 nm. One unit of enzyme activity is defined as the amount of active enzyme needed to oxidize 1.0 μmol substrate per min.

2.8. MnP purification and characterization

For purification of MnP enzyme, four steps like fractionation with ammonium sulfate, dialysis, ion exchange with DEAE-cellulose and G- 100 Sephadex gel permeation chromatography were followed [18]. Briefly, the isolated bacterium was inoculated in 100 mg/l of MO dye containing MSM broth and placed at 30 °C in BOD incubator shaker. After 24 h, aliquot (10 ml) was taken and centrifugation at 3500 ×*g* for 10 min at 4 °C to separate biomass. The supernatant obtained was saturated by using 35% ammonium sulfate at 4 °C for 24 h. The precipitate obtained was centrifuged at 8000 ×*g* for 10 min at 4 °C and the supernatant was further saturated with 65% ammonium sulfate for 24 h at 4 °C. The sample was taken, centrifuged and pellet was dissolved in sodium malonate buffer (50 mM, pH 4.5) and dialyzed with the same buffer using 12 kDa cut-off membrane. The dialysate was loaded on ion-

exchange chromatography using diethyl aminoethyl (DEAE) cellulose column. The column was washed with phosphate buffer (pH 6.5) to remove the unbounded proteins and then eluted with 0–1.0 M of NaCl linear gradient. A total of 60 cycles were done and 1.5 ml sample was obtained and used for the measurement of protein contents. The protein content was concentrated and loaded on (10 × 300 mm) Sephadex-G- 100 column. The elute was added in malonate buffer (50 mM), flow rate was set at 0.3 ml/min and absorbance was measured at 280 nm and SDS-PAGE was done in a Mini Gel Electrophoresis system. The molecular weight of protein bands was determined based on the standard protein marker (10–175 kDa) (Biokart India Pvt. Ltd., India).

2.9. Scanning electron microscopic (SEM) analysis

SEM analysis of the isolated bacterium grown in MSM broth (with and without MO dye) was done by a field emission SEM (JEOL, JSM- 6490LV). Briefly, a loopful bacterial culture was inoculated in a sterile 10 ml MSM broth and incubated in BOD incubator shaker for 24 h. Afterward, 1% of grown culture was inoculated in 100 mg/l of MO dye containing MSM broth and placed at 30 °C in a BOD incubator. After 12 h, 4 ml aliquot was collected and centrifuged and biomass was washed with phosphate buffer (0.1 M, pH 7.0) and cells were pre-fixed with glutaraldehyde 3% (v/v) and left for 6 h at 4 °C.

The pre-fixed cells were washed with phosphate buffer three times for 10 min at 4 °C and post fixed with 1% (v/v) osmium tetraoxide for 1–2 h at 4 °C. The post-fixed samples were further washed twice with phosphate buffer to remove the unreactive fixative. The samples were dehydrated with different concentrations of acetone (15, 20, 30, 40, 60, 80, 90 and 100% (v/v)) [19]. The samples were dried by using a critical point dryer and gold-coated with platinum using ion sputter coater (JEOL, Japan JRC 1600 Auto Fine Coater).

2.10. Decolorization and degradation analysis of MO dye

2.10.1. UV–visible spectroscopy analysis

The decolorization of MO dye by isolated bacterium was measured by spectrophotometer (Labtronics, LT-2201, India). During decolorization study, 4 ml (v/v) aliquot was collected from

flasks at different time intervals and centrifugation at 8000 ×*g* for 15 min at 4 °C to remove the cell biomass. The obtained supernatant was used to monitor the decolorization in the wavelength ranging from 300 to 700 nm.

2.10.2. FT-IR analysis

Different functional groups present in MO dye and its metabolites were identified by FT-IR analysis. After complete decolorization, 20 ml aliquot was collected from flasks, centrifuged and supernatant obtained was oven-dried at 50 °C for overnight. Afterward, the sample was mixed with potassium bromide (400 mg) in a ratio of 5:95 (w/w). Now, samples were ground and fused into a thin pellet prepared under vacuum condition by a PCI hydraulic press (15 tons) and fixed in sample holder. The FT-IR analysis was done by using Nicolet™ 6700 Thermo Scientific, USA spectrometer from 400 to 4000 cm^{-1} with a 16 scan speed.

2.10.3. LC-MS analysis

The LC-MS analysis was used to identify the metabolites of MO dye (AB, Sciex., USA). After complete decolorization, 50 ml aliquot was collected, centrifuged and metabolites were extract by the equal volume of dichloromethane. The extract was filtered, concentrated by passing from Na_2SO_4, dried at 40 °C in a hot air oven. The sample obtained was dissolved in methanol and filtered by a membrane filter (0.22 μm). Now, 10 μl sample was injected into C18 column (100 nm × 2.1 nm, 1.7 μm) and system conditions were set as: mobile phase A - 10 mmol ammonium acetate, mobile phase B - acetonitrile and column temperature at 30 °C. The chromatography separation was carried out by gradient elution.

The ESI-MS (Electrospray Ionisation Mass Spectrometry) was done for the detection of negative and positive ions. Helium was employed as a collision gas with 10 ml/min of flow rate. The system conditions were set as: drying temperature at 320 °C, capillary temperature at 300 °C, nebulizer pressure 35 psi, injection voltage 4500 V, capillary voltage 4000 V, scanning range 50–1500 *m*/*z*, scanning time 1 s, flow rate of dryer 9.51/min and multiplier voltage 1100 V.

