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# Biodegradation of malachite green by a novel copper-tolerant *Ochrobactrum pseudogrignonense* strain GGUPV1 isolated from copper mine waste water

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## Abstract

**Background:** Malachite green (MG) is a triphenyl methane cationic dye which is used to color fabrics and is employed as food additive, food coloring agent and medical disinfectant. MG is found to be toxic to aquatic organisms, animals including humans. Copper is a commonly found metal in environment due to anthropogenic activities. Most of the microorganisms show sensitivity toward it. This adversely affects their growth and activity. In the present study, biodegradation of MG by a copper-tolerant bacterium has been investigated. Biodegradation was confirmed by UV-Vis and FTIR spectroscopy. The metabolites generated after degradation of MG were identified by LC/MS and a plausible pathway of MG degradation has been elucidated. Microbial and phyto toxicity of generated metabolites were also evaluated.

**Results:** A strain belonging to *Ochrobactrum pseudogrignonense* strain GGUPV1 was discovered from copper mine waste water. This bacterium could tolerate as high as 50 mM copper sulfate in minimal medium. It was observed that this bacterium could degrade 400 mg/L of MG in minimal medium. Decolorization of MG was also observed in presence of copper sulfate in the medium. Degradation of MG was confirmed by UV-Vis and FTIR spectroscopy. GC/MS study indicated that metabolites generated after degradation of MG were nontoxic to *Staphylococcus aureus*.

**Conclusions:** This is the first report showing degradation of MG by *Ochrobactrum pseudogrignonense*. This strain can be successfully employed for degradation of MG.

**Keywords:** *Ochrobactrum pseudogrignonense*, Malachite green (MG), Biodegradation, Copper mine waste water

## Background

Ever-increasing demand for colored products has lead to widespread use of dyestuffs (Daneshwar et al. 2007). Various industries discharge effluents containing unused dyes directly into the water bodies causing serious threat to the environment (Zollinger 1987). In an estimate, textile dyeing effluents discharge approximately 280,000 tons of dyes worldwide every year (Jin et al. 2007). Most of the dyes are stable and thus recalcitrant to biodegradation (Robinson et al. 2001). These dyes exhibit potential

toxicity to human and animals. Hence, treatment of dyeing wastewater is of utmost importance before its safe discharging into environment (Hazrat 2010). A large number of physiochemical methods are available for treatment of dye wastewater but these methods possess a constraint due to their limited versatility, high cost, low efficiency and interference by other wastewater constituents (Banat et al. 1996). These physiochemical methods also produce a lot of sludge posing a threat as secondary pollutant (Du et al. 2011). However, biological methods are available which are eco-friendly and completely mineralize organic pollutant (Pandey et al. 2007). These methods are inexpensive, have wide range applicability, low running cost, complete mineralization of dye to

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a nontoxic compound and eco-friendly (Forgacs et al. 2004). Malachite green (MG) is a water-soluble triphenylmethane cationic dye which is used to color fabrics (Zhou et al. 2015). It is also utilized in food and medical industry (Chowdhury et al. 2011). Apart from its toxicity on aquatic and terrestrial animals, it also elicits cytotoxicity on mammalian cell and causes formation of liver tumors (Srivastava et al. 2004). Due to its ill effects, it has been banned in many countries, but in some developing countries including India, it is still being used (Hameed and El-Khaiary 2008). Therefore, there is an urgent need to develop efficient and effective method which can treat MG dye from wastewater up to an extent which is nontoxic and can be easily disposed off to the environment in an eco-friendly way.

Recent thrust in the field of bioremediation is the use of multifaceted microorganisms. These organisms perform different roles in the environment viz. apart from biodegradation, they may exhibit plant growth-promoting activities, they may degrade multiple pollutants, or they show resistance toward toxic metals (Chaturvedi et al. 2013). Copper is one of the metals, which is normally found in high amounts in water bodies due to anthropogenic activities (Giller et al. 1998). Most of the microorganisms are susceptible to high doses of copper. Thus, presence of copper in water systems severely affects growth of microorganisms, leading to a loss in their biodegradation capabilities (De La Iglesia et al. 2006). In the present investigation, a copper-tolerant bacterial strain was discovered from copper mine waste water by employing enrichment technique. Potential of the isolate to degrade MG in presence and absence of copper ions was evaluated.

