ENVIRONMENTAL BIOTECHNOLOGY

Effective bioremoval and detoxification of textile dye mixture by *Alishewanella* sp. KMK6

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Abstract *Alishewanella* sp. strain KMK6 was able to degrade mixture of textile dyes (0.5–2.0 g l^{-1}) within 8 h. An initial 28 % reduction in COD was observed immediately after decolorization at static anoxic conditions which on further incubation at shaking conditions reduced by 90 %. Partially purified azoreductase was able to utilize different azo dyes as substrates. The HPLC profile of dye degradation showed formation of metabolic products. Further FTIR analysis showed significant changes in the peaks corresponding to functional groups present in dye mixture and its degradation products. The genotoxicity assessment showed that the dye degradation products were non-toxic compared to dye mixture.

Keywords *Alishewanella* sp. KMK6 · Azoreductase · Biodegradation · Azo dyes · COD

Introduction

Azo dyes have been the largest chemical class of dyes frequently used for textile dyeing and paper printing. These industrial effluents often contain significant amount of residual dye due to inefficiency in the dyeing processes. It affects the water quality and may become a threat to public health, since certain azo dyes or their metabolites (aromatic amines) are highly toxic and potentially carcinogenic (Robinson et al. 2001; Zhang et al. 2010). It reduces the amount of sunlight to photosynthetic organisms resulting in decreased oxygen levels in aquatic ecosystems. Currently, the discharge of dye wastewaters is an

important environmental problem (Champagne et al. 2010). The major chemical pollutants present in textile wastewater are dyes containing carcinogenic amines, toxic heavy metals, pentachlorophenol, chlorine bleaching, halogen carriers, free formaldehyde, biocides, fire retardants, and softeners (Hai et al. 2007; Sharma et al. 2009). Various reports have mentioned the direct and indirect toxic effects of the dyes and metals, which can lead to the formation of tumor, cancers, and allergies besides growth inhibition of bacteria, protozoan, algae, plants, and different animals including human beings (Moawad et al. 2003).

The removal of color from the industrial wastewater is a current issue of discussion and regulation all over the world. Microbial decolorization and degradation is an eco-friendly and cost-competitive alternative to the chemical decomposition process (Sharma et al. 2009; Chan et al. 2012). Most studies on azo dye biodegradation have focused on bacteria and fungi, where bacteria are found to be more efficient (Elisangela et al. 2009). Thus, textile industries need to develop effective biological effluent treatment method as an alternative to the conventional physiochemical effluent treatment methods. Many studies have reported decolorization of single model dyes at low concentrations (Maier et al. 2004; Lin et al. 2010), but the conditions are poorly predictive of the actual decolorization efficiency of real effluents in which dyes are usually present as a mixture and often in quite high concentrations (Elisangela et al. 2009; Tamboli et al. 2010). Therefore, the selection of representative compounds to carry out a reliable and significant screenings is a key point, since model dye biotransformation cannot always be extrapolated to industrial dyes with apparent similar structure (Tamboli et al. 2010).

The objective of the present study was to evaluate the potential of *Alishewanella* sp. strain KMK6 in decolorization of textile dye mixture. The enzyme azoreductase was partially purified and evaluated for its potential to utilize different dyes as substrate.

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Material and methods

Microorganism and culture medium

The bacterial strain *Alishewanella* sp. KMK6 used was isolated from dye-contaminated soil (Kolekar and Kodam 2011) and deposited in the National Collection of Industrial Microorganisms (NCIM), Pune, India with an accession number NCIM 5295. The minimal nutrient medium containing (g l^{-1}) yeast extract, 1.0; peptone, 1.0; NaCl, 1.0 and mineral salts (mg l^{-1}): KH_2PO_4, 170.0; Na_2HPO_4, 980.0; $(NH_4)_2SO_4$, 100.0; $MgSO_4$, 4.87; MgO, 0.1; $FeSO_4$, 0.05; $CaCO_3$, 0.20; $ZnSO_4$, 0.08; $CuSO_4$, 0.016; $CoSO_4$, 0.015; and H_3BO_3, 0.006 was used for cultivation and dye degradation studies. All the chemicals used were of the highest purity available and of analytical grade.