2.11. Toxicity evaluation of untreated and bacteria treated methyl orange dye

The toxicity of untreated (UT) and bacteria treated (BT) MO dye was tested by seed germination experiment. The UT and BT MO dye samples were added to distilled water at a concentration of 100–400 mg/l. Both samples were used to prepare different concentration such as 15, 20, 30, 50, 75 and 100% (v/v) solutions. Ten healthy seeds were taken and sterilized with the mercuric chloride solution (2.0%) for 2 min [12]. The seeds were placed in between filter paper (Whatman No. 1) in Petri- plates and irrigated with 4 ml of tap water in control, UT and BT MO dye solution. The Petri-plates were placed in BOD incubator shaker, seed germination was recorded after two days while root length, shoot length and biomass was measured within one week. The toxicity tests were carried out in three sets at 28 °C and % germination was recorded as per the below equation:

$$Germination\ (\%) = \frac{No.of\ seed\ germinated}{No.of\ seeds\ sowed} \times 100$$

2.12. Statistical analysis

All the experiments were conducted in three sets. The data collected from each set of experiment was expressed as means and ± standard deviation.

3. Results and discussion

3.1. Characteristics of isolated bacterium

In present work, a total of eleven (11) morphologically distinct bacterial strains (RKS1-RKS11) were isolated, purified and screened for the MnP enzyme activity and MO dye decolorization efficiency. Out of eleven (11) bacterial strains, only four bacterial strains i.e. RKS1, RKS6, RKS8 and RKS11 produced a clear decolorization zone (yellow) around the colonies on dye amended plates within two days (Fig.1). The decolorization zone indicated the MnP enzyme activity. These bacterial strains were further screened based on their MO dye decolorization efficiency and only one bacterium (RKS6) showed the maximum decolorization (97%) of MO dye and selected for further studies.

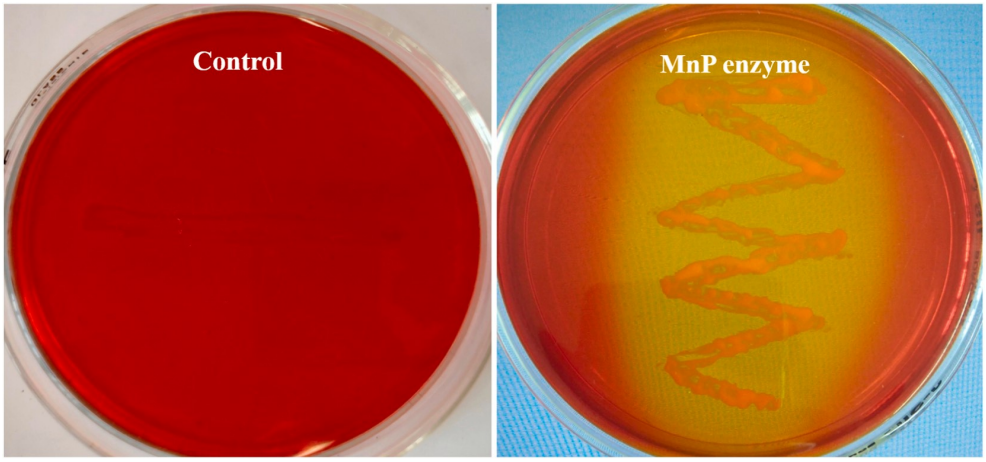


Fig. 1. Screening of the isolated bacterium capable for the degradation and decolorization of methyl orange (MO) dye.

The isolated bacterium (RKS6) showed light yellowish colonies on MSM agar plates, gram-negative, rod shaped and bacterium. It is also showed negative reactions for indole, methyl red, urease and Voges- Proskauer's test and negative reactions for oxidase, catalase, nitrate reduction, starch, citrate utilization and gelatine hydrolysis. In addition, RKS6 showed 99% similarity with *Pseudomonas aeruginosa* and hence, the RKS6 was identified as *Pseudomonas aeruginosa* with accession number MZ520730 (Fig. 2).

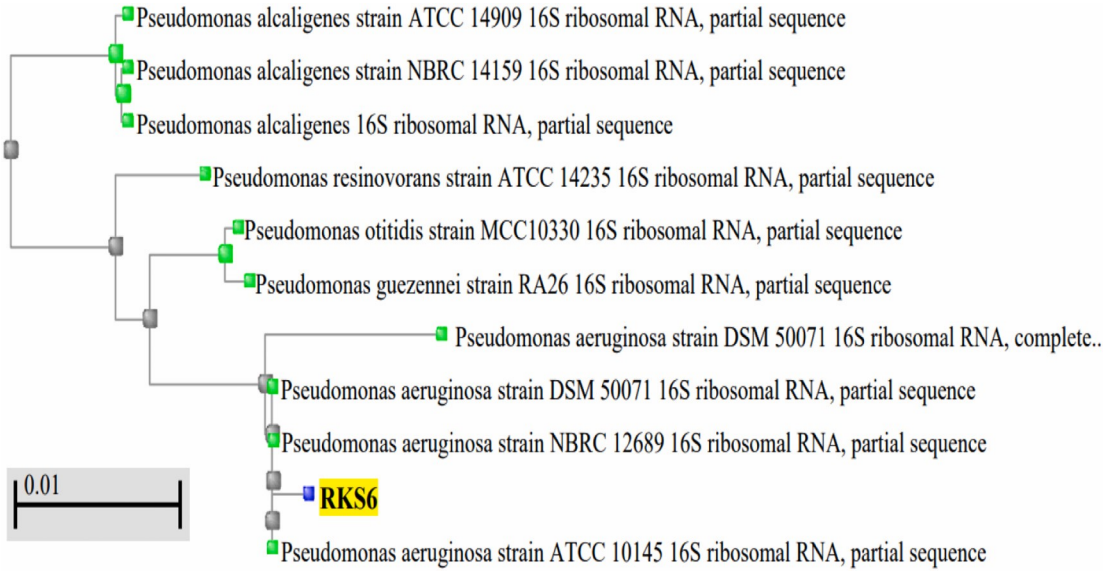


Fig. 2. Phylogenetic tree of isolated bacterium (RKS6) capable for the degradation of MO dye.

