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Decolorization and detoxification of sulfonated azo dye methyl orange by *Kocuria rosea* MTCC 1532

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ABSTRACT

Kocuria rosea (MTCC 1532) showed 100% decolorization of methyl orange (50 mg l^{-1}) under static condition. The optimum pH and temperature for dye decolorization was 6.8 and 30 °C, respectively. The *K. rosea* (MTCC 1532) showed maximum decolorization of methyl orange when growth medium containing yeast extract as compared to other substrates. The culture exhibited significant ability to decolorize repeated additions of dye, with reduction in time up to 12 h at eighth dye aliquot addition. Significant induction of reductases (NADH-DCIP reductase and azoreductase) suggests its involvement in decolorization of methyl orange. The metabolites formed after decolorization of methyl orange, such as 4-amino sulfonic acid and N,N′-dimethyl *p*-phenyldiamine were characterized using FTIR and MS. Phytotoxicity and microbial toxicity study showed the methyl orange was toxic and metabolites obtained after its decolorization was nontoxic for experimental plants (*Triticum aestivum* and *Phaseolus mungo*) and bacteria (*K. rosea*, *Pseudomonas aurugenosa* and *Azatobacter vinelandii*).

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1. Introduction

Approximately 10,000 different dyes and pigments are used industrially and over 0.7 million tones of synthetic dyes are produced annually worldwide. During processing up to 15% of the used dyestuffs is lost in the industrial effluents [1]. Azo dyes accounts for the majority of all textile dyestuffs produced and have been the most commonly used synthetic dyes in the textile, food, paper making, printing, leather and cosmetic industries [2]. In addition to their visual effect and their adverse impact in terms of chemical oxygen demand (COD), many synthetic azo dyes show their toxic, carcinogenic and genotoxic effects [3]. Thus, the wastewater must be treated before release into the natural environment.

Conventional wastewater treatment is not efficient to remove recalcitrant dyestuffs from effluents. For the removal of dyes from wastewater effluent physicochemical methods like; (adsorption, chemical precipitation, photolysis, chemical oxidation and reduction, electrochemical treatment) are not suitable due to high cost, low efficiency and in-applicability to a wide variety of dyes [4]. For biological treatment of the wastewater containing dyes, microbial or enzymatic decolorization and degradation is an ecofriendly, cost-competitive alternative to chemical decomposition process and could help to reduce this enormous water consumption [5–7].

The potentiality of the microorganisms pointed out in literature as degradation agents of several complex chemicals, including dyes as their sole source of carbon, although much attention has been focused on microbial degradation, detoxification and mineralization of dyes. To understand the metabolic pathways, by which these dyes are consumed by bacteria, having importance for the application of bacteria in the treatment of textile effluents. Decolorization of sulfonated azo dyes by using bacteria and fungus under static condition was described earlier [4,8]. The bacterial decolorization was associated with involvement of various oxidative (laccase) and reductive (NADH-DCIP reductase and azoreductase) enzymes [9,10].

In the present study we standardized various parameters such as agitation, temperature, pH different initial dye concentration, and media supplements to achieve maximum dye degradation. The intermediates formed during the degradation of methyl orange were analyzed by using UV–visible, Fourier transform infrared spectroscopy (FTIR) and gas chromatography and mass spectroscopy (GC–MS). Also proposed possible pathway for degradation of methyl orange and enzymatic system involved.

2. Materials and methods

2.1. Microorganisms and culture conditions

The strain *Kocuria rosea* (MTCC 1532) used for the present study was obtained from MTCC Chandighar, India. Pure culture was

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maintained on nutrient agar and stored at 4 °C. The nutrient broth ($g\,l^{-1}$; beef extract, 1; yeast extract, 2; peptone, 5; NaCl, 5) was used for decolorization experiment.