## Methods

### Microorganisms and culture conditions

Water samples were collected from the copper mines located at Balaghat, Malanjkhand Madhya Pradesh, India. It is an open-pit-type mine owned by Hindustan Copper limited. Geographical coordinates of Balaghat are 21° 47' 23.5248" north, 80° 47' 28.3344" east. Bacteria were isolated by enrichment technique. 5 mL of water sample was inoculated into 50 mL BSMY1 (Aksornchu et al. 2008) containing 5 mM CuSO<sub>4</sub> and incubated at 30 °C temperatures on rotary shaker at 120 rpm for 3 days. Fresh grown 1 mL of culture was transferred into BSMY1 medium containing 10, 20, 30, 40 and 50 mM CuSO<sub>4</sub> in different flasks. Finally, 0.1 mL of fresh culture growing in 50 mM CuSO<sub>4</sub> was spread on BSMY1 agar containing 50 mM CuSO<sub>4</sub> and incubated at 30 °C for 3 days. The most tolerant bacterial colony was observed and then they were used to further obtain the

pure culture. Bacteria were isolated that could tolerate the highest CuSO<sub>4</sub> concentration. For identification, the selected strain was sent to Chromas Biotech Pvt. Ltd, for 16S rDNA sequence analysis. The sequencing of rDNA was performed on payment basis. The sequence was matched against nucleotide sequences present in Gen Bank at NCBI database using BLASTn program. The nucleotide sequences showing similarity to the sequence of strain GGUPV1 were retrieved and a neighbor-joining phylogenetic tree was created using phylogeny.fr program (Dereeper et al. 2008).

BSMY1 medium contained the following (g/L); yeast extract—1, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>—0.3, MgSO<sub>4</sub>·7H<sub>2</sub>O—0.14, CaCl<sub>2</sub>·2H<sub>2</sub>O—0.2, NaCl—0.1, KH<sub>2</sub>PO<sub>4</sub>—0.05, K<sub>2</sub>HPO<sub>4</sub>—0.05, H<sub>3</sub>BO<sub>3</sub>—0.6, CoCl<sub>2</sub>·6H<sub>2</sub>O—0.17, CuCl<sub>2</sub>·2H<sub>2</sub>O—0.09, MnCl<sub>2</sub>—0.1, ZnCl<sub>2</sub>—0.22, glucose—1.0 pH-7.5

### Decolorization of MG

MG decolorization experiments 2.5 % (containing approximately  $1 \times 10^8$  CFU/mL) inoculum was transferred under sterile conditions to 50 mL BSMY1 medium taken in a 150-mL erlenmeyer flask supplemented with varying concentration of MG (50–400 mg/L). The flasks were incubated in static conditions at 30 °C for 96 h. A comparative study was also performed under shaking conditions at 120 rpm under similar conditions. Flasks containing 50 mL BSMY1 medium supplemented with 50–400 mg/L MG, without inoculum were designated as control and were kept under identical conditions. After incubation of 24 h, 10 mL culture was retrieved and centrifuged at 8000 rpm for 10 min to remove bacterial cells. Decolorization was analyzed in a UV–Vis spectrophotometer by taking absorbance at 620 nm (Parshetti et al. 2006). Percent decolorization was calculated according to Ayed et al. (2009). To study the effect of different concentrations of Cu<sup>2+</sup> ions on decolorization of MG, 10 and 20 mM CuSO<sub>4</sub> was supplemented to 50 mL BSMY1 medium containing 50 and 100 mg/L MG. The decolorization experiment was performed as stated above.

### UV–Vis spectral and FTIR analysis

Decolorization of MG was studied in UV–Vis spectroscopic analysis using Shimadzu UV–Vis spectrophotometer (UV-1800), the spectrum of MG (50 mg/L) and its degraded metabolites (50 mg/L) arising after 8 h of incubation was recorded in the range of 200–1000 nm (Chaturvedi et al. 2013). Biodegradation of MG was also monitored by Fourier transform infrared (FTIR) spectroscopy, for analysis samples were send to STIC, Cochin, Kerala, India. The analysis was carried out on payment basis (Chaturvedi et al. 2013).

### GC/MS analysis

The degraded metabolites arising after 24 h of incubation of strain GGUPV1 in BSMY1 medium containing MG (50 mg/L) were extracted with equal volume of ethyl acetate (Du et al. 2011). The ethyl acetate fraction was evaporated to dryness and was sent to SAIF, IIT Bombay, Mumbai, India for analysis. The analysis was performed on payment basis.

### Microbial and phytotoxicity studies of MG and its degraded metabolites

Microbial toxicity of MG and its degraded metabolites was studied on *Staphylococcus aureus* MTCC 737. A single colony of the strain was inoculated in 10 mL LB medium and incubated at 120 rpm at 30 °C. After overnight incubation, 100 µL culture was spread on the surface of nutrient agar plate. After spreading, four sterile filter paper discs were placed at four corners of the plates. MG (50 mg/L) and its degraded metabolites were extracted with equal volume of ethyl acetate (50 mL). The ethyl acetate fraction was evaporated to dryness. The solid residues were dissolved in 500 µL Milli-Q (MQ) water. 10 and 15 µL of MG and metabolites were placed on separate paper discs. The plates were incubated in a biological oxygen demand (BOD) incubator at 30 °C and were visually inspected (Chaturvedi et al. 2013).