Dyes

The textile dyes carmoisine, golden yellow HER, reactive blue 59, orange 2R, chocolate brown HT, orange M2R, raspberry red, navy blue HE2R, navy blue HE5R, and red BLI were generous gift from Radha Kanhaiya Dyeing Industry, Ichalkaranji, Maharashtra, India.

Decolorization studies

The strain KMK6 was cultivated for 24 h in conical flasks containing 100 ml minimal nutrient broth. After 24 h, the media were amended with the mixture of dyes viz., raspberry red, orange 2R, orange M2R, and golden yellow HER (2 g l^{-1} as mixture containing 0.5 g l^{-1} each). Aliquots (2 ml) of the culture media were withdrawn at different time intervals (2, 4, 6 and 8 h) and centrifuged at $10,000 \times g$ for 15 min in a refrigerated centrifuge (Remi C-24BL, India) to separate the bacterial cell mass. The supernatant was measured for decolorization using UV–visible spectrophotometer (Jasco A630) at 200–800 nm. All decolorization experiments were performed in the triplicates and the decolorization was expressed in terms of the percent decolorization as follows:

Decolorization (%)

$$= \frac{\text{Initial absorbance} - \text{Observed absorbance}}{\text{Initial absorbance}} \times 100$$

Effect of different carbon and nitrogen sources on dye decolorization

The effect of different carbon and nitrogen sources in minimal nutrient medium on dye degradation was studied. Different sources like corn flour and starch were used as carbon and urea, casein, soya flour and ammonium sulfate as nitrogen source (1 %) in minimal nutrient medium at pH 7.0. The

culture was grown at 37 °C, after 24 h, the dye mixture (2 g l^{-1}) was added and dye decolorization was recorded.

Decolorization of dye mixture

In order to study the effect of initial concentration of dye mixture on the dye degradation potential, the strain KMK6 was grown for 24 h and amended with the dyes viz., raspberry red, golden yellow HER, orange 2R, and orange M2R in mixture (0.5–2.0 g l^{-1}). After different time intervals, the supernatant was analyzed for percent decolorization.

Effect of dye degradation on chemical oxygen demand

To verify the reduction of organic compounds in the samples after dye decolorization, COD was monitored. Immediately after dye decolorization, the COD of the supernatant was measured as per APHA (1998) standard protocol. In order to study the fate of amines formed after dye decolorization, the flasks were further incubated in static anoxic and shaking (aerobic) conditions for further 24 h and COD was measured.

Analysis of biodegradation products

The supernatant of the dye mixture after decolorization was extracted with dichloromethane and dried over anhydrous sodium sulfate. The solvent was evaporated and the samples were used for spectral and genotoxic studies. The individual dye and degradation products were analyzed by high performance liquid chromatography (Shimadzu model 20A) on C-18 column (250×4.6 mm) by using methanol:water (80:20, v/v) as mobile phase with flow rate of 1 ml min^{-1}. The biodegraded products were characterized by Fourier transform infrared spectroscopy (Shimadzu FTIR-8400) and compared with control dye mixture. The FTIR analysis was done in the mid IR region of 500–4,000 cm^{-1} with 16 scan speed. The samples were mixed with spectroscopically pure KBr in the ratio of 5:95, sample was fixed and then analyzed.

Genotoxicity analysis

The genotoxicity analysis was carried by comet assay as described earlier (Kolekar and Kodam 2011). The in vitro analysis of the dye mixture and their degradation products was studied on coelomocytes of the earthworm, *Dichogaster curgensis*. Hydrogen peroxide was used as a positive control for DNA damage.

Enzyme studies

The biomass of *Alishewanella* sp. KMK6 was collected, washed, and resuspended in 0.1 M phosphate buffer (pH

7.4). The cell suspension was homogenized and sonicated with ultrasonicator (Sartorius, Germany) with 80 δ amplitude for 30 s with an interval of 30 s for 10 cycles. The crude lysate was centrifuged at 10,000×g for 20 min and the clear supernatant was collected and used as an enzyme source. The NADH-dichlorophenol indophenol (NADH-DCIP) reductase activity was determined as described earlier by Wakeyama et al. (1983). The azoreductase activity was performed as described by Zimmerman et al. (1982) by using carmoisine as substrate. One unit (U) of azoreductase was defined as the amount of enzyme required to degrade 1 μmol azo dye per min under appropriate conditions.

