Environment friendly degradation and detoxification of Congo red dye and textile industry wastewater by a newly isolated *Bacillus cohnni* (RKS9)

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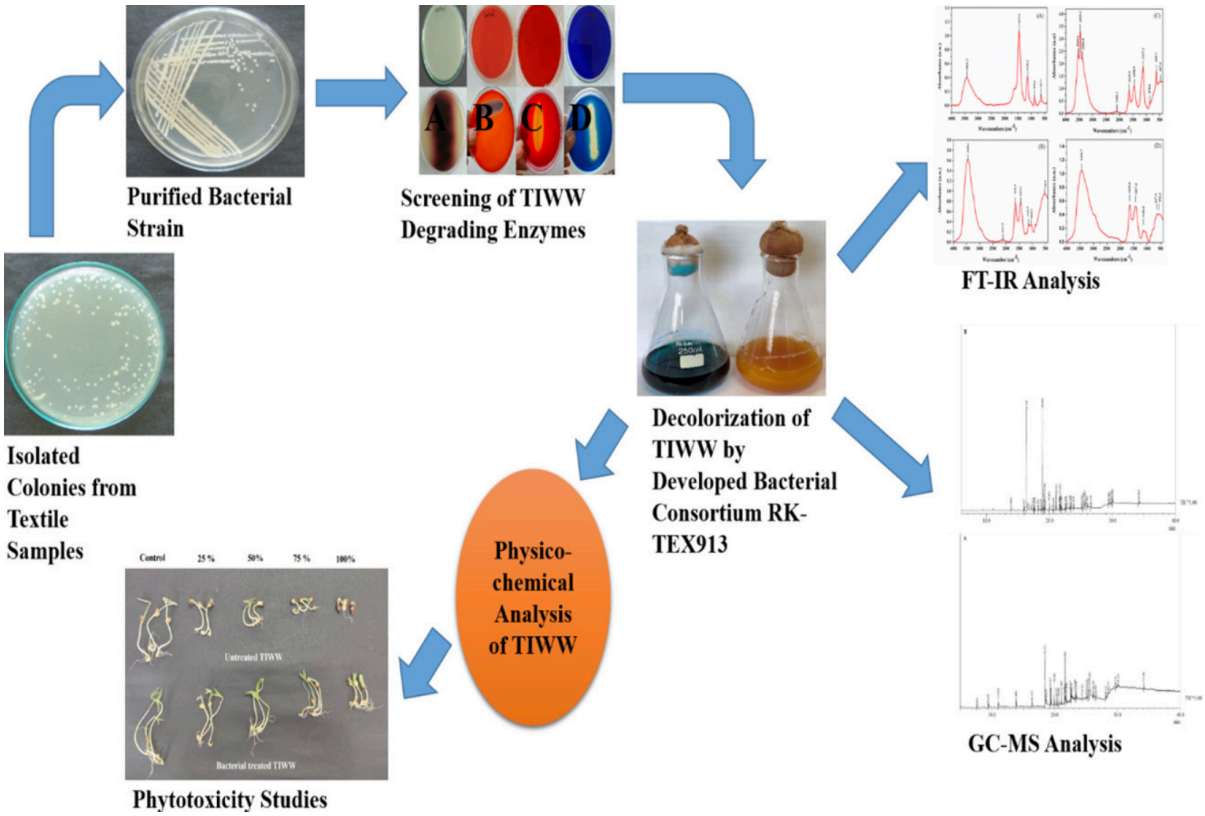
Graphic Abstract



Abstract

Textile industry wastewater (TIWW) is a major source of environmental pollution causing serious threats to all life forms and thus, it must be adequately treated before its final discharge for the safety of environment and public health. In the present study, a bacterial strain (RKS9) was isolated from textile (wastewater & sludge) sample for the effective treatment of TIWW, resulting in a significant reduction in pollution parameters such as ADMI color (93.87%), COD (77.35%), BOD (86.02%), TDS (66.75%), TOC (67.25%), TSS (60.34%), and phenol (68.55%) within 48 h. This bacterium also decolorized 99% of Congo red dye (100 mg L^{-1}) within 12 h and removed 59.76%, 40.51%, 52.71% and 26.51% cadmium, chromium, lead and nickel, respectively from the TIWW. The activities of azoreductase, laccase, lignin peroxidase (LiP) and manganese peroxidase (MnP) was monitored and metabolites produced during the treatment of dye and TIWW were also analyzed by FT-IR and GC-MS. The phytotoxicity of the untreated and treated TIWW was assessed by seed germination and seedling growth parameters of Phaseolus mungo L. and results showed a significant reduction in the toxicity of the treated TIWW, suggesting that the isolated bacterium RKS9 has a remarkable potential to effectively decolorize/detoxify TIWW.

Keywords: Textile industry wastewater Congo red dye Laccase and Lignin peroxidase Bioremediation Phytotoxicity

1. Introduction

The textile industry (TI) acts as a major source of global economy in developing countries, unfortunately, it is also a major contributor of environmental pollution and health hazards. Textile production consists of many stages such as sizing, bleaching, dyeing, printing, washing and finishing (Bilińska et al., 2019; Kishor et al., 2021). Textile industry wastewater (TIWW) contains a variety of highly toxic recalcitrant coloring pollutants (residual dyes), dissolved solids, and toxic metals, they persist in the environment for a long time and pose serious threats to the environment, animal and human health (Zhan et al., 2020). In the aquatic system, TIWW reduces photosynthetic activity and dissolved oxygen (DO) content leading to anoxic conditions that adversely affect both fauna and flora (Kishor et al., 2018; Cao et al., 2019). In the terrestrial system, it reduces soil fertility due to the accumulation of toxic pollutants and metals (Kumar et al., 2019; Kang et al., 2020; Kishor et al., 2021).

TIWW is well reported for its carcinogenic, mutagenic, genotoxic, cytotoxic and allergenic effects to all life forms (Kaur et al., 2018; Kishor et al., 2020). In many developing countries like Pakistan, China and India, TIWW is utilized for irrigation of agricultural lands, which leads to higher risks of bioaccumulation of toxic pollutants in crops and finally reached into the successive level through the biomagnification process (Noman et al., 2020; Kishor et al., 2021). Congo red (CR) is a

synthetic azo dye extensively used in TIs and the unused dye is released into the environment along with the wastewater (Kishor et al., 2021). Many textile dyes are known to be recalcitrant, highly toxic, carcinogenic, mutagenic and allergenic to all living creatures (Cao et al., 2019; Garg et al., 2020). Thus, it is necessary to adequately treat dyes and TIWW before its final discharged into the water bodies for public health protection and environmental safety.

Various physico-chemical, advanced treatment and biological methods have been utilized to treat TIWW (Bilińska et al., 2019; Kang et al., 2020; Zhan et al., 2020). Physico-chemical methods (adsorption, coagulation/flocculation, membrane filtration, ion-exchange and sedimentation) are more effective in color removal but are costly and generate large amount of sludge as secondary waste that affects water/soil properties and living beings (Kumar et al., 2019; Ceretta et al., 2020). While advanced treatment methods (ozonation, photo-fenton, electro-fenton, photo-catalytic, sono-catalytic, electro-coagulation and electro-oxidation) are capable to degrade and decolorize TIWW in short duration of time (Kishor et al., 2021). However, these methods are both expensive and technically demanding complicated procedures with the generation of large amounts of sludge as secondary pollutant (Kaur et al., 2018; Ağtaş et al., 2020; Kang et al., 2020).

In contrast, biological methods are environment friendly, low cost and globally accepted methods effectively used to treat different wastewaters (Kishor et al., 2018; Ceretta et al., 2020). Biological methods employ different types of microbes (bacteria, fungi, yeast, actinomycetes and algae) but out of these, bacteria are considered as the most effective agents in wastewater treatment due to their fast growth, immense environmental adaptability and biochemical versatility (Chen et al., 2018; Bilińska et al., 2019; Garg et al., 2020). Bacteria can degrade, decolorize, detoxify and mineralize various pollutants by using different metabolic pathways (Cao et al., 2019; Kishor et al., 2020). Many bacterial strains have been utilized by various researchers to decolorize dyes and synthetic dyes wastewater (Kumar et al., 2019; Garg et al., 2020). However, few studies reported on the decolorization of real TIWW. For example, Kurade et al. (2012) used *Brevibacillus laterosporus* MTCC 2298 in the treatment of real textile wastewater and achieved 67% decolorization after 48 h. *Pseudomonas* sp. SUK1 resulted in 32% decolorization of textile dye effluent within 48 h (Jadhav et al., 2010). *Providencia* sp. SDS decolorized up to 84% of textile effluent (undiluted) within 48 h (Phugare et al., 2011). Saratale et al. (2009) used *Micrococcus glutamicus* NCIM-2168 strain in the degradation of textile dyes wastewater and observed 63% decolorization and 48% and 42% reduction in total organic carbon (TOC) and chemical oxygen demand (COD), respectively within 72 h under static condition. However, these studies had been emphasized only decolorization with no discussion of the degradation by-products and their toxicity in the environment. Whereas the present study reports not only the isolation and characterization of a bacterial strain effective in the degradation

and decolorization of CR dye and TIWW, but also the characterization of the parent compounds and their metabolites bas well as evaluation of the toxicity of TIWW before and after bacterial treatment for its safe disposal into the environment.