Phytotoxicity of MG and its degraded metabolites was accessed by estimating the germination of seeds of *Vigna radiata* in presence of ethyl acetate extract of MG and its degraded metabolites (after evaporation of ethyl acetate, the solid residues were suspended in 20 mL MQ water). The seeds were surface sterilized with 2 % mercuric chloride for 20 min, and were washed thrice with double distilled water. Twenty seeds were placed on the surface of sterile cotton containing 20 mL MG and its metabolites separately and all plates were incubated at 20 °C in presence of 70 % humidity. In control plates, MQ water (20 mL) was used in place of MG solution. After 7 days of incubation, seed germination percentage and radical/plumule lengths were calculated (Du et al. 2011).

## Results and discussion

### Isolation of copper-tolerant microorganism

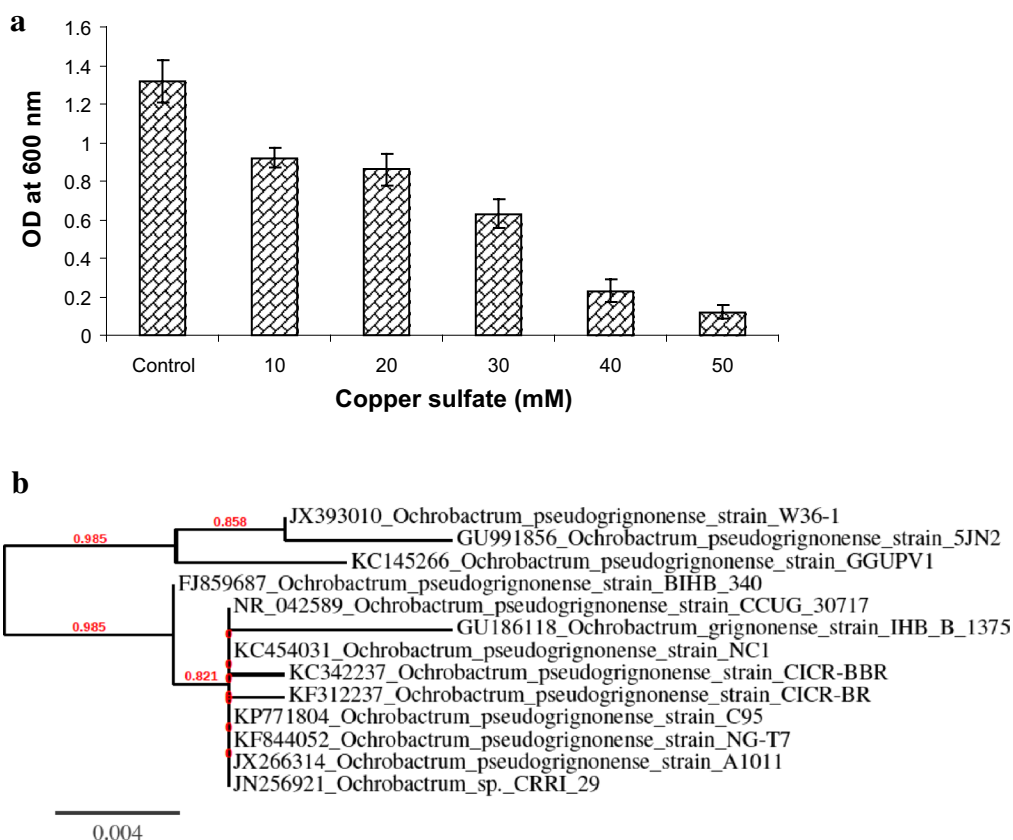
By employing enrichment technique in BSMY1 medium containing varying concentrations of CuSO<sub>4</sub>. A single bacterial colony was recovered which could tolerate as high as 50 mM CuSO<sub>4</sub>. Growth of this isolate in presence of varying concentration of copper sulfate is represented in Fig. 1a. It is evident that with increasing concentration of CuSO<sub>4</sub>, a gradual decrease in growth was observed. The decrease in growth was more pronounced in higher concentration of CuSO<sub>4</sub>, i.e., 40 and 50 mM. The possible mechanism of bacterial resistance to copper ions has

been attributed to retention of copper ions in periplasmic space of the bacterium and its removal from cytoplasm with the help of various enzymes (Anand et al. 2006). The strain was sent for identification on the basis of 16S rDNA sequencing; it was identified as *Ochrobactrum pseudogrignonense* strain GGUPV1 (Accession No. KC145266). The phylogenetic tree of the isolate is represented in Fig. 1b.

### Decolorization of MG

Synthetic dyes when discharged in water systems impart color to the water thus inhibit penetration of light in the water body (Daneshwar et al. 2007; Jin et al. 2007). They also interfere with transfer of oxygen in water leading to a decrease in dissolved oxygen (DO), and increase in chemical oxygen demand (COD), biological oxygen demand (BOD). This situation often leads to death of aquatic organisms (Banat et al. 1996; Chowdhury et al. 2011). Therefore, identification of microorganisms, which decolorize these dyes, is of utmost importance (Du et al. 2011). Decolorization potential of the strain GGUPV1 was studied in minimal medium BSMY1 containing varying concentration of MG (50–400 mg/L). Initially, comparison in decolorization was made under shaking and static conditions. The results are depicted in Fig. 2a. It is evident that under static conditions, decolorization of 50 and 100 mg/L MG after 24 h of incubation of dye was highest as compared to under shaking conditions. There are many reports which confirm that bacterial isolates show high rates of MG decolorization under static conditions as compared to shaking conditions. Parshetti et al. (2006) have reported complete decolorization of 50 mg/L MG by *Kocuria rosea* MTCC 1532 under static conditions. The apparent reason for this observation is availability of reduced electron carriers such as NADH<sub>2</sub> to reduce MG under static conditions, whereas under shaking conditions both O<sub>2</sub> and MG competes with these electron carriers leading to a decrease in decolorization (Du et al. 2011; Parshetti et al. 2006).