3.2. Effects of environmental parameters on degradation and decolorization of methyl orange dye

In present work, the different environmental parameters like pH, temperature, dye concentration and static and shaking conditions were optimized to achieve the maximum decolorization and mineralization of MO dye by RKS6 is shown in Fig. 3.

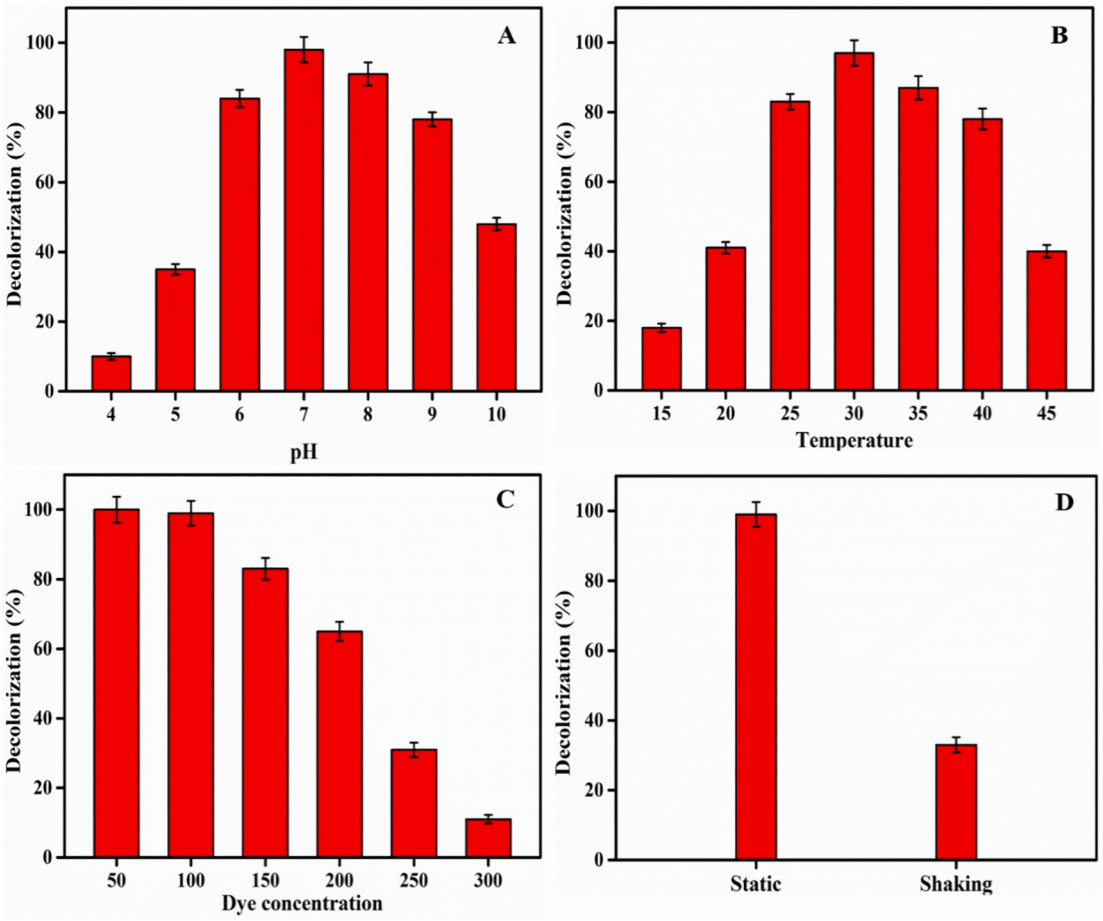


Fig. 3. Effects of (A) pH, (B) temperature, (C) dye concentration and (D) shaking and static condition on degradation of MO dye.

3.3.1 pH

It is an important factor, which play an important role in microbial growth, reproduction, enzymatic machinery and biodegradation efficiency of cells. The effect of pH (4–10) on decolorization of MO dye by RKS6 was studies in 150 ml conical flask as shown in Fig. 3A.

Results revealed that the decolorization of MO dye increases rapidly between pH 6–8 and below pH 6 and above 8, the decolorization efficiency decreased rapidly. The maximum decolorization (98%) was observed at pH 7 indicating that neutral condition favours for bacterium activity. At pH 4, 5, 6 and 8, 10%, 35%, 84% and 91% decolorization was recorded, respectively while at pH 9 and 10, 78% and 48% decolorization were recorded, respectively.

Kishor et al. [8], reported maximum degradation of MB dye at pH 7. The effective decolorization of MO dye by *B. stratosphericus* was reported at pH 7 [2]. Bunti´c et al. [16], also found the maximum degradation of CV and safranin dyes at pH 7. Thus, higher pH values may decrease decolorization due to decrease in enzymatic activity and bioactivity [16], while at lower pH values, H^+ ions inhibit dye cations and thus, causes reduction in decolorization efficiency [2].