Thus, this study aimed to isolate a bacterial strain for the effective degradation and decolorization of CR dye and TIWW, analyze the physico-chemical parameters before and after the bacterial treatment of TIWW, characterize the parent compounds and their metabolites by Fourier-Transform Infrared Spectroscopy (FT-IR) and Gas Chromatography–Mass Spectrometry (GC–MS) as well as evaluate the toxicity of the TIWW before and after the bacterial treatment via the seeds germination and seedlings growth of *Phaseolus mungo* L.

2. Material and methods

2.1. Chemicals

The chemicals (glutaraldehyde, hydrogen peroxide, acetic acid, sodium acetate, mercuric chloride and sodium hydroxide) used in this study were procured from *M/s* S D fine chemicals Ltd., Mumbai, India. Different solvents (acetone, ethyl acetate and methanol) were procured from *M/s* Spectrochem Ltd., Mumbai, India. The guaiacol, phenol red (PR), azure B (AB) and Congo red (CR) dyes were procured from *M/s* Sisco Research Laboratories Ltd., Mumbai, India. Bacterial media and salts (nutrient broth, beef extract, peptone, agar, glucose, dextrose, starch, calcium chloride, sodium chloride, copper sulfate and magnesium sulfate) were procured from Hi-media Laboratories Ltd., Mumbai, India. *Phaseolus mungo* L. seeds were collected from the local market of Lucknow, India. All the chemicals used in this study were of analytical grade.

2.2. Collection of textile industry wastewater and sludge samples

The wastewater and sludge samples were collected in pre-sterilized clean containers from the discharge site of Handloom Bhandar situated at Unnao City (26.48 N, 80.43 E), Uttar Pradesh, India. Unnao is a heavily industrialized city of India, which significantly discharge a large amount of wastewater that causes severe threats on the nearby water bodies and soil ecosystems. The collected wastewater and sludge samples were transported to laboratory, stored at 4 °C and used for physico-chemical analysis, bacterial isolation, biodegradation studies, pollutants and metabolite characterization as well as phytotoxicity evaluation tests.

2.3. Isolation and screening of potential bacterial strain capable for the dye and TIWW decolorization

The sludge and TIWW samples were used to isolate bacterial strains that can potentially degrade CR by following the enrichment/ acclimatization method (Cao et al., 2019). Briefly, 15 ml (v/v) of TIWW and 5.0 gm (w/v) of sludge sample were placed in 250 ml conical flask having 80 ml of

sterilized MSM (mineral salt medium) broth. The composition of MSM broth was (g/l): glucose 10; peptone 5; Na_2HPO_4 2.4; K_2HPO_4 2.0; NH_4NO_3 0.1; $MgSO_4$ 0.01 and $CaCl_2$ 0.01 with pH 7.2. The flasks were added with 100 mg l^{-1} of CR, methylene blue (MB), methyl red (MR), crystal violet (CV) and reactive black (RB) dyes followed by incubation at 32 ± 1 °C, 85 rpm in incubator shaker (LI-BODS-10, LABARD). After 15 days, 15 ml (v/v) of acclimatized culture was collected and added in 85 ml of fresh MSM medium followed by incubation at 85 rpm in incubator shaker at 32 ± 1 °C. After 4 days, 1.0 ml of this enrichment culture was serially diluted (10^{-1} to 10^{-6}), 30 l of each dilution was spread on MSM agar plates and incubated at 32 ± 1 °C. The MSM agar medium was composed of (g/L): glucose 10; peptone 5; Na_2HPO_4 2.4; K_2HPO_4 2.0; NH_4NO_3 0.1; $MgSO_4$ 0.01; $CaCl_2$ 0.01 and agar 15; and pH 7.2. After 24 h, the morphologically distinct colonies were selected, pick up and purified by repeated plate streaking method.

All the selected isolates (RKS1-RKS13) were screened for laccase, manganese peroxidase (MnP), lignin peroxidase (LiP) and azoreductase enzyme activity using guaiacol, phenol red, azure B and Congo red dyes, respectively. All dyes were sterilized with membrane filter 0.22 µm (Millipore Ltd, Bedford., MA). All media were sterilized by autoclaving at 121 °C for 20 min and pH was adjusted to 7.2 (pH system 361, Systronics., India). The sterilized MSM agar medium containing 70 µl (1 M) of guaiacol, 100 mg l^{-1} of phenol red, 100 mg l^{-1} of azure B and 100 mg l^{-1} of CR dyes was inoculated with a loopful culture of each isolated bacterium followed by incubation at 32 ± 1 °C. After six days, only one isolate (RKS9) producing clear decolorization zone on all four different substrates was selected for further study.

2.4. Characterization and identification of isolated bacterium

The characterization (morphological and biochemical) of the selected bacterium was made according to the "Bergey's Manual of Determinative Bacteriology" (Whitman et al., 2012). Further molecular identification was performed following the 16S rRNA gene sequencing analysis. The genomic DNA was isolated from the overnight grown cultures (Atashpaz et al., 2010). Genomic DNA (~5 ng) sample was used to amplify 16S rRNA gene using the universal primers (27F) 5-AGAGTTTGATCMTGGCTCAG-3 and (1492R) 5-CGGTTACCTTGTTACGACTT-3 (Narde et al., 2004). The reaction mixture (50 µl) was prepared by adding: template DNA (100 ng), PCR buffer (1X), dNTP each (200 µM), $MgCl_2$ (3.0 mM), primer (25 pmol), DNA polymerase (2.5 units) and rest of the autoclaved deionized water was added to make final volume. The thermocycling steps: denaturation (94 °C) 30 cycles for 1 min, annealing at 45 °C and extension at 72 °C were performed for 2 min on a Veriti® 96-Well Thermal Cycler, Applied Biosystems, USA. The amplified PCR product was purified by using a gel extraction kit (Merk, Biosciences, India) followed by BLAST analysis to identify the bacterial isolate

using the online option available at https://www.ncbi.nlm.nih.gov/BLAST.cgi (Bharagava et al., 2018). The phylogenetic tree was prepared using MEGA software version 5.0 and the obtained 16S rRNA sequences was also submitted in GenBank databases under the accession number MW406977 for bacterium RKS9.

2.5. Decolorization studies of CR dye and TIWW with isolated bacterium

For CR dye decolorization, 2.0 ml (v/v) of bacterium culture (1.4×10^6 CFU ml^{-1}) was added in 98 ml of sterile MSM broth (pH 7.2) supplemented with 100 mg l^{-1} of CR dye followed by incubation at 32 ± 1 °C under static condition. After 3, 6, 9 and 12 h, 4 ml samples were taken followed by centrifugation at 8000 x g for 10 min to separate the bacterial biomass. The bacterial biomass was dissolved in 2.0 ml distilled water, added in 98 ml of sterile MSM broth (pH 7.2) amended with 100 mg l^{-1} of CR dye and placed in incubator at 32 ± 1 °C under static condition. Afterwards, the different time intervals (3, 6, 9 and 12 h), 4 ml aliquot was taken and centrifuged at 8000 x g for 10 min at 4 °C. The obtained supernatant was utilized to monitor the decolorization of dye at 497 nm using a double beam UV–visible spectrophotometer (Labtronics, LT-2201, India). The % decolorization was calculated following the formula as below:

$$Decolorationsation\ (\%) = \frac{Absorbance\ (R1) - Absorlabce\ (R2)}{Absorbance\ (R1)} \times 100$$

Where R1 is the initial absorbance, R2 is the final absorbance of CR dye decolorization.

For the TIWW decolorization, 20 ml (v/v) of bacterium culture (1.4×10^6 CFU ml^{-1}) was inoculated in 80 ml of undiluted filter-sterilised (Whatman No.1) TIWW (pH 7.2) and the flasks were incubated at 32 ± 1 °C, 100 rpm. After 12, 24, 36 and 48 h, an appropriate amount of samples were taken from flasks followed by centrifugation at 8000 x g for 10 min at 4 °C. The bacterial biomass was dissolved in distilled water and inoculated in 80 ml of undiluted filter (Whatman No.1) sterilized TIWW (pH 7.2). The flasks were incubated in incubator shaker at 32 ± 1 °C and 100 rpm. Afterward, the different time intervals (12, 24, 36 and 48 h), 4 ml aliquot was taken and centrifuged at 8000 x g for 10 min at 4 °C. The decolorization of TIWW was monitored following the American Dye Manufacturing Institute (ADMI 3WL) tristimulus filter method (Kurade et al., 2012). The uninoculated flask was used as a control and only culture medium (without CR dye/TIWW and bacterium culture) was used as a blank during the spectrophotometry analysis of samples. The percent decolorization was calculated following the below formula and expressed in terms of ADMI color removal.

$$ADMI\ removal\ \% = \frac{ADMI\ (R1) - ADMI(R2)}{ADMI(R1)} \times 100$$

whereis the initial ADMI absorbance andis the final ADMI absorbance.