A time course study of MG (50–400 mg/L) decolorization was performed under static conditions (Fig. 2b). It is evident that 50–200 mg/L MG was completely decolorized after 48 h of incubation, whereas 300 mg/L MG was completely decolorized after 72 h of incubation and 400 mg/L of MG was incompletely decolorized after 96 h of incubation. There are several reports of degradation of high levels of MG by bacterial strains. This strain was able to mineralize 400 mg/L of MG in minimal medium, which was higher than previous reports made by Parshetti et al. (2006) in which *K. rosea* could mineralize 50 mg/L of MG in semisynthetic medium. Also, a strain of *Sphingomonas paucimobilis* was able to decolorize 50 mg/L of MG in minimal medium (Ayed et al. 2009).



**Fig. 1** **a** Growth of strain GGUPV1 in presence of different concentrations of  $\text{CuSO}_4$ , **b** phylogenetic tree of the strain GGUPV1

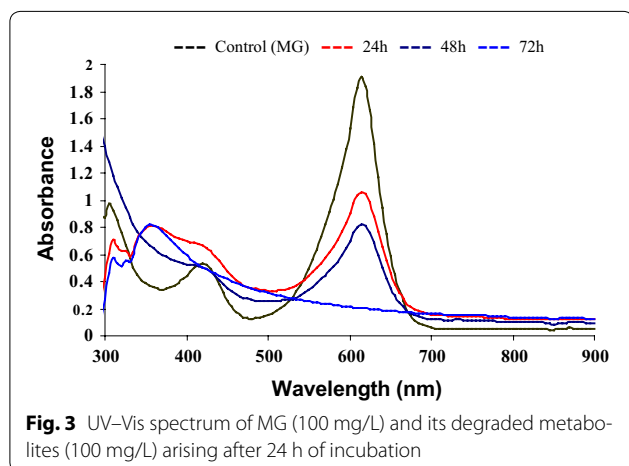
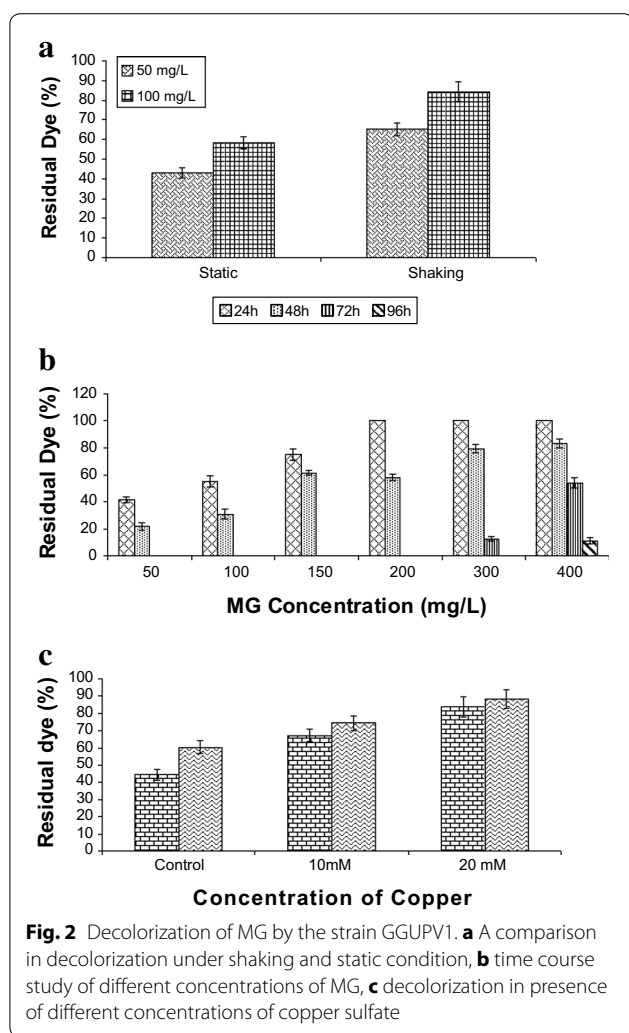
Decolorization of MG was also studied in presence of 10 and 20 mM  $\text{CuSO}_4$  after 24 h of incubation (Fig. 2c). This concentration was selected because reduction in bacterial growth was less as compared to higher concentrations. It was observed that in presence of copper ions, significant reduction in decolorization of MG took place. The reduction of decolorization was more prominent at 20 mM concentration as compared to 10 mM. The apparent reason for this observation could be toxicity of  $\text{CuSO}_4$  to bacterial cells. Similar result was also reported by Du et al. (2011) in *Pseudomonas* sp. strain DY1 in which pronounced reduction in MG decolorization was observed in presence of  $\text{Cu}^{2+}$  ions.