3.2.2. Temperature

The effect of temperature (15–45 °C) on MO dye decolorization was studied in a temperature control BOD incubator shaker (LI-BODS-10, Labard., India). In present work, the decolorization efficiency of MO dye decreases below and above 30 °C. Minor decrease in decolorization efficiency was recorded at 25 and 35 °C. It was also observed that decolorization efficiency drastically decreases at 15 and 45 °C. The maximum decolorization (97%) was recorded at 30 °C and only 18% decolorization was recorded at 15 °C. At 20, 25 and 35 °C, the decolorization was recorded up to 41%, 83%, and 87%, respectively followed by 78% and 54% at 40 and 45 °C, respectively as shown in Fig. 3B.

Our results are well supported by various workers as Bunti´c et al. [16], reported the optimum decolorization of CV and safranin dye at 30 °C. Kishor et al. [5], also reported similar pattern of MB dye decolorization at 30 °C. Therefore, at lower temperature, the decrease in decolorization efficiency might be due to the decrease in microbial growth and enzymatic activities [8,16] while the enzyme activity decreases at higher temperature due to the denaturation of enzymes [5].

3.2.3. Dye concentration

In TIWW, the concentration of dyes varies from ~60–250 mg/l, hence, the decolorization of MO dye was performed at varied concentrations (50–300 mg/l) and it was recorded that decolorization of MO dye by RKS6 decreases with increase in dye concentration as shown in Fig. 3C. At 50 mg/l concentration, complete decolorization was achieved while at 100–150 mg/l dye concentrations, 98–83% decolorization was recorded, respectively. At 200–250 mg/l dye concentration, 65–31% decolorization was recorded and only 11% decolorization could be achieved at 300 mg/l dye concentration. This decrease in decolorization efficiency might be due to the inhibition of microbial growth and metabolic activity of bacteria at higher dye concentration [16]. Same findings were also reported on the decolorization of reactive blue EFAF by *E. profundumstrain* by Bose et al. [20] and decolorization of MB dye by *Bacillus albus* [8].

3.2.4. Static and shaking condition

In present study, almost complete decolorization (99%) was achieved at static condition within 12 h while at shaking condition (100 rpm), only 33% decolorization was achieved by RKS6 within same conditions as shown in Fig. 3D. Similarly, Kurade et al. [21], reported 98% decolorization of scarlet RR dye at static condition within 18 h. Kishor et al. [12], also achieved 99% decolorization of congo red dye by *Bacillus cohnni* at static condition.

MO dye is a synthetic azo dye, which contain azo bond and it's decolorization is initiated with the reduction of azo bonds by enzymatic activities [1,5]. Under aerobic condition, the cleavage of azo bonds is inhibited as the enzymatic activities get reduced significantly because of the blockage of transfer of electrons from NADH to azo bonds of dyes [5,21,22].

3.3. Decolorization and TOC removal of MO dye by isolated bacterium

In present work, the RKS6 showed more than 99% decolorization of MO dye within 12 h at optimized conditions as shown in Fig. 4A and B. Similarly, Shyamala et al. [23], reported 88% decolorization of MO dye by *Bacillus* sp. TVU-M4 within 32 h. Navas et al. [13], also observed 33% degradation of MO dye by *Thermus* sp. after 24 h.

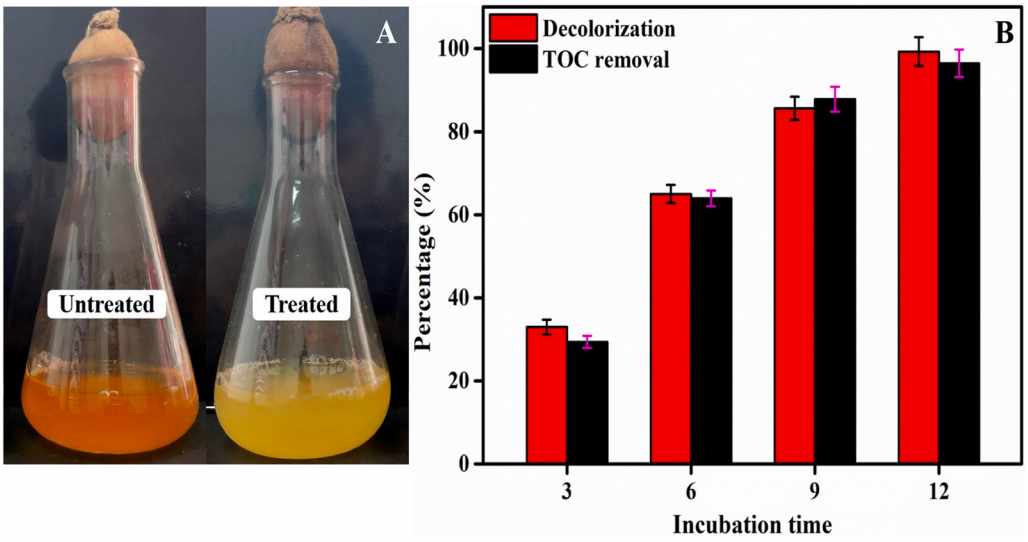


Fig. 4. MO dye decolorization in liquid medium (A) and TOC removal and decolorization of MO dye (B) by isolated bacterium at different incubation time.