2.2. Dyes and chemicals

ABTS (2,2-azinobis (3-ethylbenzothiazolin-6-sulfonic acid)) was purchased from Sigma–Aldrich (Mumbai, India). Yeast extract and glucose were obtained from Hi Media laboratory (Mumbai, India). Catechol, n-propanol, and other fine chemicals were purchased from Sisco Research Laboratories (India). Methyl orange was obtained from Sd. Fine Chemicals Limited (Biosar, India). All chemicals were of the highest purity and of an analytical grade.

2.3. Decolorization experiments

All decolorization experiments were performed in triplicates. A loopful of microbial culture was inoculated in 250 ml Erlenmeyer flask containing 100 ml nutrient broth (pH 6.5) and incubated at 30 °C for 24 h at static condition. Dye was added at concentration of 50 $mg\,l^{-1}$ under sterile condition and after 24 h (3 ml) of the culture media was withdrawn at different time intervals. Aliquot was centrifuged at 5000 rpm for 15 min to separate the bacterial cell mass, clear supernatant was used to measure decolorization. The percentage of decolorization was calculated as follows.

$$\%\,\text{decolorization} = \frac{\text{initial absorbance} - \text{observed absorbance}}{\text{initial absorbance}} \times 100$$

Decolorization performance of methyl orange (50 $mg\,l^{-1}$) by *K. rosea* was studied at varying temperatures (10, 20, 30, 40 and 50 °C) and at various increasing dye concentration (10, 30, 50, 70 and 100 $mg\,l^{-1}$) in nutrient broth at static condition. Studies on effect of various carbon and nitrogen sources were carried out in semi-synthetic medium (dye concentration 50 $mg\,l^{-1}$) at 30 °C in static condition. 10% inoculum of optical density 1.0 (620 nm) grown in nutrient broth for 24 h was used for inoculation of semi-synthetic medium. To study the effect of carbon and nitrogen sources on decolorization of methyl orange, semi-synthetic medium having following composition was used ($g\,l^{-1}$): methyl orange, 0.050; $(NH_4)_2SO_4$, 0.28; NH_4Cl, 0.23; KH_2PO_4, 0.067; $MgSO_4{\cdot}7H_2O$, 0.04; $CaCl_2{\cdot}2H_2O$, 0.022; $FeCl_3{\cdot}6H_2O$, 0.005; yeast extract, 0.2; NaCl, 0.15; $NaHCO_3$, 1.0 and 1 $ml\,l^{-1}$ of a trace element solution containing ($g\,l^{-1}$) $ZnSO_4{\cdot}7H_2O$, 0.01; $MnCl_2{\cdot}4H_2O$, 0.1; $CuSO_4{\cdot}5H_2O$, 0.392; $CoCl_2{\cdot}6H_2O$, 0.248; $NaB_4O_7{\cdot}10H_2O$, 0.177; $NiCl_2{\cdot}6H_2O$, 0.02 with different carbon and nitrogen sources (1% each) such as glucose, sucrose, lactose, maltose, molasses (carbon source), yeast extract, NH_4Cl, peptone (nitrogen source). Further decolorization of repeated addition of dye (50 $mg\,l^{-1}$) aliquots to culture media was also studied in nutrient broth at static condition.

2.4. Enzyme assays

2.4.1. Preparation crude enzyme

K. rosea was grown in 100 ml nutrient broth at 30 °C for 24 h, centrifuged at 10,000 × g for 20 min before addition of dye. These cells (75 $mg\,ml^{-1}$) were suspended in potassium phosphate buffer (50 mM, pH 7.4) for sonication (sonics-vibracell ultrasonic processor company name), keeping sonifier output at 40 A maintaining temperature at 4 °C and giving 7 strokes, each of 1 s with 1 min interval. This extract was used as enzyme source without centrifugation [5]. Similar procedure was followed to the control cells (0 h) and the cells obtained after complete decolorization (72 h).