#### UV-Vis and FTIR analysis

In biological systems, decolorization of MG occurs by two processes, absorption or adsorption by bacterial/fungal cells and degradation. UV-visible spectroscopy is a valuable technique to differentiate between these two processes. UV-Vis spectrum of MG shows a single prominent peak at 615 nm. During absorption or adsorption, the intensity of this peak decreases with course of time but during degradation, there is disappearance of

this peak with concomitant appearance of some new peaks, which correspond to various metabolites produced during degradation of MG. MG degradation was studied by recording UV-Vis spectra (200–1000 nm) of ethyl acetate extract of MG (100 mg/L) and its degraded metabolites (100 mg/L) extracted after 24, 48 and 72 h of degradation (Fig. 3). The spectra of MG showed a characteristic major peak at 615 nm and two minor peaks at 420 and 305 nm, respectively. The spectra of degraded metabolites showed disappearance of the major-minor peaks and appearance of new peaks at 355 nm, respectively, indicating degradation of MG by strain GGUPV1. These results are in accordance with previous reports (Du et al. 2011), which demonstrate that after degradation of MG by *Pseudomonas* strain DY1, two new peaks appeared in the UV-Vis spectrum.

Degradation of MG was also confirmed by FTIR analysis of MG and its degraded metabolites (Fig. 4a, b). FTIR spectrum of MG showed distinct peaks in the fingerprint region (1500–500  $\text{cm}^{-1}$ ), which corresponds to mono- and para-substituted benzene rings and were distinct to MG. The peaks were observed at 714, 828  $\text{cm}^{-1}$ , corresponding to aromatic ring structure, 937  $\text{cm}^{-1}$ ,



corresponding to amine group, and  $1514\text{ cm}^{-1}$ , which corresponds to nitro aromatic group, respectively. Also, peaks at  $1167\text{ cm}^{-1}$  corresponds aromatic C–N

stretching vibrations. The peaks at  $1365$  and  $828\text{ cm}^{-1}$  were observed for  $\text{CH}_2$  scissoring and C55O stretching band (Fig. 4a).

The degraded metabolites showed characteristic peak at  $3431\text{ cm}^{-1}$  corresponding to C55O overtone,  $1654\text{ cm}^{-1}$  for aromatic ketones, and  $1458\text{ cm}^{-1}$  for aromatic group. Reduction of peaks at  $714$ ,  $937$ , and  $1514\text{ cm}^{-1}$  indicated loss of aromaticity of metabolites (Fig. 4b). These results are in accordance with previous reports of Chaturvedi et al. (2013), Ayed et al. (2009), and Kalyani et al. (2009), and confirming degradation of MG by strain.