The degradation and mineralization of MO dye was determined in terms of TOC reduction [1]. The RKS6 showed 96% TOC reduction at optimized conditions as shown in Fig. 4B. The decolorization and TOC removal from MO dye might be due to the dye degradation and mineralization by RKS6, which utilizes dye molecules and its metabolites as C and N sources [1,5]. Saratale et al. [1], also reported 62% TOC reduction from scarlet R dye solution by a consortium within 72 h.

Further, after treatment study, the heat-killed bacterium cell mass was collected, centrifuged and pellet was washed with ethanol and acetone and colorless cell biomass was obtained indicating that no biosorption was occurred during MO dye decolorization process. Hence, it indicated that decolorization of dye was occurred because of the metabolic activity of RKS6 not by adsorption. The decolorization was not observed in flasks without inoculated RKS6.

3.4. Enzyme characteristics

Different enzymes present in microorganisms are only responsible for the decolorization and mineralization of textile pollutants [5,12,19]. MnP (EC 1.11.1.13) is a ligninolytic enzyme that has a wide potential to oxidize or decompose pollutants into non-toxic and inorganic

compounds [5,19]. MnP enzyme can decolorize, degrade, detoxify and mineralize the textile dyes [5]. For example, more than 76% decolorization of azo orange IV dye was reported by MnP enzyme at 35 °C [24]. MnP enzyme produced by *Streptomyces microflavus* was also capable to decolorize CV and safranin dyes within 24 h [16]. MnP enzyme also degrades and mineralizes TIWW pollutants [5,12,19].

In this study, the RKS6 also showed the MnP enzyme activity during the MO dye decolorization. Hence, this MnP enzyme was characterized by SDS-PAGE analysis resulting one protein band corresponding to ~53 kDa as shown in Fig. 5A. During decolorization study, it was observed that MnP enzyme activity increased with increase in incubation time. After 12 h, 286 U/mg of MnP enzyme activity was recorded at 30 °C as shown in Fig. 5B. Rekik et al. [25], have also isolated and purified MnP enzyme from *Trametes pubescens* during the treatment of textile dyes. It was found to have 55. 2 kDa molecular weight, 221 U/mg of MnP activity and 95% decolorization of AB dye within 24 h. Zhang et al. [9], also purified MnP enzyme (45 kDa) produced by *Cerrena unicolor* BBP6 during the decolorization of MO dye and denim bleaching wastewater.

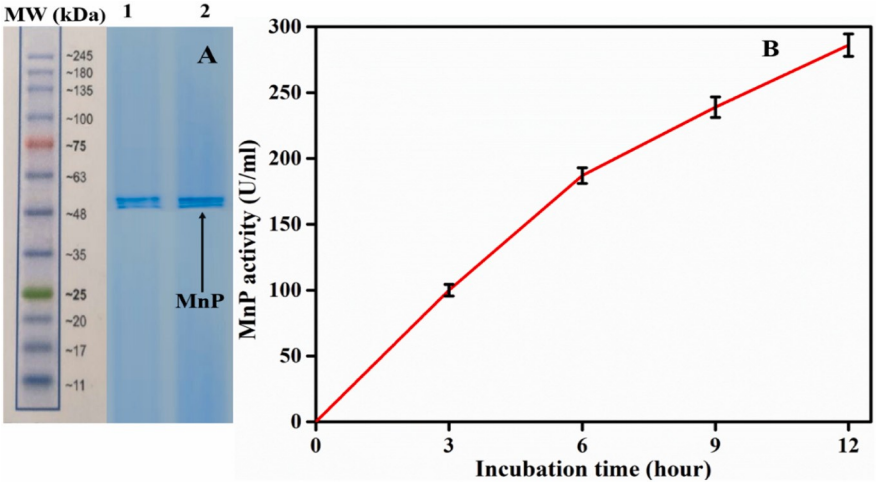


Fig. 5. SDS-PAGE profile of purified MnP enzyme (A) produced by isolated bacterium and its activity (B) during the decolorization of MO dye.

3.5. Effects of methyl orange dye on bacterial cell morphology

In this work, *P. aeruginosa* was selected as an effective organism for the decolorization of MO dye. The results of SEM analysis of bacterial cultures grown in MSM broth (with and without MO dye) during the degradation process as shown in Fig. 6. The bacterial cells grown in MSM broth (control) without MO dye were appeared as large and rod- shaped cells with a smooth surface (Fig. 6A) while the bacterial cells grown in MO dye containing MSM broth showed changes in cells surface morphology like small, rough, coagulate, wrinkled surface as well as increase in number of cells as compared to control (Fig. 6B). The changes occurred in bacterial cell surface morphology might be because of the stress conditions and dye adsorbed on the bacterial cell surface [19].

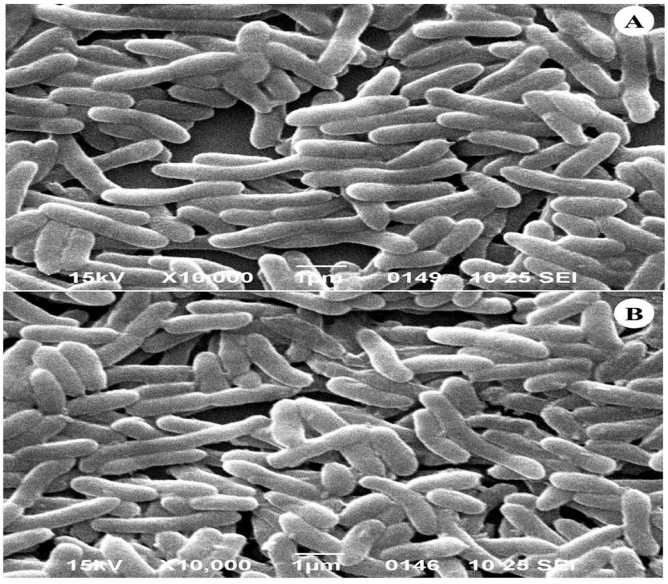


Fig. 6. SEM analysis of isolated bacterium growing in MSM medium (A) and MO dye during treatment process (B).