Purification of azoreductase enzyme

The strain KMK6 was grown in minimal nutrient broth at 37 °C for 24 h and was amended with dye mixture (1 gl^{-1}). After the complete decolorization of the dye; broth was subjected to centrifugation at 10,000×g for 20 min at 4 °C and cell mass was collected. The cell mass was suspended (75 mg ml^{-1}) in 0.1 M sodium phosphate buffer (pH 7.4) and incubated along with 1 % Triton X-100 and 0.1 mM phenylmethylsulfonyl fluoride with continuous stirring for 30 min at 37 °C. This solution was homogenized and disrupted by using ultrasonicator at 4 °C giving ten strokes each of 80 δ amplitude for 30 s with the interval of 30 s. The crude enzyme was centrifuged at 10,000×g for 30 min at 4 °C. The cell lysate was fractionated by ammonium sulfate at 40 % saturation to remove impurities, followed by 70 % saturation to precipitate azoreductase. The precipitated protein was collected by centrifugation at 10,000×g for 30 min at 4 °C, and the pellet was dissolved in 10 ml of phosphate buffer (pH 7.4). The solution was desalted by overnight dialysis against phosphate buffer (10 mM, pH 7.4). After centrifugation, the clear supernatant was applied on DEAE cellulose column (1× 18 cm) pre-equilibrated with 50 mM sodium phosphate buffer (pH 7.4). The column was washed with 100 ml of the same buffer and eluted using a linear gradient of NaCl (0.0–1.0 M). The flow rate was maintained at 0.5 ml min^{-1}, and a total of 30 fractions each of 2.0 ml were collected. Each fraction was then assayed for their protein content (A$_{280}$ nm) and azoreductase activity. The pooled active fractions were dialyzed against 10 mM sodium phosphate buffer (pH 7.4) and concentrated using a freeze dryer (Operon, Korea) and applied on centricon centrifugal filter devices with 30 kDa nominal molecular weight limit and the fraction that contains the protein of molecular weight less than 30 kDa was used for further studies. The partially purified enzyme was subjected to azoreductase activity as mentioned earlier by using different textile dyes (carmoisine, raspberry red, navy blue HE2R, chocolate brown HT, reactive blue 59, golden yellow HER, navy blue HE5R, red BLI, orange 2R, and orange M2R) as substrates.

Statistical analysis

The data were expressed as mean±SEM. The data obtained for degradation and genotoxic studies were analyzed by one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparisons test. While the data obtained for enzymatic studies was compared with parametric t-test for twin samples. Readings were considered significant when P was ≤0.05. All statistical analyses were carried out using GraphPad Prism software (Version 5.0, USA).

Results

Effect of different carbon and nitrogen sources on dye decolorization

The decolorization efficiency of strain KMK6 was observed by using low cost media containing additional carbon and nitrogen sources. Additional supplement of starch and corn showed 95–98 % dye decolorization within 8 h. The substitution of peptone to starch in media showed 96 % decolorization whereas, substitution of peptone to corn showed relatively less (78 %) decolorization (Fig. 1a). The decolorization efficiency of the strain KMK6 was increased with the addition of nitrogen sources like peptone and yeast extract. The yeast extract substituted with ammonium sulfate in combination with peptone showed 90 % decolorization within 8 h. The medium containing yeast extract, ammonium sulfate and peptone showed 55 % which is significantly less, while the medium containing urea and peptone showed only 20 % dye decolorization. It was observed that the strain *Alishewanella* sp. KMK6 was able to grow in all low cost media containing soya flour, corn, and ammonium sulfate and showed decolorization efficiency of 90 % (Fig. 1b).

Effect of initial concentration of dyes

The effect of initial dye concentration on the decolorization ability of the strain KMK6 was studied by inoculation of the culture to minimal nutrient medium supplemented with different concentrations of dye mixture, 0.5–2.0 gl^{-1}. The dye decolorization at the concentration range of 0.5–1.75 gl^{-1} was found to be 85–88 % in 8 h, while at 2.0 gl^{-1}, it was 80 %. The dye decolorization ability of the strain is promising even at high concentrations (Fig. 1c).