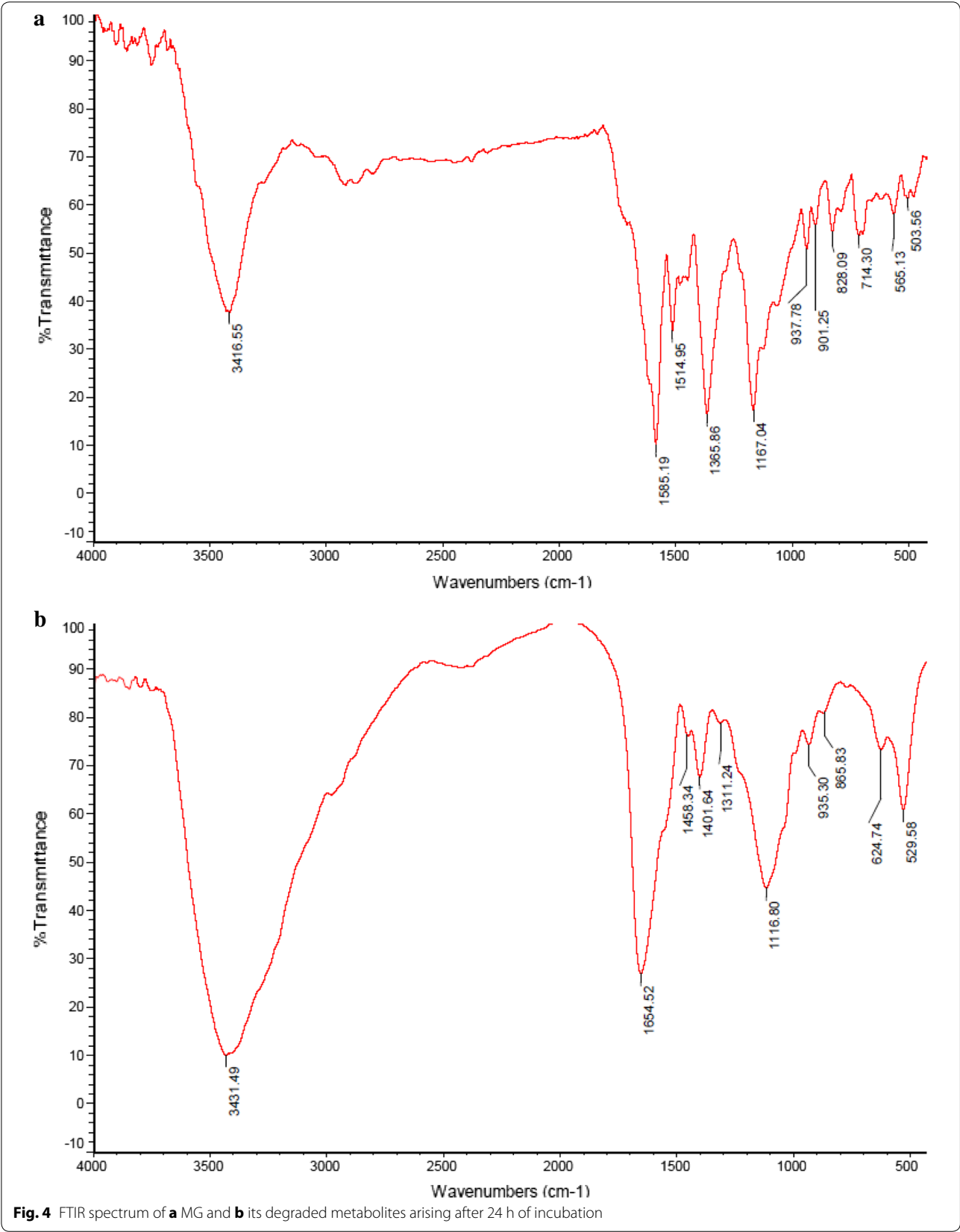
### GC/MS study

The metabolites generated after degradation of  $50\text{ mg/L}$  of MG were identified by GC/MS analysis (Fig. 5). The metabolites methanone, [4-(dimethyl amino)phenyl] phenyl ( $m/z$  148), phenol, 3-(demethylamino) ( $m/z$  136) and phenol, 3,5-demethoxy ( $m/z$  154) were identified. Previous studies have demonstrated that degradation of MG is facilitated by action of multiple enzymes such as MG reductase, Mn peroxidase and lignin peroxidase. (Parshetti et al. 2006; Ayed et al. 2009). Based on these available data, a pathway was proposed (Fig. 6). According to this pathway, MG is first reduced to lueco MG, which then is cleaved to produce methanone, [4-(dimethyl amino)phenyl]phenyl and phenol, 3-(demethylamino).

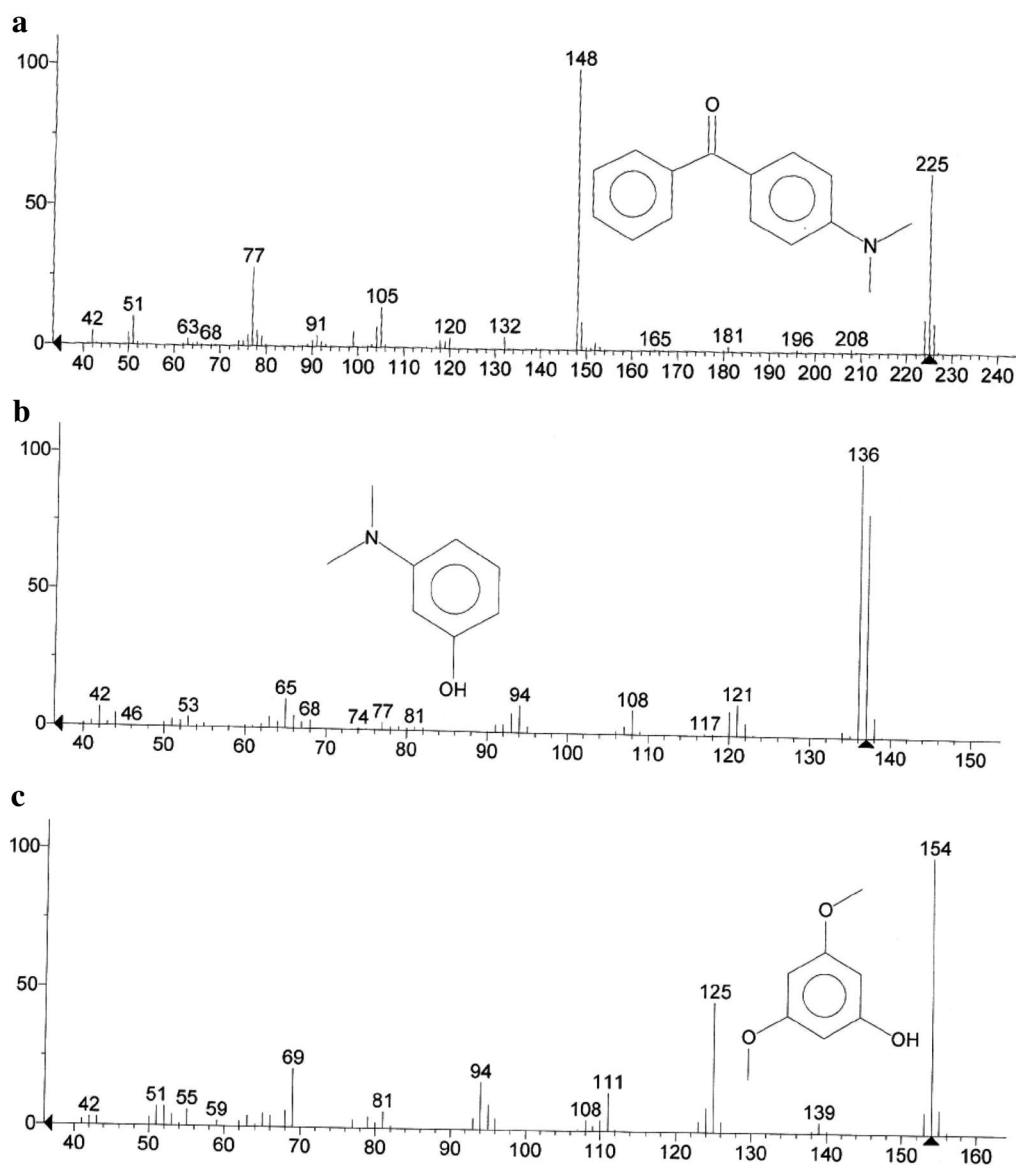
### Microbial and phytotoxicity of MG and its degraded metabolites