2.4.2. Enzyme activities

Activities of lignin peroxidase, laccase, and tyrosinase were assayed spectrophotometrically in cell free extract. Laccase activity was determined in a reaction mixture of 2 ml containing ABTS (10%) in 0.1 M acetate buffer (pH 4.9) and measured increase in optical density at 420 nm [11]. Tyrosinase activity was determined in a reaction mixture of 2 ml, containing catechol (0.01%) in 0.1 M phosphate buffer (pH 7.4) at 410 nm [6]. Lignin peroxidase activity was determined by monitoring the formed propanaldehyde at 300 nm in a reaction mixture of 2.5 ml containing 100 mM n-propanol, 250 mM tartaric acid, 10 mM H_2O_2 [12]. All enzyme assays were carried out at 30 °C where reference blanks contained all components except the assayed enzyme. One unit of enzyme activity was defined as amount of enzyme required to increase 1.0 unit of absorbance min^{-1}.

The NADH-DCIP reductase activity was determined by using a procedure reported earlier [13]. The assay mixture contained 50 μM DCIP, 28.57 mM NADH in 50 mM potassium phosphate buffer (pH 7.4) and 0.1 ml of enzyme solution (cell free extract) in a total volume of 5.0 ml. The DCIP reduction was calculated using the extinction coefficient of 19 $mM^{-1}\,cm^{-1}$.

Azoreductase activity in cell fractions was assayed by the modification of earlier method [14]. The reaction mixture (0.35 mM NADH, 24 mM methyl orange, and 250 ml cell extract) and both controls A (24 mM methyl orange) and B (0.35 mM NADH and 24 mM methyl orange) had 3.0 ml of total volume in 100 mM sodium–potassium phosphate buffer (pH 7.5). The decreasing absorbance of NADH at 340 nm was monitored using a Hitachi U-2800 spectrophotometer in 10 min. One unit of enzyme activity was defined as the amount of enzyme that reduced 1 μmol of NADH min^{-1}. The activity of aminopyrine *N*-demethylase was determined using the procedure reported earlier [15]. The assay of aminopyrine N-demethylase activity included incubations in 50 mM N-2-Hydroxymethyl piperazine-N-2 ethane sulfonic acid buffer (pH 7.8), containing NADPH-generating system (NADPH 2.6 mM, glucose 6-phosphate 12.5 mM, glucose 6-phosphate dehydrogenase 4 units) and 0.5 ml of enzyme solution (cell free extract) of incubation medium. After the addition of 0.4 ml of aminopyrine (80 mM) to the incubation medium, the reaction mixture was incubated at 37 °C for 10 min and adding 1 ml of ice cold 20% trichloroacetic acid solution terminated the reaction. The amount of formaldehyde liberated was determined colorimetrically using Nash reagent. All enzyme assays were run in triplicate and average rates were calculated to represent the enzyme activity.

2.5. Microbial toxicity study

The toxicity of methyl orange (500 and 1000 ppm) and its degradation product (ethyl acetate extracted and dried, 500 and 1000 ppm) was studied in relation to an organism exploited for decolorization (i.e., *K. rosea*), in addition, toxicity effects were studied for a phosphate-solubilizing bacterium *Pseudomonas aeruginosa* and a nitrogen-fixing bacterium *A. vinelandii*. The toxicity effect was measured in terms of a zone of inhibition (diameter, in cm) after 24 h of incubation at 30 °C.

2.6. Phytotoxicity study

The ethyl acetate extracted products of methyl orange degradation were dried and dissolved in 5 ml distilled water to make a final concentration of 100 ppm. The phytotoxicity study was carried out (at room temp.) in relation to *Triticum aestivum* and *Phaseolus mungo* (10 seeds of each) by watering separately 5 ml sample of control methyl orange (100 ppm) and metabolites obtained after its decolorization (100 ppm) per day. Control set was carried out using distilled water at the same time. Germination (%) and length of plumule (shoot) and radicle (root) was recorded after 7 days.