Effect of static and shaking conditions on COD

The COD removal was observed to be 28 % immediately after decolorization at static anoxic conditions. Further incubation of decolorized media up to 24 h reduced the COD by 66 % at static anoxic, whereas it reduced by 90 % under shaking (aerobic) conditions (Fig. 1d).
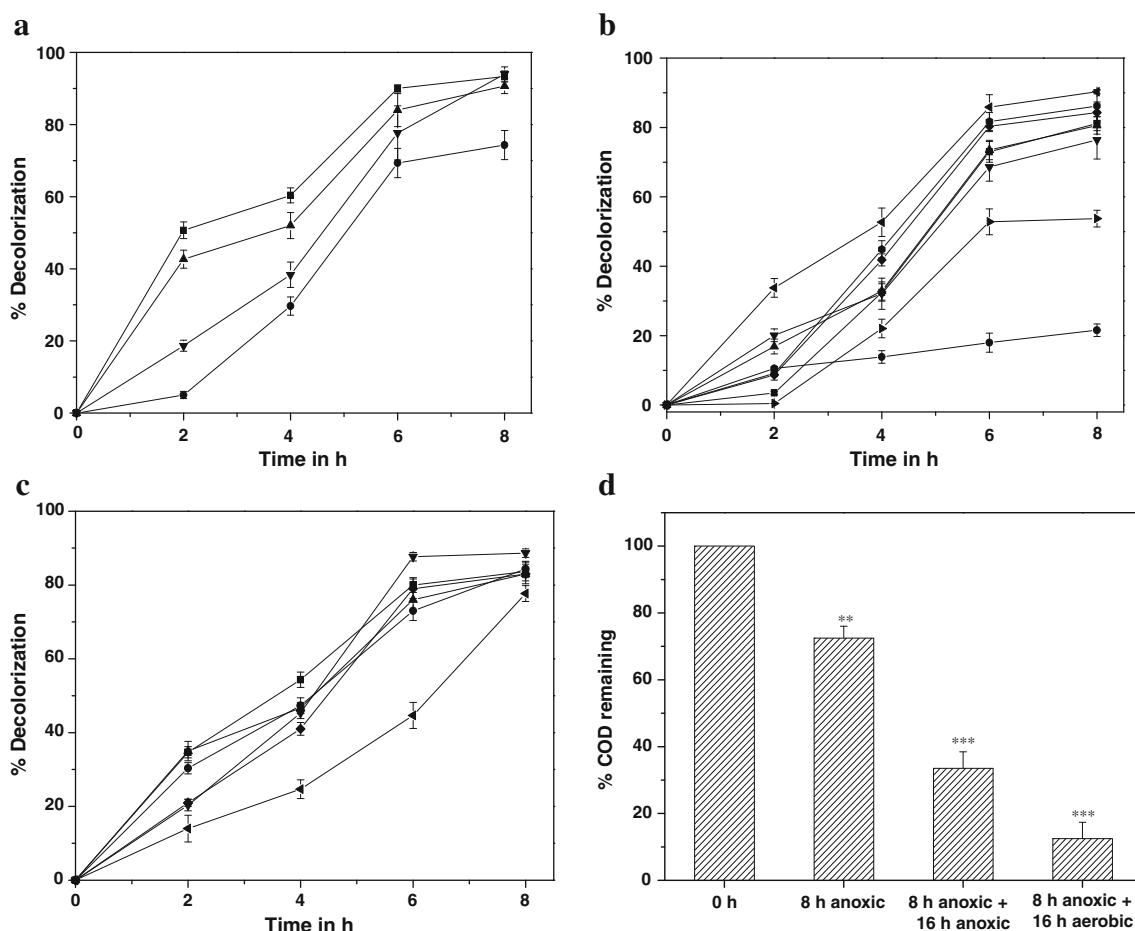
Fig. 1 a Decolorization of mixture of dyes (2.0 g L^{-1}) in presence of different carbon sources: corn + yeast extract + peptone, (*square*); corn + yeast extract, (*circle*); starch + yeast extract + peptone, (*upright triangle*); starch + yeast extract, (*inverted triangle*). **b** Decolorization of mixture of dyes (2.0 g L^{-1}) in the presence of different nitrogen sources: urea + yeast extract + peptone, (*square*); urea + peptone, (*circle*);soya flour + yeast extract + peptone, (*upright triangle*); soya flour + peptone, (*inverted triangle*); casein + yeast extract + peptone, (*rhombus*); casein + peptone,