3.6. Decolorization and degradation of methyl orange dye by isolated bacterium

3.6.1. UV–visible spectrophotometer analysis

The UV–Visible spectroscopy is an important analytical tool used to analyse the dye decolorization. The dye decolorization might be associated with its degradation and if the major absorption peak for dye completely disappears or new absorption peak appears, it indicated that dye is completely degrade or new metabolite is produced, respectively [8,16]. In present work, the UV–visible spectra of untreated MO dye sample showed a maximum absorption peak at 464 nm, but after treatment this peak disappeared indicating that dye is completely degraded.

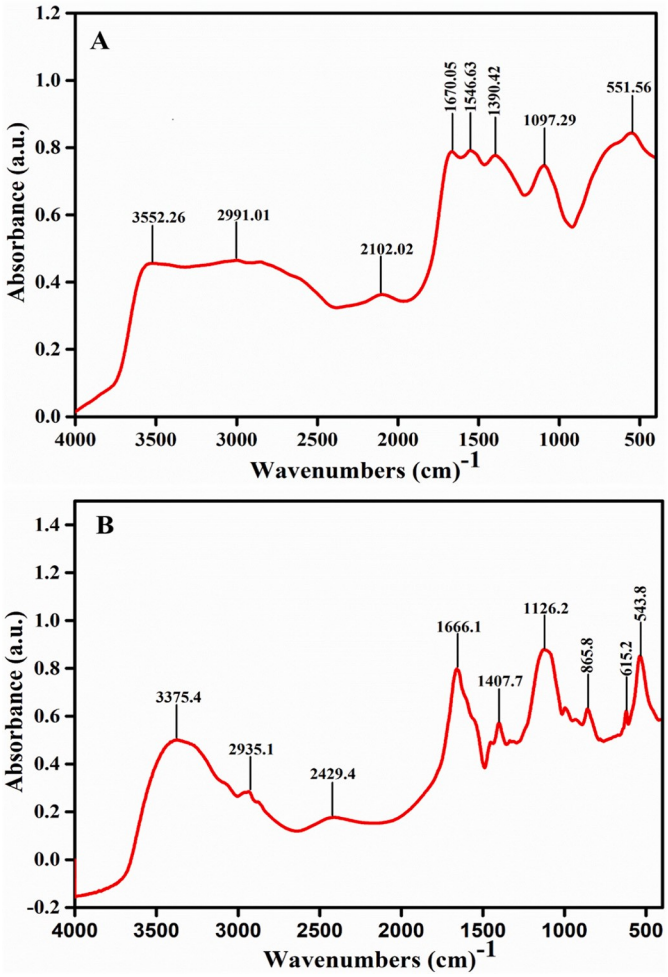


Fig. 7. FT-IR spectrum of untreated (A) and treated MO dye (B) by isolated bacterium.

3.6.2. FT-IR analysis

FT-IR is a basic method used to characterize the functional groups present in MO dye and its metabolites. The FT-IR spectrum of MO dye sample showed absorption peak at 3552.2 cm^{-1} corresponding –NH group of azo bond [5]. The peak recorded at 2991.6 cm^{-1} might be for $^{-}$C–H group in aromatic hydrocarbons. The peak recorded at 2102.3 and 1670.5 cm^{-1} corresponded to C^{-}–C and C^{-}N cm^{-1} stretching, respectively [12]. The peak obtained at 1546.6 cm^{-1} corresponded to NO_2 stretching (Fig. 7A). The peak recorded at 1390.4 cm^{-1} corresponded to SO_2 stretching [21]. The peak recorded at 1097.2 and 551.5 cm^{-1} corresponded for C–F and $^{-}CH_2$ group, respectively [16].

The FT-IR spectrum of treated metabolites of MO dye sample showed peak at 3375.4 cm^{-1} for –OH group, 2935.1 cm^{-1} for –CH group, 2429.4 cm^{-1} for –SH stretch, 1666.1 cm^{-1} for C^{-}N group, 1407.7 cm^{-1} for –OH group, 1126.2 cm^{-1} for C–N group, 865.8 cm^{-1} for –CH group, 615.2 cm^{-1} for C^{-}–C–H bond and 543.8 cm^{-1} for C^{-}O group [4,12,16]. In our study, it was recorded that some peaks get completely disappeared, some get transformed while some new peaks appeared in comparison to control indicating that MO dye is degraded by the isolated bacterium as shown in Fig.7B.

3.6.3. Possible degradation pathways of MO dye

The metabolites of MO dye were further identified by LC-MS analysis. Based on the parent structure of MO dye and metabolites produced during the treatment process, the possible degradation pathways of MO dye as shown in Fig. 8. The degradation of MO dye was initiated with the demethylation and addition of hydroxyl groups to rings. In LC-MS, the peak at RT 3.58 min with molecular mass of 304 *m*/*z* corresponding to parent MO dye molecule [26,27]. During degradation, in first step, the demethylation of MO dye takes place leading to the formation of 4-[(4- aminophenyl) diazenyl] benzene sulfonate (RT = 3.15 and *m*/*z* = 276) (A) [28,29]. In second step, product (A) was converted into 4, 2- ((dihydroxymethyl) hyrazono-4) 5-benzene sulfonate (RT = 20.15 and *m*/*z* = 233) (B) due to the cleavage of azo group and aromatic ring [30]. In third step, demethylation and dihydroxylation of product (B) takes places leading to the formation of 4-(triazan-2-yl) benzene sulfonic (RT = 6.95 and *m*/*z* = 173)

(C) [30]. In last step, product C get finally degraded and mineralized into simpler molecules (i.e. water and carbon dioxide).