The toxicity of MG and its degradation products are major environmental concern. Microbial toxicity was studied by employing the conventional plate assay technique in the bacterium *S. aureus* MTCC 737. The results clearly showed that in the presence of MG ( $5$  and  $10\text{ }\mu\text{L}$ ), inhibitory zones were formed indicating toxicity of MG to the bacterium. When the bacterium was grown in presence of ethyl acetate extract of degraded metabolites ( $5$  and  $10\text{ }\mu\text{L}$ ), no zone of inhibition was observed. It indicated nontoxic nature of degraded metabolites to the bacterium. Similar results have also been reported by Chaturvedi et al. (2013), Ayed et al. (2009), which prove that the metabolites produced after degradation of MG are less toxic to microorganisms.

Usually, synthetic dyes and their degraded metabolites are disposed in water bodies without any treatment. Often this water is used for irrigation purposes. Therefore, phytotoxicity of MG and its degradation products was also evaluated. Phytotoxicity studies of MG and its degraded metabolites were performed with mung bean (*Vigna radiata*), which is a most widely grown pulse crop in India. The results are depicted in Table 1. It is clearly



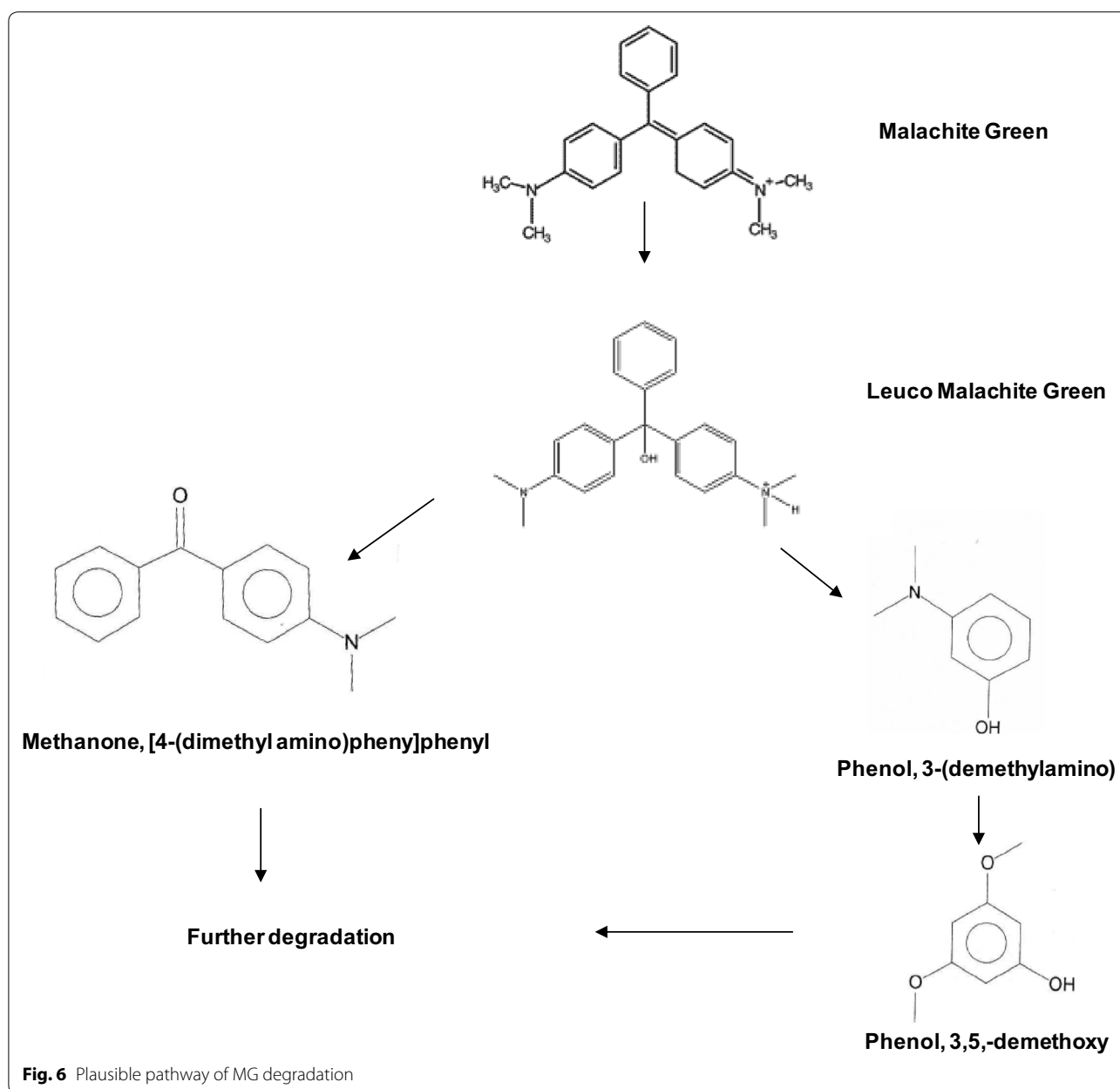
**Fig. 4** FTIR spectrum of **a** MG and **b** its degraded metabolites arising after 24 h of incubation