2.7. Analysis of metabolites formed after decolorization of methyl orange

The culture media was centrifuged after complete decolorization of methyl orange at 7000 rpm for 20 min. The metabolites presents in culture supernatant were extracted using equal volume of ethyl acetate, dried over anhydrous Na_2SO_4 and evaporated to dryness in rotary evaporator. The crystals obtained were dissolved in small volume of methanol and used for further analysis. The Fourier transform infrared spectroscopy (FTIR) analysis was done in the mid IR region of 400–4000 cm^{-1} with 16 scan speed. The samples were mixed with spectroscopically pure KBr in the ratio of 5:95, pellets were fixed in sample holder, and the analyses were carried out. Rotary vacuum evaporated sample was dissolved in methanol and gas chromatography mass spectroscopy (GC–MS) analysis of metabolites were carried out using a Shimadzu 2010 MS Engine, equipped with integrated gas chromatograph with a HP1 column (60 m long, 0.25 mm id, nonpolar). Helium was used as carrier gas at a flow rate of 1 ml min^{-1}. The injector temperature was maintained at 280 °C with oven conditions as: 80 °C kept constant for 2 min^{-1} increased up to 200 °C with 10 °C min^{-1} raised up to 280 °C with 20 °C min^{-1} rate. The compounds were identified on the basis of mass spectra and using the NIST library.

2.8. Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparison test.

3. Results and discussion

3.1. Decolorization experiment

The strain *K. rosea* completely 100% decolorized methyl orange (50 mg l^{-1}) under static condition. There was no decolorization at shaking condition. These observations suggest that the decolorization performance of *K. rosea* is better at static condition where depletion in oxygen content is followed. The reason for decreased decolorization at shaking condition could be competition of oxygen and the azo compounds for the reduced electron carriers under aerobic condition [8]. Micro-aerobic condition was probably more supportive in the degradation of the azo dye than aerobic condition [16].

Maximum decolorization ability was observed at 30 °C (tested temp. 10, 20, 30, 40 and 50 °C) (data not shown). To confirm whether this decolorization was due to microbial action or change in pH, the change in pH was recorded which was in the range of 6.5–6.8.

3.2. Effect of initial dye concentration on decolorization

The textile effluent contains variable concentration of textile dyes. It was approximate about 20–200 mg l^{-1}. In order to check the decolorization efficiency of *K. rosea*, we exposed them for various concentration of methyl orange. The percentage of decolorization was decreased with increasing dye concentration (Fig. 1). The percent decolorization and time required at mg l^{-1} dye concentration was 10 (100%, 48 h), 30 (100%, 60 h), 50 (100%, 72 h), 70 (73%, 120 h) and 100 (40%, 120 h) in 100 ml batch culture of *K. rosea* in nutrient broth. The percentage decolorization was decreased beyond the dye concentration 50 mg l^{-1} even at longer time (120 h). Reduction in the decolorization may result from the toxicity of the dye to bacteria and/or inadequate biomass concentration for the uptake of higher concentrations of dye [5,7].

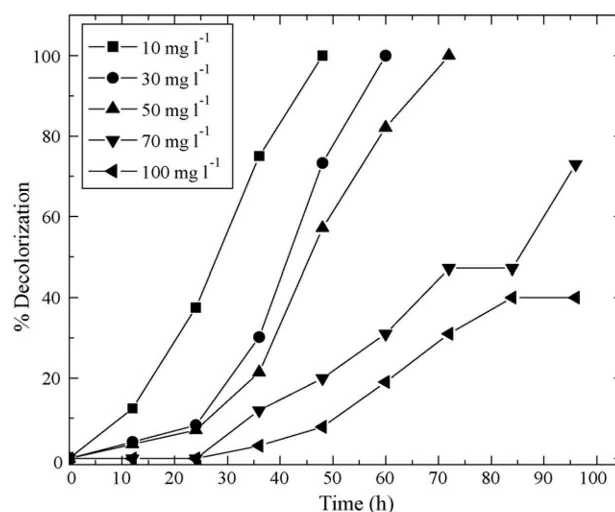

Fig. 1. Effect of increasing concentration of dye on decolorization performance of *Kocuria rosea*. (■) 10 mg l^{-1}; (□) 30 mg l^{-1}; (♦) 50 mg l^{-1}; (▲) 70 mg l^{-1}; (△) 100 mg l^{-1}.