(*triangle rotated counterclockwise*); ammonium sulfate + yeast extract + peptone, (*triangle rotated clockwise*); ammonium sulfate + peptone, (*circle*). **c** Decolorization of dye mixture at increasing initial concentration 0.5– 2.0 g L^{-1}: 0.5 g L^{-1}, (*square*);0.75 g L^{-1}, (*circle*); 1 g L^{-1}, (*upright triangle*);1.50 g L^{-1}, (*inverted triangle*); 1.75 g L^{-1}, (*rhombus*);2 g L^{-1}, (*triangle rotated counterclockwise*). **d** COD removal during biodegradation of dye mixture (2.0 g L^{-1}). **$P<0.001$; ***$P<0.0001$ (significantly different from 0 h by one-way ANOVA with Tukey–Kramer comparison test)

Chromatographic and spectroscopic analysis of biodegradation product

The UV–visible spectrophotometric analysis of culture supernatant showed decolorization of dye mixture after 8 h of incubation at 37 °C (Fig. 2a). The HPLC analysis of the degraded products of the dye mixture was performed by measuring the absorbance at the λ_{max} of the respective dyes. The dye orange 2R showed the peaks at 6.6 and 6.2 min while the product of dye mixture showed peaks at 6.8 and 3.0 min at λ_{max} of orange 2R. Orange M2R showed peaks at 6.0 min, whereas degradation product showed similar peak decreased by 60 %. The dye raspberry red showed peak at 9.0 min, while in the degradation product, it showed three peaks at 8.7, 7.3, and 6.0 min. In case of golden yellow HER, the parent dye showed peak at 6.8 min which almost

disappeared in the degradation product and new peaks appeared at 8.7, 8.2, and 2.9 min. The results indicate that there was biotransformation of dyes and formation of new metabolic products.

The FTIR spectrum of dye mixture (Fig. 2b) showed peaks at 3,500–3,304 cm^{-1} (-NH stretch), 2,250 cm^{-1} (alkynes), 1,606–1,480 cm^{-1} (aromatic), 1,372 cm^{-1} (S=O sulphonyl), 1,230 cm^{-1} (O–H stretch), and 685 cm^{-1} (chlorides). The degradation product after 24 h showed peaks at 3,390 cm^{-1} (O–H) stretch indicating the removal of amine from the mixture of dyes. The peaks at 2,857, and 3,054 cm^{-1} showed the formation of alkanes and aldehydes, peak at 2,685 cm^{-1} for O–H stretching, 1,664 cm^{-1} for C=O group and broad peak at 745–895 cm^{-1} showed for aromatic vibrations. These changes in the FTIR spectrum confirm the biodegradation of dye mixture by the strain KMK6.
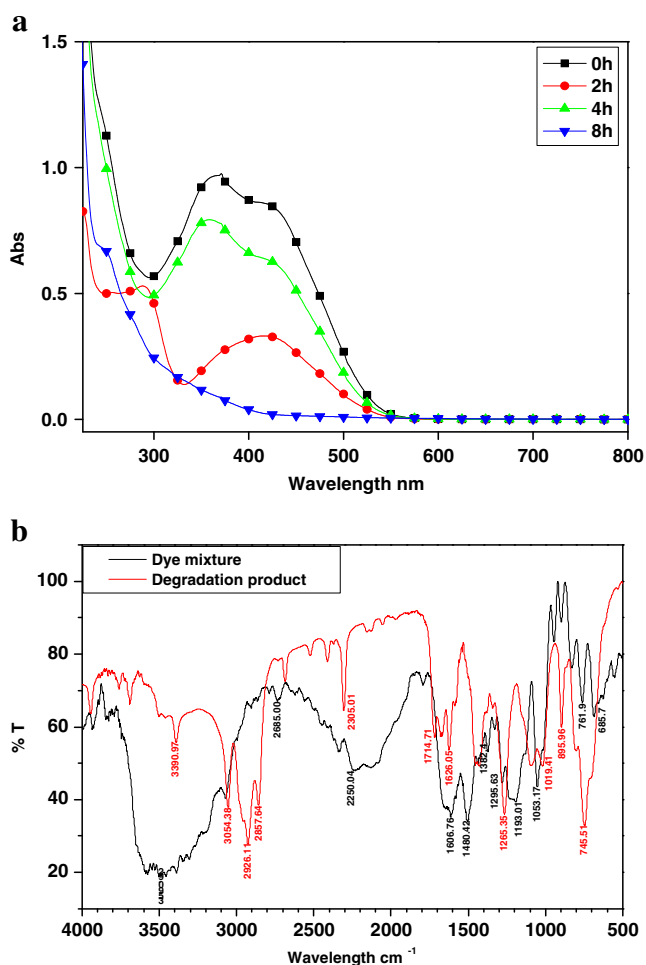
Fig. 2 **a** UV–visible spectrum of dye mixture during decolorization. **b** FTIR spectrum of the dye mixture and degraded product