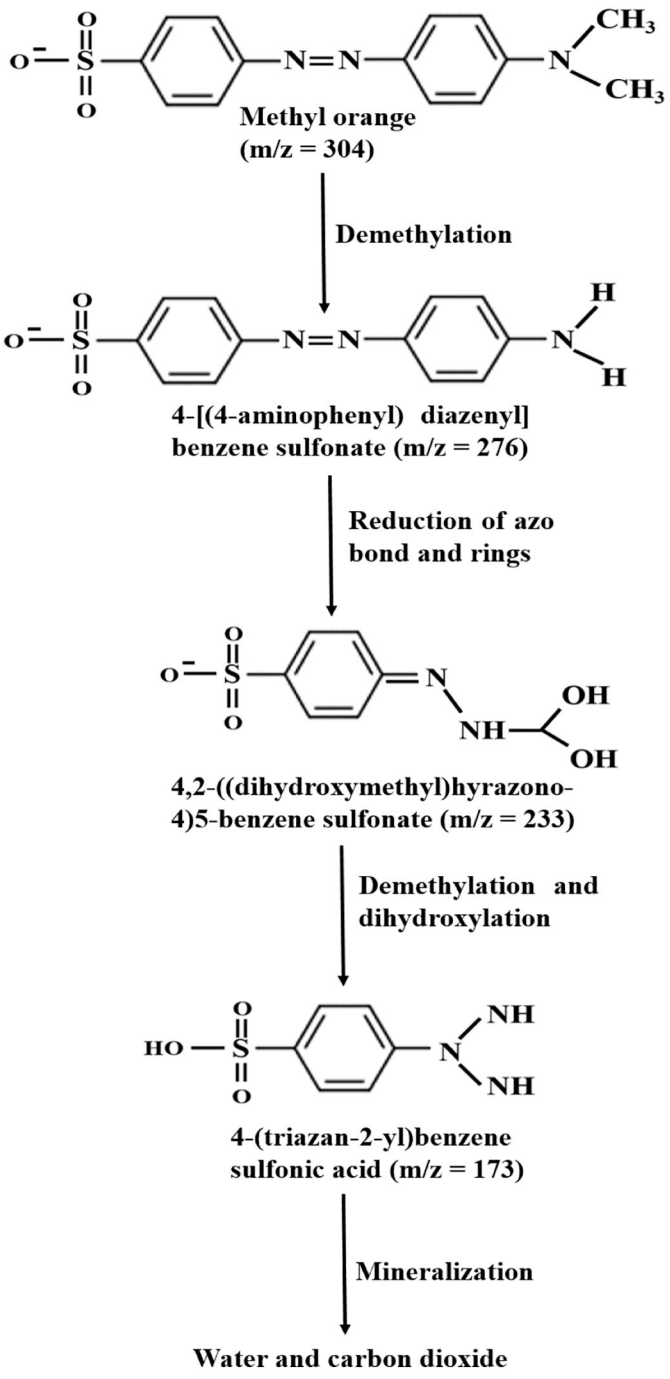


Fig. 8. Possible pathways of MO dye degradation by isolated bacterium.

3.7. Toxicity of methyl orange dye before and after bacterial treatment

Mung bean is a sensitive plant widely used by researchers as a test model plant to evaluate the toxic effects of pollutants. In the present work, the solution of different concentration (15, 20, 30, 50, 75 and 100%) of UT and BT MO dye was made and evaluated for their toxic effects on mung beans plants. The seeds irrigated with 15%, 20% and 30% UT MO dye showed 100% germination and maximum root length, soot length and biomass. But the seeds irrigated with 50% and 75% concentration of untreated dye showed 20% and 30% inhibition in seed germination and negative impacts on root length, soot length and biomass production in comparison to control as shown in Fig. 9 while the seeds irrigated with 100% concentration of UT dye showed 50%, 97%, 98% and 86% inhibition in seed germination, root length, shoot length and biomass production, respectively as compared to control as shown in Table 2.

Table 2. Toxicity effects of untreated and treated MO dye on mung beans.