3.3. Effect of carbon and nitrogen sources on decolorization of methyl orange

The efficacy of *K. rosea* to decolorize methyl orange in presence of additional carbon and nitrogen sources (1%) was tested in order to obtain efficient and faster decolorization. In synthetic medium only 8.3% decolorization was observed within 48 h (Table 1). The maximum percent of decolorization was observed with yeast extract (90%), while less decolorization was observed with other supplements of carbon and nitrogen source within 48 h. With glucose as a carbon source, the culture showed negligible decolorization (6%) where as presence of lactose, maltose and sucrose showed 43.33%, 33.33% and 23.7% decolorization, respectively, decolorization was not observed with molasses. Peptone and NH_4Cl as a nitrogen source exhibited only 15% decolorizing ability, So from these results, we observed that presence of nitrogen source yeast extract in synthetic media showed maximum decolorization of methyl orange by *K. rosea*. Similar result was also reported by bacterial consortium RVM in which when yeast extract was omitted from the medium there is no decolorization of reactive violet 5 [17].

3.4. Decolorization repeated additions of dye aliquots

Further study was carried out to test the ability of our culture to decolorize repeated additions of dye aliquots. Upon 100% decolorization of first dye (50 mg l^{-1}) aliquot within 72 h, second dye aliquot was added, which was also decolorized (100%) within next 72 h. The culture caused 100% decolorization of a third, fourth, and

Table 1
Effect of carbon and nitrogen sources (in semi-synthetic media) on decolorization of methyl orange by *K. rosea*.

Media	%Decolorization
SM	8.3 ± 0.41
SM + yeast extract	90 ± 4.50
SM + molasses	ND
SM + NH_4Cl	15 ± 0.75
SM + lactose	43.33 ± 2.16
SM + maltose	33.33 ± 1.66
SM + sucrose	23.7 ± 1.18
SM + glucose	6 ± 0.30
SM + peptone	15.38 ± 0.76

SM: semi-synthetic media; ND: no decolorization.

Table 2
Enzyme activities profile in cells before (control) and after decolorization.

Enzyme assay	Control	After decolorization
DCIP reductase[a]	17.80 ± 0.268	26.82* ± 0.366
Azoreductase[b]	20.2 ± 0.15	40.6** ± 0.13
Laccase[c]	0.009 ± 0.002	0.006* ± 0.001
Tyrosinase[c]	0.021 ± 0.006	0.015* ± 0.013
Lignin peroxidase[c]	0.011 ± 0.001	0.007* ± 0.005
Aminopyrine N-demethylase[d]	0.013 ± 0.008	0.010* ± 0.02

Values are mean of three experiments SEM (±; standard error of mean). Significantly different from control cells at $^{*}P < 0.05$, $^{**}P < 0.001$ by one-way ANOVA with Tukey–Kramer multiple comparisons test.
[a] μg DCIP reduced min^{-1} mg^{-1} of protein.
[b] μmol NADH reduced min^{-1} mg^{-1} of protein.
[c] Units ml^{-1} min^{-1}.
[d] nmoles of formaldehyde librated min^{-1} mg^{-1} of protein.

fifth aliquot, which was subsequently added, within 60 h, 24 h, and 18 h of addition, respectively. Further sixth, seventh, eight aliquot additions caused 100% decolorization only within 12 h. The reduction in time for methyl orange decolorization by *K. rosea* in next constitutes cycle might be due to acclimatization of this bacterial strain to the particular dye [8]. The culture exhibited the ability to decolorize repeated additions within less time, which is significant for its commercial application.