2.6. Analysis of enzymatic activity during TIWW decolorization by isolated bacterium

During decolorization study, samples were withdrawn at regular intervals followed by centrifugation at 8000 x g, 4 °C for 10 min. The supernatant was used to determine the activity of extracellular enzymes. For intracellular enzyme activity, the cell biomass was suspended in potassium phosphate buffer (50 mM, pH 7.4) followed by sonication by a sonifier set at 50 amp and giving 7 strokes each of 30 s, with 3 min interval at 4 °C (Sonics-Vibracell Ultrasonic Processor, USA). The sample was centrifuged at 8000 x g for 15 min at 4 °C and used as source of crude intracellular enzymes. The activities of laccase, MnP, LiP and azoreductase enzymes were measured by using a double beam UV–Vis spectrophotometer.

For azoreductase enzyme activity, the reaction mixture (3.6 ml) was contained 1.7 ml potassium phosphate buffer (50 mM, pH 7.0), 0.8 ml methyl red (152 mM), 100 µM of NADH (20 mM) and 1.0 ml of crude enzyme (Balapure et al., 2015). The reaction mixture was incubated at 25 °C for 4 min followed by the addition of NADH and the absorbance was recorded at 440 nm.

The laccase activity was measured following the methodology of Bharagava et al. (2018) with slight modifications. The reaction mixture (4.0 ml) containing 1 ml of guaiacol (2 mM), 2.5 ml of acetate buffer (10 mM, pH 5) and 0.5 ml of crude enzyme extract was incubated for 2 h at 25 °C. The oxidation of guaiacol was measured by spectrophotometer at 420 nm.

The MnP enzyme activity was measured following the methodology of Arora and Gill (2001) in which the reaction mixture (4 ml) was contained 1.0 ml phosphate buffer (pH 7.0), 0.5 ml of $MnSO_4$ (1 mM), 1 ml phenol red (0.1 mM) and 1.0 ml of crude enzyme extract. The reaction was started by adding 0.5 ml of H_2O_2 O (1 mM) and stopped by adding 40 µl of NaOH (5 M) to the reaction mixture. The samples were taken at every 4 min and activity was recorded at 610 nm.

Lignin peroxidase (LiP) activity was measured following the methodology of Buntić et al. (2017) with few modifications. The reaction mixture (3.5 ml) was prepared by adding 1.3 ml of sodium tartrate buffer (pH 3.0, 125 mM), 0.5 ml of azure B dye (0.160 mM) and 1.2 ml of centrifuged bacterial supernatant. The reaction was started by adding 0.5 ml of H_2O_2 (2 mM) and the activity was measured at 310 nm. One unit of enzyme activity is defined as the amount of enzyme required to reduce 1 µM of substrate per minute.

2.7. Physico-chemical analysis of untreated and bacteria treated TIWW

The untreated and treated TIWW samples were analyzed for pH, color, BOD, COD, TDS, TSS, TS, SS and toxic metals such as Cd, Cr, Pb, and Ni as per the standard protocols of APHA (2005). Besides these parameters, the electric conductivity (EC), phenol, sulfate, total phosphate (TP) and total nitrogen (TN) were also measured. The pH of TIWW was measured by a digital desktop pH meter (Systronics-361, India). BOD, COD and TP were measured following the 5-days dilution method, open reflux method, and TOC-V_{csn} analyzer (Shimadzu, Japan), respectively whereas TDS, TSS and TS were determined by the drying method. The total phenol, TN and sulfate were measured by 4-aminoantipyrene method, Vanadomolybdo phosphoric acid colorimetric method and $BaCl_2$ precipitation method, respectively. The concentration of metals was measured using the Inductively Coupled Plasma Spectrophotometer (Thermo Electron; Model IRIS Intrepid II XDL, USA) following the standard methods of APHA (2005).

2.8. Characterization of untreated and bacterial treated TIWW and dye samples by FT–IR and GC–MS analysis

2.8.1. Sample preparation

Untreated and bacterial treated TIWW (100 ml (v/v)) and dye samples were collected and centrifuged at 8000 x g for 10 min at 4 °C to separate cell biomass and other suspended particles. The parent compounds and their metabolites were extracted from the supernatant following the liquid–liquid extraction method using equal volume of ethyl acetate and samples (Bharagava et al., 2018). The extract was dried over anhydrous sodium sulfate and evaporated to dryness in a rotary evaporator (Rotavapor RE 120 Buchi, Flawil., Sweden) and used in FT–IR analysis. For the GC–MS analysis, the dry residues were dissolved in HPLC grade dichloromethane (DCM), filtered through (0.22 µm) membrane filter (Millipore Ltd, Bedford., MA) prior to injection into the column.

2.8.2. FT–IR analysis

The dried samples were mixed with 400 mg of pure KBr in a ratio of 5:95, the mixture was finely ground and fused into a thin pellet prepared under vacuum conditions by using a PCI hydraulic press with capacity of 15 tons and fixed in a sample holder for analysis. The samples were analyzed by using a Nicolet™ 6700 Thermo Scientific, USA, Spectrophotometer in the mid-infrared (IR) region from 400–4000 cm^{-1} with a 16 scan speed.

2.8.3. GC-MS analysis

Ethyl acetate extract (5 µl) was injected into a PE-5MS column (0.18 mm diameter, 20 m long, 0.18 mm film thickness). The column temperature was kept at 80 °C (2 min), 50–280 °C (10 °C min^{-1})

and hold time (7 min). Helium gas was employed as carrier gas and the flow rate was programmed at 1.0 ml min^{-1}. The temperature of injection port and MS transfer line were set at 280 °C and 290 °C, respectively. The parent chemicals and their metabolites were characterized on the basis of RT (Retention Time), fragmentation pattern, and mass spectra by using the NIST Library (National Institute of Standards and Technology) (version 1.10 beta, Shimadzu).

2.9. Toxicity assessment of untreated and treated TIWW

The toxicity of the untreated and treated TIWW was evaluated in terms of seed germination and seedlings growth parameters using the seeds of *P. mungo* L. For this, different concentrations, 25, 50, 75 and 100% (v/v) of untreated and bacterial treated TIWW were prepared. Tap water was used as control. Ten (10) uniform-sized healthy seeds of *P. mungo* L. were selected, surface sterilized with 2.0% $HgCl_2$ solution for 2 min to remove fungal contamination and washed with sterile distilled water (Bharagava and Chandra, 2010). Ten seeds were placed in each sterilized Petri-dishes in between Whatman No. 1 filter paper (Whatman, England) and 5 ml of each concentration of untreated and bacteria treated TIWW samples were used for irrigation of seeds (Bharagava et al., 2018) and incubated at 28 °C in a BOD incubator for 7 days. The % of seed germination was recorded after two days and the root and shoot length was calculated after seven days of incubation period. The experiment was conducted in triplicate. The % of seed germination was recorded using the following formula:

$$Germination\ \% = \frac{Number\ of\ seeds\ germinated}{Number\ of\ seeds\ sowed} \times 100$$

2.10. Statistical analysis

The experiments were carried out in triplicates to minimize the experimental errors and the results obtained from each set of experiments were expressed as mean and standard deviation.

3. Results and discussion

3.1. Characteristics of isolated bacterium

Initially, thirteen (RKS1-RKS13) morphologically different bacterial strains were selected, purified and screened, based on the enzyme production capability. Out of the 13 isolates, only one isolate was found capable to produce clear decolorization zone around the colonies on dyes amended MSM agar plates after 2–6 days at 32 ± 1 °C. The bacterial strain RKS9 produced a clear yellow and

white decolorization zone around colonies grown on phenol red and azure B dye amended MSM agar plates, respectively (Fig. 1C & D). The strain RKS9 also produced dark reddish and white decolorization zone around the colonies grown on guaiacol and CR dye amended MSM agar plates, respectively (Fig. 1A & B). The production of the dark reddish zone, white zone, yellow zone and white zone around colonies indicated the laccase, azoreductase, MnP and LiP enzyme activity and were selected for further study.

Bacterial strain RKS9 produces circular white colonies on MSM agar plates and was gram positive, aerobic, motile, rod-shaped, and endospore-forming strain. It showed positive reactions for catalase, oxidase, nitrate reduction, citrate utilization and methyl red and negative reactions for gelatin hydrolysis, starch hydrolysis, indole, H_2S production, urease test and Voges–Proskauer's test.

Further, the 16S rRNA gene sequencing analysis revealed that the isolated bacterium RKS9 has 99% similarity with *Bacillus cohnni* and thus, this bacterium was identified as *B. cohnni* with accession number MW406977 (Fig. 2).

3.2. CR dye and TIWW decolorization by isolated bacterium

In the present study, the isolated bacterium RKS9 was found capable to decolorize CR dye up to 99.67% within 12 h (Fig. 3A). After the separation of bacterial biomass, this bacterium showed complete decolorization of CR dye within the same time period; this compared well to other studies. For example, Haq et al. (2018) reported that *S. liquefaciens* showed more the 90% decolorization of azure B dye. D'Souza et al. (2017) also reported that *Acaligens* species showed 76% decolorization of CR dye within 24 h.