Conc. of MO dye	No. of Seeds Sown	No. of Seeds Germinated		% Germination		Root length (cm)		Shoot length (cm)		Biomass (gm)		Root and shoot ratio	
		UT MO dye	BT MO dye	UT MO dye	BT MO dye	UT MO dye	BT MO dye	UT MO dye	BT MO dye	UT MO dye	BT MO dye	UT MO dye	BT MO dye
15%	10	10	10	100%	100%	2.56 ± 0.12	2.69 ± 0.15	5.59 ± 0.29	5.75 ± 0.11	1.84 ± 0.22	2.27 ± 0.11	0.45 ± 0.41	0.46 ± 1.36
20%	10	10	10	100%	100%	2.79 ± 0.23	2.91 ± 0.28	5.21 ± 0.17	5.26 ± 0.16	1.63 ± 0.10	2.59 ± 0.12	0.53 ± 1.17	0.55 ± 1.75
30%	10	10	10	100%	100%	1.96 ± 0.11	2.34 ± 0.17	4.11 ± 0.15	4.57 ± 0.12	1.85 ± 0.14	2.07 ± 0.16	0.47 ± 0.73	0.51 ± 1.42
50%	10	8	10	80%	100%	1.28 ± 0.17	2.25 ± 0.25	3.10 ± 0.26	3.85 ± 0.18	0.88 ± 0.12	1.88 ± 0.21	0.41 ± 0.65	0.58 ± 1.39
75%	10	7	10	70%	100%	0.61 ± 0.12	2.33 ± 0.30	1.16 ± 0.10	3.67 ± 0.13	0.64 ± 0.13	1.54 ± 0.10	0.52 ± 1.20	0.63 ± 1.31
100%	10	5	9	50%	90%	0.02 ± 0.01	2.29 ± 0.11	0.52 ± 0.15	3.71 ± 0.14	0.45 ± 0.19	1.68 ± 0.09	0.03 ± 0.06	0.62 ± 0.78
Control	10	10	10	100%	100%	3.84 ± 0.15	3.84 ± 0.15	6.50 ± 0.19	6.50 ± 0.19	2.70 ± 0.20	2.70 ± 0.20	0.59 ± 0.78	0.59 ± 0.79

All the values are means of triplicates ($n = 3$) ± SD.
*MO; methyl orange: Conc.: concentration; UT: untreated; BT: bacterial treated.

But, the seeds irrigated with 15–75% concentration of bacterial treated MO dye showed 100% germination and good root length, shoot length and biomass production as compared to untreated MO dye as shown in Fig. 9. Whereas the seeds irrigated with 100% concentration of bacterial treated MO dye showed 90% seed germination, 56% root length, 51% soot length and 59% biomass production as compared to untreated MO dye (100%). This study indicated that the bacteria treated MO dye solution was less or non-toxic to plants as

compared to untreated MO dye. A similar pattern was also reported by various workers [4,12,16].

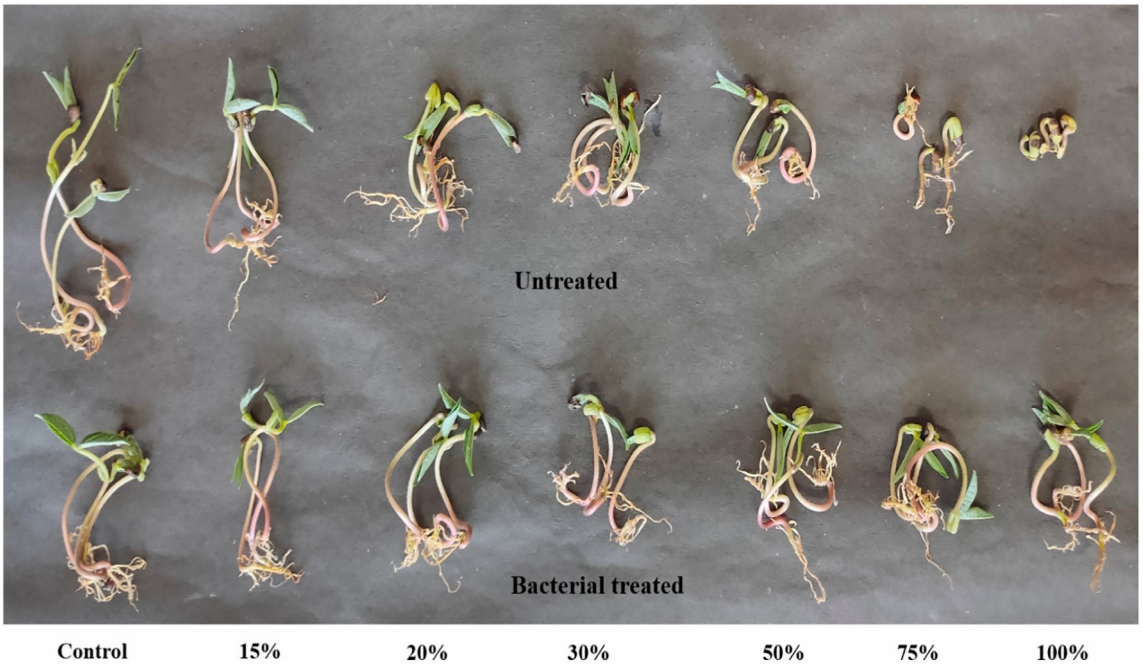


Fig. 9. Toxicity profile of untreated and bacterial treated MO dye at different concentration (15%, 20%, 30%, 50%, 75% and 100%) on mung beans.

4. Conclusion

In this work, a MnP enzyme producing bacterial strain (RKS6) was isolated from TIWW and sludge and characterized based on the 16S rRNA gene sequencing analysis as *Pseudomonas aeruginosa*. RKS6 showed more than 99% decolorization of MO dye along with 96% reduction of TOC within 12 h at optimized conditions. *P. aeruginosa* also produced MnP enzyme during MO dye decolorization and found to have ~53 kDa molecular weight. The produced metabolites were analyzed by FT-IR and LC-MS analysis and found that bacterium was high effective for the complete mineralization of dye molecule into water and carbon dioxide. Further, bacterial treated dye solution was found less toxic and showed 90% seeds germination with good biomass production indicating that isolated bacterium *P. aeruginosa* has a high efficiency for the complete mineralization and detoxification of MO dye.

Declaration of competing interest

On behalf of all authors, the corresponding author declares that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at

https://doi.org/10.1016/j.jwpe.2021.102300.

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