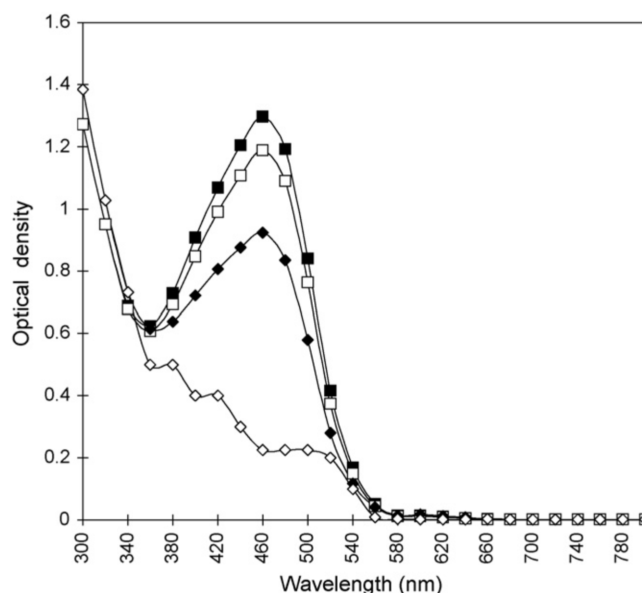

Fig. 2. UV–visible spectral changes observed during decolorization of methyl orange. (■) 0 h; (□) 24 h; (⧫) 48 h; (◊) 72 h.

3.5. Enzyme analysis

In order to get additional insight in to decolorization mechanism, lignin peroxidase, laccase, tyrosinase, aminopyrine *N*-demethylase, DCIP reductase, and azo reductase enzyme activities were monitored over time.

In present study, significant increase in the enzyme activities of azoreductase and NADH-DCIP reductase were observed over period of methyl orange decolorization by *K. rosea* than tyrosinase, lignin peroxidase, aminopyrine *N*-demethylase and laccase enzyme activities (Table 2). This suggests the reductases are (azoreductase and NADH-DCIP reductase) involved in decolorization of methyl orange. Generally azo dyes can be cleaved symmetrically and asymmetrically with an active site available for an enzyme to excite the molecule [8,18]. Reductive biodegradation takes place upon action of enzymes such as azo reductases. The azo dye reduction in anaerobic incubation is non-specific process in which reducing equivalents from either biological or chemical source are transferred to the dye. It was stated that the reducing equivalents formed during anaerobic oxidation of carbon sources, are used for the reduction of azo bond and there are reports concerning the degradation of organic nitrogen containing compounds under static anoxic condition [8,18]. The involvement of NADH-dependent azoreductases from *Pseudomonas luteola*, *Escherichia coli*, *Pigmentiphaga kullae* K 24, *Enterobacter agglomerans* and *Bacillus* sp. strain SF was described earlier [2,19–22]. *E. coli* azoreductase was able to decolorize a wide range of structurally complex sulfonated azo dyes [23].

3.6. Microbial toxicity studies

The microbial toxicity studies revealed, zone of inhibition was observed with control methyl orange with all bacterial strains studied whereas its degradation products showed comparatively less growth inhibition (Table 3). These findings suggest that the degradation products were less toxic to an exploited bacterium *K. rosea*, a phosphate-solubilizing bacterium *P. aeruginosa*, and nitrogen-fixing bacterium *A. vinelandii*, compared with the control methyl orange.

Table 3
Microbial toxicity studies of methyl orange and metabolites obtained after its decolorization.

Bacteria	Diameter of inhibition zone (cm)			
	Methyl orange (500 ppm)	Degradation product (500 ppm)	Methyl orange (1000 ppm)	Degradation product (1000 ppm)
K. rosea	0.5	N.I.	1.3	N.I.
P. aurugenosa	0.5	0.2	0.7	0.3
A. vinelandii	0.7	0.1	1.0	0.2

Table 4
Toxicity of methyl orange and its degradation products extracted (100 ppm) for the *Sorghum vulgare* and *Phaseolus mungo*.