In case of TIWW, the isolated bacterium RKS9 resulted 93.87% ADMI color removal from the real TIWW (Fig. 3B). After the separation of bacterial biomass, this bacterium showed 95% ADMI color removal from the real TIWW within 48 h. The higher decolorization efficiency of the RKS9 strain for CR dye rather than TIWW might be due to the structural complexities of various pollutants (dyes) present in TIWW (Cao et al., 2019). The degradation and decolorization of textile effluent by isolated *Bacillus* sp. had been reported by Okareh et al. (2017). During treatment process, the increased decolorization efficiency of isolated bacterial strain might be associated with the diversified metabolic activities and also the bacterial cells are supposed to use pollutants as C and N source resulting in the degradation and decolorization of wastewater pollutants (Chen et al., 2018; Cao et al., 2019; Garg et al., 2020; Kishor et al., 2021).

After decolorization process, the biomass of heat-killed bacteria was taken, washed with different eluents like ethanol, ethyl acetate and acetone and the colorless biomass showed that no biosorption was occurred during the decolorization of CR dye and TIWW. Thus, it indicated that the

decolorization was occurred only because of the metabolic activities and not by the physical adsorption process (Kishor et al., 2021). The decolorization was not observed in the control flasks throughout the experiment.

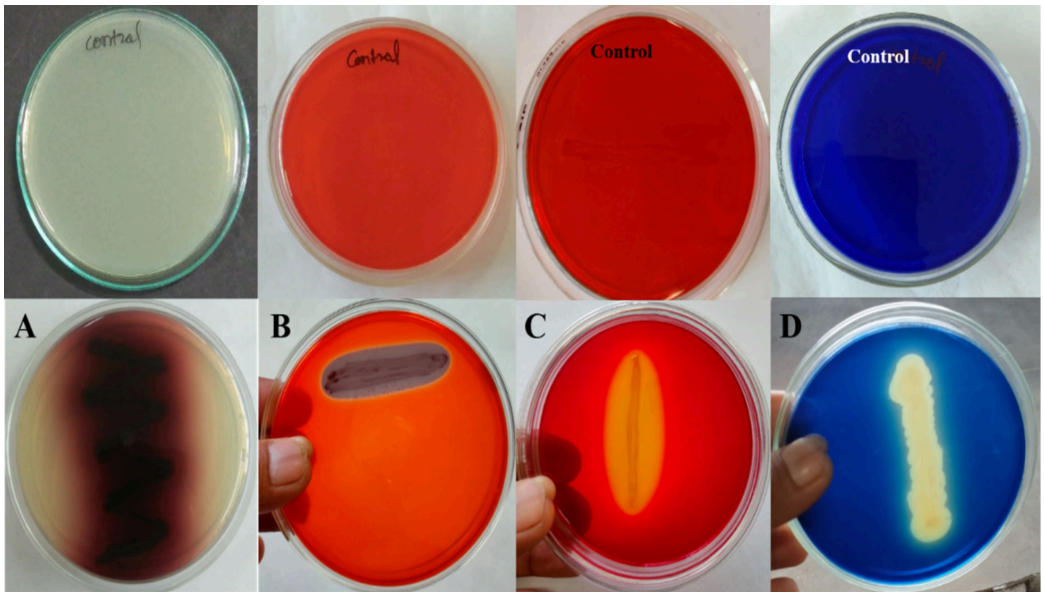


Fig. 1. Screening of Laccase (A), Azoreductase (B), Manganese Peroxidase (C) and Lignin Peroxidase (D) enzyme producing bacterial strains on MSM agar plates amended with Guaiacol, Congo red, Phenol red and Azure B dye, respectively.

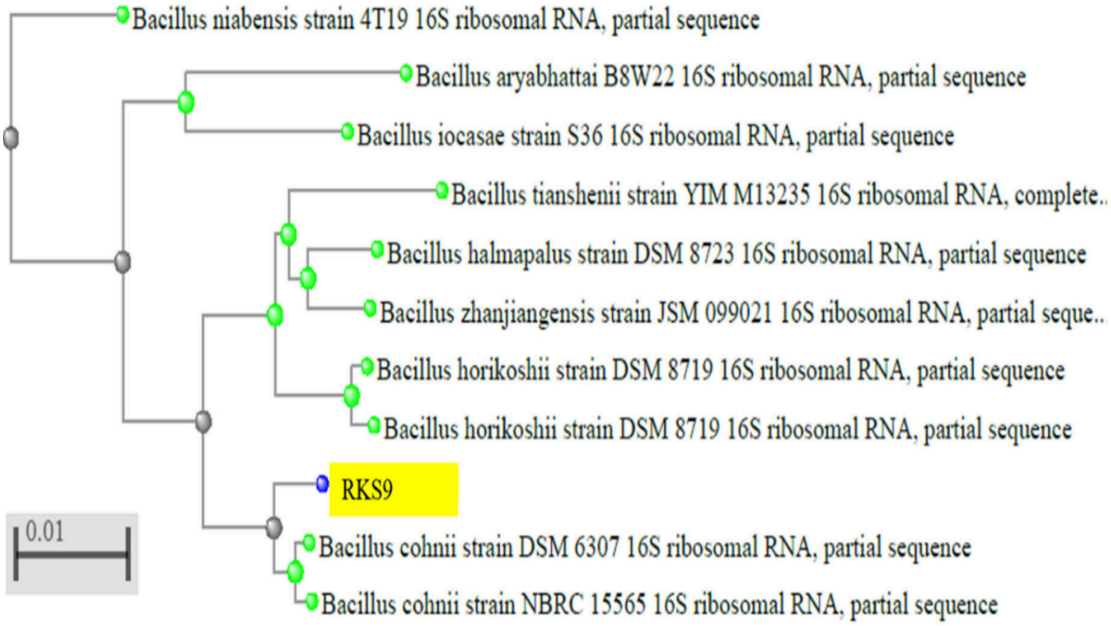


Fig. 2. Phylogenetic tree of the isolated bacterium capable for decolorization of Congo red dye and textile industry wastewater.

3.3. Enzyme activities during the TIWW decolorization by isolated bacterium

Different enzymes such as laccase, azoreductase, MnP and LiP were detected during the decolorization of TIWW. These enzymes are known to convert various pollutants present in industrial wastewaters into non/less toxic products (Buntić etal., 2017, Cao et al., 2019). The cumulative action of all these enzymes might be responsible for the effective decolorization of CR dye and TIWW within 12–48 h. The activity of MnP, azoreductase, LiP and laccase enzymes increases with increase in incubation time (Fig. 4). The maximum enzyme activity for laccase, LiP, MnP and azoreductase was recorded during the decolorization at 36, 36, 12 and 24 h of incubation period, respectively.

Laccase and LiP enzymes showed higher activity up to 17 U ml^{-1} and 15 U ml^{-1}, respectively as compared to MnP 9 U ml^{-1} and azoreductase 7 U ml^{-1}. The detailed of activities for all enzymes during the decolorization of TIWW has been showed in Fig. 4. The participation of all above reported enzymes in decolorization of different dyes and wastewaters is well documented by various workers (Bharagava et al., 2018; Cao et al., 2019; Garg et al., 2020). Laccase and LiP enzymes may be the key agents for the decolorization of CR dye and TIWW. Similarly, Haq et al. (2018) reported more than 90% decolorization of AB dye by a LiP producing *Serratia liquefaciens* strain and TIWW decolorization by laccase and LiP producing *Streptomyces microflavus* strain (Buntić et al., 2017). Bharagava et al. (2018) also reported 99% decolorization of CV dye by laccase and LiP producing *Aeromonas hydrophila* strain.

3.4. Physico–chemical characteristics of untreated and bacteria treated TIWW

The physico–chemical characteristics of untreated and bacteria treated TIWW are summarized in Table 1. Results revealed that untreated TIWW was dark blue in color having high pH, temperature, COD, BOD, TDS, TSS, TS, TOC, EC, TN, TP, phenol, sulfate, and heavy metals content (Table 1). The recorded values of all parameters in this study were found to be above the recommended standard discharged limit for industrial wastewaters (National Environmental Quality Standards) (Hussain et al., 2019; Central Pollution Control Board 2010).