**Fig. 5** Metabolites detected in GC/MS analysis **a** methanone, [4-(dimethyl amino)phenyl]phenyl ( $m/z$  148), **b** phenol, 3-(dimethylamino) ( $m/z$  136) and **c** phenol, 3,5-dimethoxy ( $m/z$  154)

evident that MG is toxic to *V. radiata*, in presence of MG seed germination was significantly reduced ( $40 \pm 0.57$ ) when compared to control ( $86 \pm 1.53$ ). In the presence of degraded metabolites, the germination was higher ( $70 \pm 0.57$ ) indicating reduced toxicity of metabolites. Apart from germination, radical/plumule length also showed a drastic reduction in presence of MG. In presence of degraded metabolites, the reduction was not significant. These results demonstrate that MG is potentially toxic to *V. radiata* whereas its degraded metabolites are to a lesser extent. Our findings are consistent with

previous reports showing that MG is toxic to microbes and plants, its degradation by microbes leads to a significant reduction in its toxicity (Chaturvedi et al. 2013; Parshetti et al. 2006; Ayed et al. 2009). The results of cytotoxicity and phytotoxicity analysis are in accordance with earlier findings. Du et al. (2011) reported that degraded product formed by biodegradation of MG by *Pseudomonas aeruginosa* NCIM 2074 are non toxic. Similarly Parshetti et al. (2006) reported that biodegradation product of MG formed by action of *Kocuria rosea* MTCC 1532 is nontoxic.



**Table 1** Phytotoxicity study of malachite green (MG) and its degradation product

Sample name	Control (dis- tilled water)	Control with mala- chite green (MG)	Metabolites gen- erated after deg- radation with the strain GGUPV1
Germination (%)	86 ± 1.53	40 ± 0.57	70 ± 0.57
Shoot length (cm)	8.93 ± 0.152	6.53 ± 0.412	7.03 ± 0.152
Root length (cm)	5.23 ± 0.30	2.6 ± 0.40	4.13 ± 0.321

All the values are mean of germinated seeds of three experiments along with standard deviation

## Conclusions

In the present study, a copper-tolerant bacterium identified as *Ochrobactrum pseudogrignonense* strain GGUPV1 was discovered from copper mine waste water. This strain was able to degrade 400 mg/L MG in minimal medium. This bacterium was also able to decolorize 100 mg/L MG in presence of 10 and 20 mM CuSO<sub>4</sub>. Biodegradation was confirmed by UV–Vis and FTIR spectroscopy. GC/MS analysis led to propose a pathway of MG degradation operative in this bacterium, which involved formation of metabolites such as methanone, [4-(dimethyl amino)

phenyl]phenyl ( $m/z$  148) and phenol, 3-(demethylamino) ( $m/z$  136). Further, the degraded metabolites were non-toxic to *S. aureus* MTCC 737 and *V. radiata*.

#### Authors' contributions

VC carried out the isolation of bacterium, and degradation, microbial and phytotoxicity experiments. PV designed the whole study and prepared the manuscript. Both authors read and approved the final manuscript.

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#### Competing interests

The authors declare that they have no competing interests.

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