Parameters studied	*Triticum aestivum*			*Phaseolus mungo*		
	Distilled water	Methyl orange (100 ppm)	Extracted metabolite (100 ppm)	Distilled water	Methyl orange (100 ppm)	Extracted metabolite (100 ppm)
Germination (%)	100	80	100	100	70	90
Plumule (cm)	13.58 ± 1.67	2.3** ± 0.24	9.34** ± 0.40	5.12 ± 0.40	0.48* ± 0.21	3.18* ± 0.32
Radical (cm)	6.47 ± 1.13	0.22** ± 0.04	4.21** ± 0.41	3.87 ± 0.34	0.12** ± 0.03	2.20** ± 0.21

Values are mean of germinated seeds of two experiments, SEM, significantly different from the control (seeds germinated in distilled water) at $^{*}P < 0.05$, $^{**}P < 0.001$ by one-way ANOVA with Turkey–Kramer multiple comparison test.

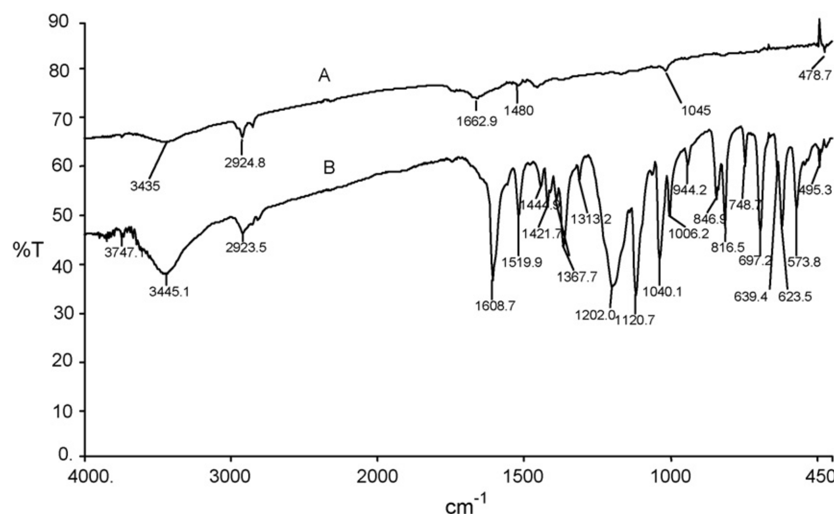

Fig. 3. FTIR analysis of methyl orange (A) and metabolites obtained after its decolorization (B).

3.7. Phytotoxicity studies

We observed that seed germination (%) and length of plumules and radicles were significantly higher in the degradation products than in the control dye (Table 4). Phytotoxicity study revealed the germination (%) of the both *T. aestivum* and *P. mungo* seeds was less in presence of methyl orange treatment as compare to its degradation product and distilled water. The methyl orange itself significantly affected the length of plumule and radical than its degradation product, indicates less toxicity of the degradation products and water. Similarly, earlier report showed products of methyl orange degradation were less toxic than the original azo dye [24]. This study indicates the detoxification of methyl orange by a *K. rosea*.

Table 5
GC–MS data for metabolites obtained after decolorization of methyl orange.

No. from Fig. 4	Metabolites	Retention time (min)	Mass peak (*m*/*z*)	Mass spectrum
II	4-Amino sulfonic acid	16.10	177	Inten.(x100); peaks: 28, 36, 39, 52, 65, 74, 80, 92, 93, 108, 109, 173, 177; m/z
III	N,N′-dimethyl *p*-phenylenediamine	19.12	136	Inten.(x100); peaks: 15, 28, 39, 52, 60, 65, 77, 93, 107, 121, 127, 136; m/z