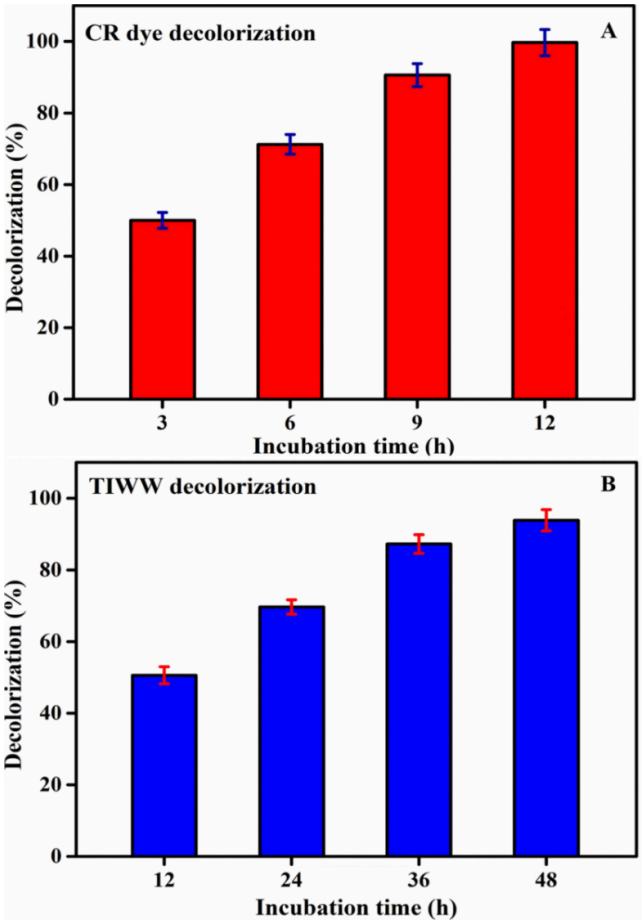


Fig. 3. Decolorization of Congo red dye **(A)** and textile industry wastewater **(B)** by isolated bacterium *B. cohnni* MW406977 within 12 and 48 h.

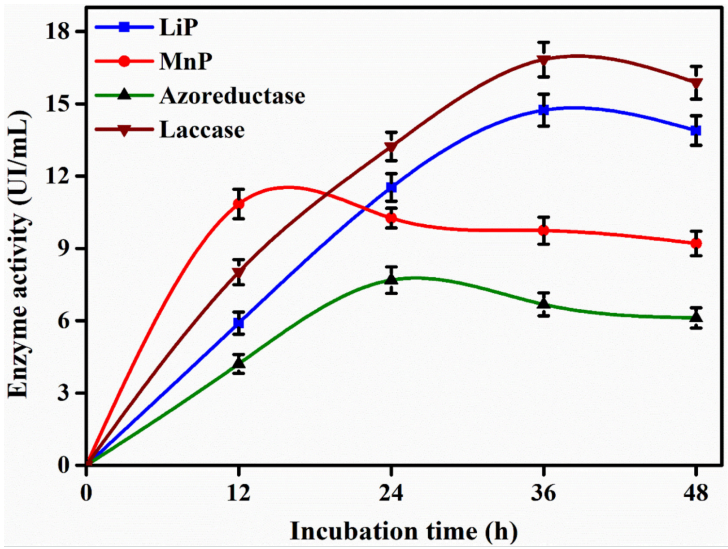


Fig. 4. Laccase, manganese peroxidase, lignin peroxidase and azoreductase enzyme activity during the decolorization of textile industry wastewater within 48 h at 32 ± 1 °C.

The dark blue color and alkaline pH (9.7) of the TIWW might be because of the presence of various dyes and chemicals used in industries (Kurade et al., 2012; Kishor et al., 2021). The raised EC (5.93 us m^{-1}) could result from the salts and chemicals present in the TIWW (GilPavas et al., 2018; Hussain et al., 2019). The high TSS (464 mg l^{-1}) and TDS (7165 mg l^{-1}) content could be attributed to the presence of organic and inorganic matters (Hussain et al., 2019; Chandanshive et al., 2020). The elevated COD (1733 mg l^{-1}) and BOD (694 mg l^{-1}) values probably caused by the presence of complex mixture of dyes, volatile organic compounds, acids, phthalate, detergents, surfactant, bases, and softeners used in textile industries (Kurade et al., 2012; GilPavas et al., 2018; Kishor et al., 2021). The excess chloride (1691.66 mg l^{-1}) and TN (9.63 mg l^{-1}) content in TIWW might be due to the presence of sodium chloride, hydrochloric acid, chlorine gas and urea (Hussain et al., 2019; Oktem et al., 2019). The use of pentachlorophenol and nonylphenols in dyeing process contributed to high phenol content (1.348 mg l^{-1}) in TIWW (Oktem et al., 2019; Hussain et al., 2019). Sulfur compounds like sodium sulfate increased the level of sulfate (1575 mg l^{-1}) in TIWW (Kurade et al., 2012; Oktem et al., 2019). Heavy metals detected in TIWW were associated with various textile dyes and additive chemicals used in textile industries (Hubadillah et al., 2020). Thus, if this untreated/partially treated TIWW discharged into water bodies, it could cause serious pollution in water bodies and soil and pose significant threats to public health (Hubadillah et al., 2020).

Color is the major polluting parameter in TIWW leading to the reduction in penetration power of sunlight and photosynthetic activity of aquatic plants, resulting in the low oxygen level in aquatic water bodies disturbing both aquatic fauna and flora (Kishor et al., 2018; Bharagava et al., 2018). The high BOD and COD values result a decline in DO content in receiving water bodies, leading to anoxic conditions that adversely affect both fauna and flora and cause toxicity to aquatic system (Kishor et al., 2021). Phenolic compounds are reported as highly toxic that damage the heart, liver, lung and kidneys in human/animals (Kumar et al., 2019). TS, TDS and TSS contents pose negative impact on water bodies and soil fertility by fluctuating the ion composition and salinity (Hussain et al., 2019; Kishor et al., 2020). The high values of nitrogen, phosphate and sulfate are the main cause of eutrophication and damage the normal ecological functioning of water bodies. In addition, heavy metals found in TIWW also reported to cause carcinogenic, mutagenic, cytotoxic, allergic threats to all life forms and serious threats in water and soil ecosystem (Khandare and Govindwar, 2015; Kishor et al., 2021).

After the bacterial treatment, the dark blue-colored TIWW turned into transparent/colorless due to the reduction of chromophore group present in dyes (Kishor et al., 2021). The treatment of TIWW by the isolated bacterium RKS9 effectively reduced COD, BOD, TDS, TSS, TOC, TS, sulfate,

phenol, TP, chloride, TN and surfactant up to 77.35%, 86.02%, 66.75%, 60.34%, 67.25%, 64.78%, 68.55%, 20.63%, 13.41%, 44.23%, and 83.24%, respectively within 48 h (Table 1). This bacterium also removed heavy metals like cadmium, chromium, lead and nickel up to 59.76%, 40.51%, 52.71 and 26.51%, respectively (Table 1).

Similarly, Kurade et al. (2012) found 89%, 68% and 74% reduction in ADMI, BOD and COD values, respectively from textile industry wastewater by bacteria-yeast consortium within 48 h. The pH (7.9), COD (2130 mg l^{-1}), BOD (210 mg l^{-1}), TDS (820 mg l^{-1}), chloride (412 mg l^{-1}), sulfate (345 mg l^{-1}), Ni (0.54 mg l^{-1}) and Pb (0.48 mg l^{-1}) were reported in treated textile wastewater (Phugare et al., 2011). Saratale et al. (2010) reported 5567 mg l^{-1} of ADMI, 1156 mg l^{-1} of COD, 12586 mg l^{-1} of TOC, 225 mg l^{-1} of TN and 89 mg l^{-1} of TP in bacteria treated textile wastewater. Jadhav et al. (2010) also found 1440 mg l^{-1} of COD and 141 mg l^{-1} of BOD in treated textile wastewater by bacteria within 48 h. The decrease in various parameters might be associated with the utilization of different pollutants as C and N sources by the bacterial culture during the treatment process (Garg et al., 2020; Kishor et al., 2021). The heavy metals were either bioaccumulated inside the bacterial cells or bind to lipopolysaccharide (LPS) of the extracellular membrane (Chandra and Singh, 2012). Overall, the findings of this study showed that the isolated bacterium *B. cohnni* could be used for the effective treatment of TIWW as compared to previous studies reported by various researchers (Saratale et al., 2010; Phugare et al., 2011; Kurade et al., 2012).

3.5. Characteristics of untreated and treated dye and TIWW

The FT-IR analysis of untreated dye samples showed many peaks at 3467.2, 1587.1, 1442.7, 1359.1 and 829.1 cm^{-1} indicating the presence of N H, N H, C H, N O and Si CH bonds, respectively. The absorption peaks at 1201.4, 1052.6, 873.1 and 749.8 cm^{-1} indicated the presence of C N, S O, O H and C S bonds, respectively (Fig. 5A) (Haq et al., 2018). But, after bacterial treatment, several new peaks appeared at 3450.1, 2117.8, 1641.7 and 1443.5 cm^{-1} indicating the presence of N H, C ≡H, C N and C H bonds, respectively (Kurade et al., 2012; Bharagava et al., 2018). The peaks at 1141.7, 1043.5 and 538.9 cm^{-1} represent -SO_2, N H and C O bending, respectively (Fig. 5B) (Chandanshive et al., 2018; Haq et al., 2018). However, the peaks appeared at 875.6 and 627.7 cm^{-1} in untreated dye sample could not be detected in treated samples indicating that the compounds corresponded by these peaks were degraded by bacterium during the treatment process (Kurade et al., 2012). In addition, some new peaks at 2117.8, 1641.7, 1043.5 and 538.9 cm^{-1} were detected, suggesting the biotransformation of CR dye molecules into metabolites by the isolated bacterium (Kurade et al., 2012; Haq et al., 2018).