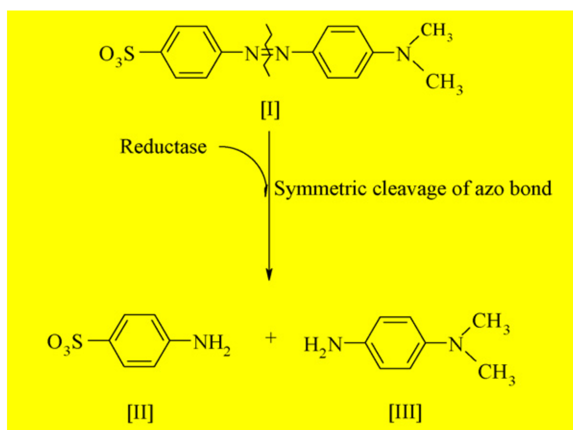

Fig. 4. Proposed pathway for degradation of methyl orange using *K. rosea*. [I] = methyl orange; [II] = 4-amino sulfonic acid; [III] = N,N′-dimethyl *p*-phenylenediamine.

3.8. Biodegradation analysis

UV–visible scan (300–800 nm) of culture supernatants withdrawn at different time intervals indicated decolorization and decrease in dye concentration from batch culture (Fig. 2). Absorbance at maximum wavelength (λ_{max}) observed at 460 nm (0 h) was decreased with small shift towards lower wavelength after complete decolorization of dye (72 h) indicates the formation of other metabolites. The nature of degradation product was confirmed from FTIR analysis (Fig. 3). Comparison of FTIR spectrum of control dye with metabolites extracted after complete decolorization clearly indicated the biodegradation of the parent dye compound by *K. rosea*. FTIR spectra of control methyl orange display peak at 2923 cm^{-1} for asymmetric $–CH_3$ stretching vibrations, peak at 1444 cm^{-1} and 1421 cm^{-1} for C=C–H in plane C–H bend, peak at 1006 cm^{-1}, 944 cm^{-1} and 846 cm^{-1} for ring vibrations and peak at 816 cm^{-1} for disubstituted benzene ring, this confirms aromatic nature of the dye. The peak at 1608 cm^{-1} for –N=N– stretch and peak at 1201 cm^{-1} and 1120 cm^{-1} for –C–N confirms the azo nature of the dye. Peaks at stretch 697 cm^{-1}, 623 cm^{-1} and 573 cm^{-1} for –C–S– stretching vibrations and peak at 1367 cm^{-1} for S=O stretching vibrations confirms the sulfonic nature of dye. The FTIR spectra 72 h extracted metabolites display the peak at 3435 cm^{-1} for N–H bend, peak at 2924 cm^{-1} for asymmetric $–CH_3$ stretching vibrations, peak at 1662 cm^{-1} for C=C aromatic stretching vibrations, peak at 1480 cm^{-1} for ring vibrations and peak at 1045 cm^{-1} for S=O stretching vibrations. This indicates the formation of sulfonated aromatic amines from methyl orange degradation by *K. rosea*.

We have proposed a pathway for degradation of methyl orange by *K. rosea* on the basis of enzyme action and GC–MS data. Initial step in the decolorization of methyl orange involves azoreductase mediated symmetric cleavage of azo bond which results into formation of 4-amino sulfonic acid and N,N-dimethyl *p*-phenylenediamine (Fig. 4). These two metabolites were identified using GC–MS, which supports the proposed pathway (Table 5). This suggests the *K. rosea* degrade azo dye methyl orange via symmetric cleavage of its azo bond by azo reductase.

4. Conclusions

The present study indicates potential of *K. rosea* to 100% decolorize and degrade methyl orange at static condition. The culture has ability to decolorize methyl orange in repeated additions within less time, which is significant for its commercial application. As decolorization progress the inducible nature of azoreductase and NADH-DCIP reductase suggest there involvement in the dye degradation. Degradation products were nontoxic for plants (*Sorghum vulgare* and *P. mungo*) and microorganisms (*K. rosea*, *P. aeruginosa*, and *A. vinelandii*) compared with the control dye. Overall findings suggested the ability of *K. rosea* for the decolorization of azo dye and ensured the ecofriendly degradation of methyl orange.

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