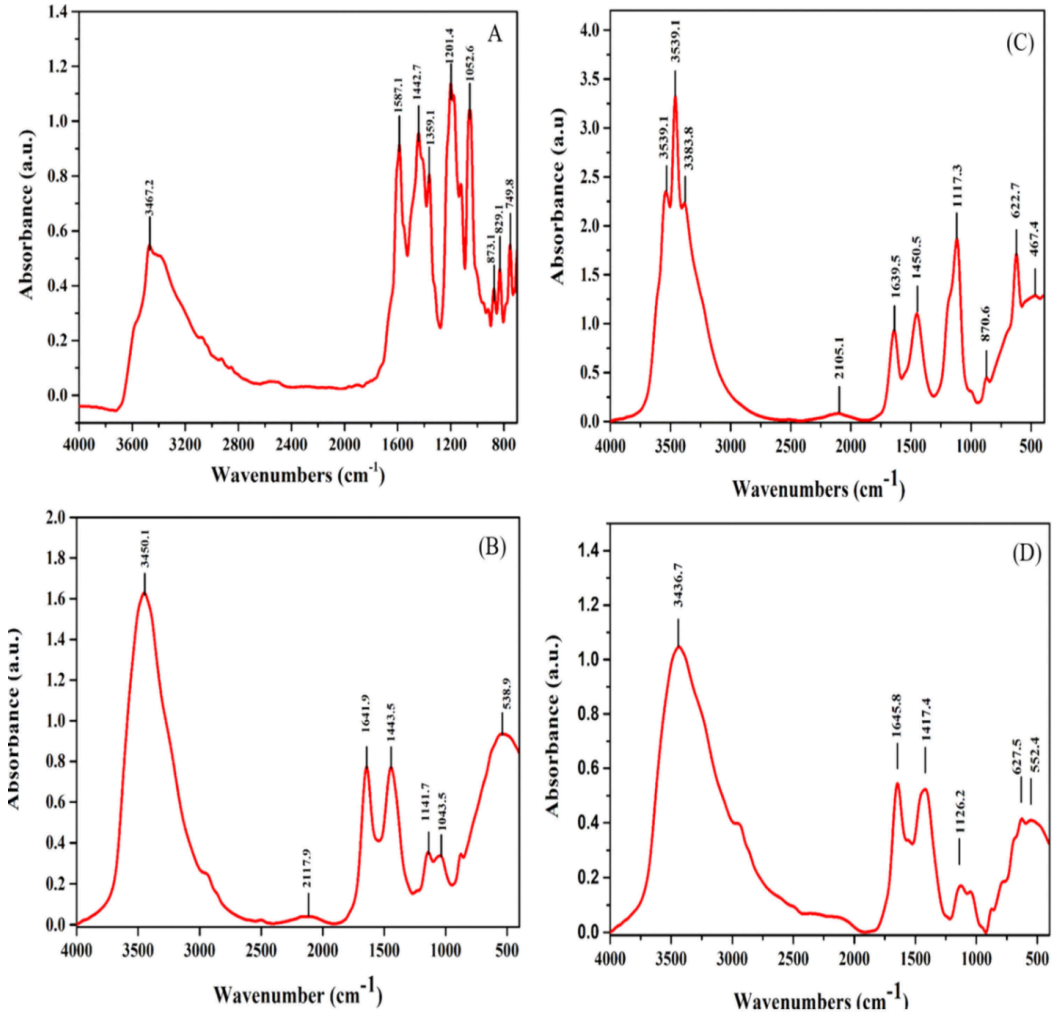


Fig. 5. FT-IR spectra of untreated (A) and treated (B) Congo red dye and untreated textile industry wastewater pollutants (C) and their metabolites (D).

Table 1. Characteristics of untreated and bacteria treated textile industry wastewater.

Characteristic	Permissible limit		Recorded value		% Reduction
	CPCB (2010)	NEQS	Untreated TIWW	Treated TIWW	
pH	6.0–9.0	6–10	9.7 ± 0.1	7.3 ± 0.05	24.97
Temperature (°C)	< 35	40	40 ± 1	36 ± 0.36	10
EC (us/m)	0.85	NG	5.93 ± 0.65	1.53 ± 0.20	74.19
Color	NG	NG	Dark blueish	Transparent/ Colorless	–
BOD (mg l^{-1})	30	80	694.33 ± 3.05	97.03 ± 0.41	86.02
COD (mg l^{-1})	250	150	1733 ± 5.29	393 ± 3.51	77.35
TOC (mg l^{-1})	NG	NG	3762 ± 9.84	1232 ± 5.50	67.25
TS (mg l^{-1})	NG	NG	7076 ± 4.58	2492 ± 4.8	64.78
TDS (mg l^{-1})	2100	3500	7165 ± 6.24	2382 ± 2.64	66.75
TSS (mg l^{-1})	100	150	464 ± 2.64	184 ± 6.55	60.34
Sulfate (mg l^{-1})	1000	600	1575 ± 7	1250 ± 6.24	20.63
Chloride (mg l^{-1})	1000	1000	1691.66 ± 5.03	1527 ± 4	9.73
Total nitrogen (mg l^{-1})	NG	40	9.63 ± 0.15	5.37 ± 0.05	44.23
Total phosphate (mg l^{-1})	NG	NG	8.2 ± 0.1	7.1 ± 0.3	13.41
Phenol (mg l^{-1})	1.0	0.1	1.348 ± 0.09	0.424 ± 0.02	68.55
Surfactant	NG	NG	11.46 ± 0.25	1.92 ± 0.04	83.24
Heavy metals (mg l^{-1})					
Cadmium (Cd)	0.05	0.1	0.84 ± 0.11	0.339 ± 0.02	59.76
Chromium (Cr)	2.0	0.1	1.54 ± 0.24	0.916 ± 0.03	40.51
Lead (Pb)	0.1	NG	0.184 ± 0.08	0.087 ± 0.006	52.71
Nickel (Ni)	3.0	1.0	1.32 ± 0.009	0.97 ± 0.020	26.51

While in case of the untreated TIWW, the peak appeared at 3539.1 cm^{-1} indicated the presence of –OH group (O H stretching) corresponding to alcohol and phenol derivatives. The peaks appeared at 3460.5 and 3383.8 cm^{-1} indicated –N H and –OH group corresponding to primary amines and phenolic compounds, respectively (Bharagava et al., 2018; Navada and Kulal, 2019). The peak

appeared at 1639.5 cm^{-1} may be for C O and NH_2 group in primary amides, 1450.5 cm^{-1} for N=N-O group of azoxy compounds. The peaks appeared at 1117.3 and 870.6 cm^{-1} indicated CS bond of thiocarbonyl compounds and -CH_2 group, respectively (Cao et al., 2019). The peaks observed at 622.7 and 467.4 cm^{-1} characterized by the presence of C-OH stretching of alcohols and CH_2 of vinyl compounds, respectively. But, after the bacterial treatment, the peaks appeared at 3436 and 1645 cm^{-1} indicated the presence of NH stretching of aromatic amines and C N bonding of oximes, respectively (Okareh et al., 2017; Chandanshive et al., 2017). The peaks appeared at 1417.4, 1126.2 and 627.6 cm^{-1} corresponded to –OH group in carboxylic acid, C S in thiocarbonyl compounds and –OH group of alcohols, respectively (Okareh et al., 2017; Bharagava et al., 2018). The FT–IR spectra of the untreated and bacterial treated TIWW are represented in Fig. 5C & D. The band at 552.4 cm^{-1} corresponded to –OH stretching in alkanes. However, a significant change in the FT-IR spectrum, disintegration of major peaks and appearance of new peaks in the treated TIWW suggested biodegradation of the TIWW pollutants (Phugare et al., 2011; Kurade et al., 2012).

The compounds in the untreated and treated TIWW identified by GC–MS analysis are presented in Table 2. The major peaks observed in the untreated TIWW at RT 9.43, 11.03, 16.41, 18.45, 19.34, 19.42 and 19.96 indicated the presence of undecane, dodecane, heptadecane, phenol, 1, 2-benzendicarboxylic acid, bis (2-methylpropyl) ester, pentachlorophenol and hexadecanoic acid, methyl ester, respectively. In addition, many other peaks at RT 20.32, 21.60, 21.89, 23.34, 25.35, 26.18 and 30.12 also appeared in the untreated TIWW indicating the presence of 1, 2-benzendicarboxylic acid dibutyl ester, 1, 9-octadecenoic acid, methyl ester, methyl stearate, hexadecanoic acid, 1 (hydroxymethyl)-1, 2-ethanediyl ester, 1H-indole, 5-methyl based and 1, 2-benzendicarboxylic acid, decane, 1,9-bis[(trimethylsilyl)oxy]-, respectively (Fig. 6A).

The dye components such as hexadecanoic acid, methyl ester, 1H-indole, 5-methyl and 9, 12-octadecenoic acid, methyl ester are used as coloring agents at dyeing and printing stage. Dyes are well reported to cause carcinogenic, mutagenic, genotoxic, cytotoxic and allergic reactions to plants, rates, fishes, microbes, mollusks and animals (Bharagava et al., 2018, Garg et al., 2020). These also severely damage aquatic life due to the reduction in dissolved oxygen content and decrease the penetration power of sunlight (Kaur et al., 2018, Ceretta et al., 2020). The methyl stearate is applied as a lubricant during the textile manufacturing process and is reported to cause the toxic effects in aquatic environments. Many waxes such as dodecane, undecane and heptadecane are used at scouring stage as a phase change materials and protective agents to protect the textile products from water, intensive heat, nuclear radiation, chemicals and biological agents. Phenolic compounds like phenol and pentachlorophenol are used as fungicide, algaecide, insecticide, molluscicide and anti-fouling paint ingredients during the finishing of textile products. These compounds have been

reported as highly toxic to aquatic life, increased incidence of breast cancer and disrupt endocrine system in mammals (Kumar et al., 2019, Garg et al., 2020). In addition, the phthalates like 1, 2-benzendicarboxylic acid, bis (2-methylpropyle) ester, 1, 2-benzendicarboxylic acid dibutyl ester and 1, 2-benzendicaboxylic acid also found in TIWW as these are used as additive adhesives and printing inks reagents at the printing stage of the textile manufacturing process. Phthalates are well known as carcinogenic, endocrine disrupters and aquatic toxicants affecting the reproductive system and impair fertility to all life forms (Liang et al., 2017).

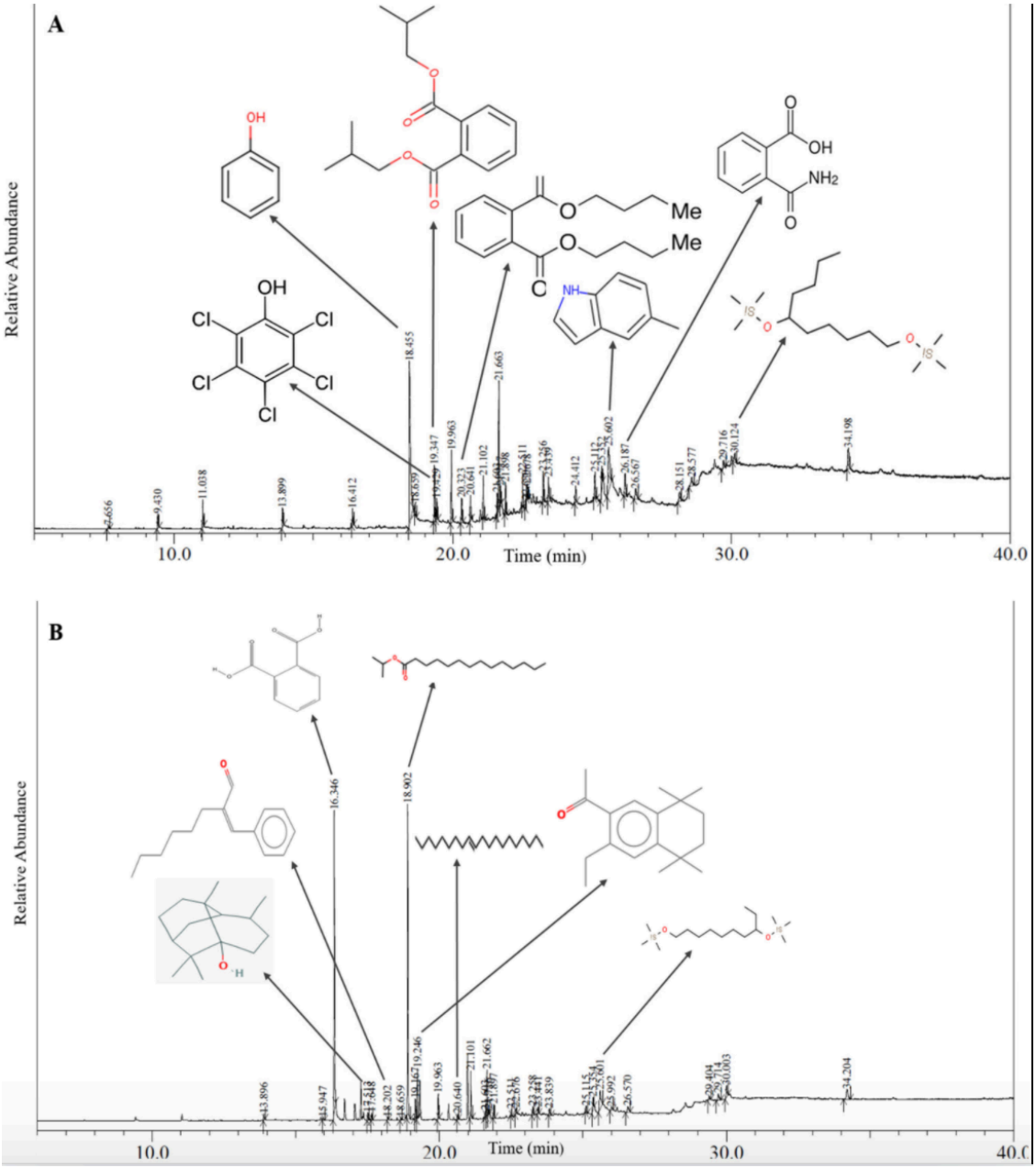


Fig. 6. GC-MS chromatograms of compounds extracted with ethyl acetate from untreated (A) and treated (B) textile industry wastewater.

After bacterial treatment, it was recorded that compounds of untreated TIWW were mineralized and some compounds were transformed into new metabolic products by the isolated bacterium. The degraded metabolites such as 2-buten-1-ol, 2-ethyl-4-(2, 2, 3-trimethyl-3-cyclopenten-1-yl)- (RT: 15.94), hexadecanoic acid, methyl ester (RT: 19.96), octanal, 2-(phenyl ethylene)-(RT 18.20), 1,2-Benzendicarboxylic acid, diethyl ester (RT: 16.34), 1, 6-methanonaphthalen-1(2H)-ol, octahydro (RT: 17.51), 7-acetyl-6-ethyl-1, 1, 4, 4-tetramethyltralin (RT: 19.24), decane 1, 9-bis[(trimethylsilyl)oxy]-(RT: 25.60), 9-Eicosene (RT 20.64), Isopropyl myristate (RT: 18.90), (E), 3-methyl-4-(2, 6, 6-trimethyl-2-cyclohexen-1-Y (RT: 17.64) and 9-octadecenamide (RT: 30.00) (Fig. 6B). The two organic compounds such as dodecane (RT: 11.03) and hexadecanoic acid, methyl ester (RT: 19.96) could not be degraded/metabolized by the isolated bacterium. However, the possible degradation pathway of TIWW pollutants is shown in Fig. 7.

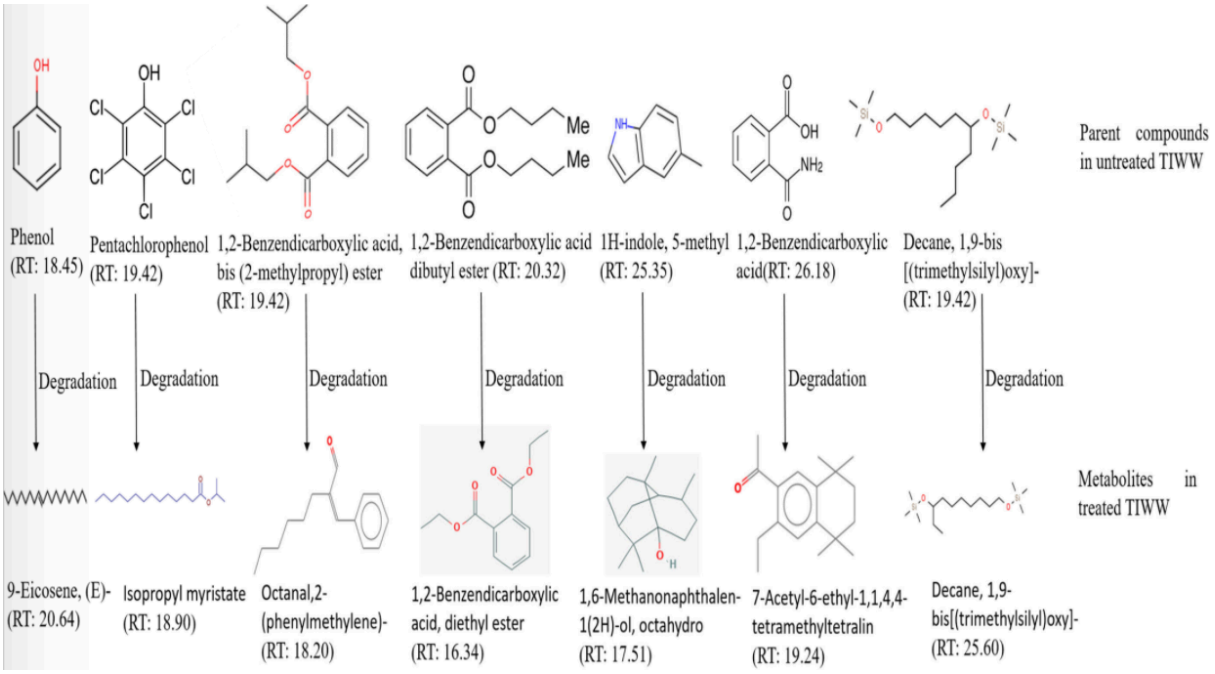


Fig. 7. Possible pathways of textile industry wastewater pollutants degradation.

3.6. Toxicological effects of untreated and treated TIWW on seed germination and seedling growth parameters

The untreated TIWW is toxic to environment; if it is directly used to irrigate the agricultural land it could inhibit various physiological and biochemical processes of seed germination and seedling growth parameters, but after treatment, the inhibitory effects on seed germination and seedling growth parameters were alleviated. It was observed that 25% (v/v) untreated TIWW has no inhibitory effects on seed germination, but higher concentration (50%, 75% and 100% (v/v)) showed 20%, 40% and 60% inhibition in seed germination, respectively as compared to control samples.

After bacterial treatment, it showed 100% seed germination at 25%–75% TIWW concentration and only 10%–20% seed germination was inhibited by bacteria treated TIWW (100%). The effects of untreated and treated TIWW on seed germination, shoot lengths and root lengths are shown in Table 3. It was observed that 25% of untreated TIWW showed maximum seedling growth (shoot length and root length) (Table 3). Whereas seeds irrigated with 50%, 75% and 100% concentration of untreated TIWW showed inhibitory effects on root and shoot length. The 100% concentration of untreated TIWW showed maximum inhibition in root length (96%), shoot length (93%) and seed germination (60%) as compared to control. The results clearly indicated that untreated TIWW was highly toxic for seed germination, which might be because of a variety of pollutants. On the other hand, when seeds were irrigated with 25, 50, 75 and 100% (v/v) of bacteria treated TIWW, it showed good elongation of root and shoot length (Fig. 8) and thus, it was observed that bacteria treated TIWW has improved the seed germination (40%), root length (45%) and shoot length (62%) in comparison to the untreated TIWW. These findings were also supported by various other researchers (Kurade et al., 2012, Haq et al., 2018, Bharagave et al., 2018).

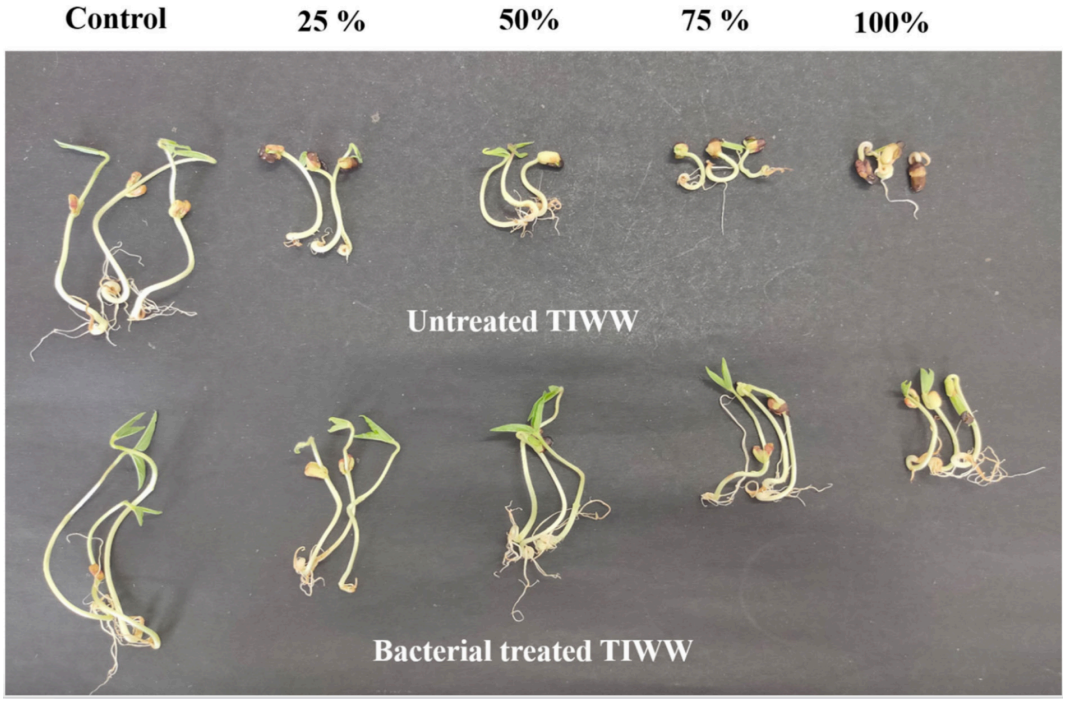


Fig. 8. Effect of untreated and bacteria treated textile industry wastewater on seed germination and seedlings growth parameters of *Phaseolus mungo* L.

Table 2. Compounds identified in untreated and bacteria treated textile industry wastewater by GC-MS analysis.

Compounds	Retention Time	Peak Area	Untreated TIWW	Bacteria Treated TIWW
Undecane	9.43	1.20	+	–
Dodecane	11.03	2.01	+	+
2-Buten-1-ol, 2-ethyl-4-(2,2,3-trimethyl-3-cyclopenten-1-yl)-	15.94	0.36	–	+
1,2-Benzendicarboxylic acid, diethyl ester	16.34	29.08	–	+
Heptadecane	16.41	1,27	+	–
1,6-Methanonaphthalen-1(2H)-ol, octahydro	17.51	0.66	–	+
3-Methyl-4-(2,6,6-trimethyl-2-cyclohexen-1-Y	17.64	0.65	–	+
Octanal,2-(phenylmethylene)-	18.20	0.29	–	+
Phenol	18.45	23.91	+	–
Isopropyl myristate	18.90	28.79	–	+
7-Acetyl-6-ethyl-1,1,4,4-tetramethyltetralin	19.24	5.51	–	+
1,2-benzendicarboxylic acid, bis (2-methylpropyl) ester	19.34	4.20	+	–
Pentachlorophenol	19.42	1.77	+	–
Hexadecanoic acid, methyl ester	19.96	5.37	+	+
1,2-benzendicarboxylic acid dibutyl ester	20.32	2.05	+	–
9-Eicosene, (E)-	20.64	0.50	–	+
9,12-octadecenoic acid, methyl ester	21.60	1.29	+	–
Methyl stearate	21.89	2.37	+	–
Hexadecanoic acid, 1 (hydroxymethyl)-1,2-ethanediyl ester	23.34	2.34	+	–
1H-indole, 5-methyl	25.35	2.24	+	–
Decane, 1,9-bis[(trimethylsilyl)oxy]-	25.60	4.29	–	+
1,2-Benzendicarboxylic acid	26.18	2.86	+	–
Decane, 1,9-bis[(trimethylsilyl)oxy]-	30.12	18.5	+	–
9-Octadecenamide	30.00	1.81	–	+

Where, – is absent; + is present.

Table 3. Phytotoxicity evaluation of untreated and bacteria treated textile industry wastewater by using seeds of *Phaseolus mungo* L.

Conc. of TIWW (%)	No. of Seeds Sown	No. of Seeds Germinated		% Germination		Shoot length (cm)		Root length (cm)	
		Untreated TIWW	Treated TIWW	Untreated TIWW	Treated TIWW	Untreated TIWW	Treated TIWW	Untreated TIWW	Treated TIWW
25 %	10	10	10	100 %	100%	5.3 ± 0.1	6.4 ± 0.1	2.6 ± 0.2	3.2 ± 0.4
50%	10	8	10	80 %	100%	2.5 ± 0.3	5.8 ± 0.1	1.5 ± 0.2	2.6 ± 0.3
75%	10	6	10	60 %	100%	1 ± 0.4	5.2 ± 0.2	0.5 ± 0.3	2.2 ± 0.4
100%	10	4	8	40 %	80%	0.5 ± 0.1	4.5 ± 0.2	0.2 ± 0.08	1.6 ± 0.6
Control	10	10	10	100 %	100%	7.3 ± 0.2	7.3 ± 0.2	3.6 ± 0.3	3.6 ± 0.4

4. Conclusion

In present study, one bacterial strain RKS9 capable to decolorize dye and TIWW was isolated, characterized and identified as *B. cohnni* (accession number MW406977). This bacterium bears a versatile property to efficiently decolorize and detoxify both dye and TIWW resulting 99.43%, 90.38%, 86.02% and 68.55% reduction in color, COD, BOD and phenol, respectively during the treatment of TIWW. The enzymatic study suggested the involvement of manganese peroxidase (MnP), azoreductase, lignin peroxidase (LiP) and laccase enzymes in the degradation and decolorization of dye and TIWW. The degradation of dye and TIWW pollutants was confirmed by the FT-IR and GC-MS analysis. The toxicity studies also demonstrated a significant reduction in the toxicity of TIWW after treatment allowing 80% seed germination in comparison to untreated TIWW allowing only 40% seed germination. Thus, this study suggested that the isolated bacterium *B. cohnni* can be effectively applied as a better alternative for the treatment of dyes and TIWW and the treated TIWW can be used as a liquid fertilizer in agricultural land after making proper dilution.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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