Genotoxicity assessment

The genotoxicity of dye mixture and its degraded products was carried out by comet assay. This assay demonstrated the extent of DNA damage in terms of tail profile in earthworm coelomocytes (Fig. 3). The DNA damage (tail length, %DNA in tail, olive tail moment) in the dye-exposed cells was significantly high as compared to control and degradation products. While DNA damage in the cells exposed to H_2O_2 was significantly high than that of treated with dye and their degraded products (Table 1).

Enzyme studies

The induction in the activities of NADH-DCIP reductase and azoreductase in the cells exposed to dye mixture was significantly high as compared to unexposed cells. The azoreductase, a key enzyme in the biodegradation of azo dyes, showed six-fold induction in cells exposed to dye as compared to control cells. Similarly, NADH-DCIP reductase was found

to be induced by 4.5-fold in the cells exposed to dye as compared to unexposed cells (Table 2).

Purification of azoreductase and assessment of its activity with different dyes

The azoreductase enzyme was partially purified from the cell free extract of *Alishewanella* sp. KMK6. The crude cell lysate showed the initial activity of 0.131 nmol mg^{-1} min^{-1}, which increased after each purification step and finally to 9.366 nmol mg^{-1} min^{-1} of specific activity and 71-fold purification. This partially purified enzyme was further used to study the decolorization of different dyes. The azoreductase enzyme showed decolorization of different dyes and the activity was found to be in the range 0.60–3.09 nmol min^{-1} mg^{-1} (Table 3). The maximum enzyme activity was observed for reactive blue 59 as substrate whereas minimum was observed for golden yellow HER, showing difference in substrate specificity.

Discussion

Textile industry wastewater is composed of mixtures of different dyes and cause harmful effects when they are released in effluents (Klepacz-Smolka et al. 2010). The present study gives more focus on the investigation of the potential microorganism and its use in the removal of dyes in mixture. The *Alishewanella* sp. strain KMK6 was able to decolorize textile dye individually (Kolekar and Kodam 2011) and in mixture at a significantly high rate as compared to those achieved by other individual isolates (Senan and Abraham 2004; Machado et al. 2006; Anastasi et al. 2010; Tamboli et al. 2010).

The composition of nutrient medium used for dye decolorization was five times diluted as compared to the previous reports (Elisangela et al. 2009; Galai et al. 2010; Phugare et al. 2011). The mixture of seven dyes containing Red HE8B, Red M5B, Remazol Red, Orange 3R, Rubine, Golden Yellow HER, and Direct Blue GLL at a concentration of 0.8 g l^{-1} was decolorized within 48 h by using consortium of *Sphingobacterium* sp. ATM, *Bacillus odysseyi* SUK3, and *Pseudomonas desmolyticum* NCIM 2112 and was found to be efficient than individual strains (Tamboli et al. 2010). The decolorization of dye mixture was also reported earlier with consortium (Senan and Abraham 2004), where the dye concentration was 100 mg l^{-1} and only 35 % of decolorization was observed in 7 days. Many fungi were tested for the decolorization and degradation of dye mixtures and in these experiments, often lower dye concentrations were used and the time required was 17 days (Machado et al. 2006). *Bjerkandera adusta* was successful in decreasing the color of an artificial effluent containing 0.17 g l^{-1} of dyes within 8 days (Anastasi et al. 2010). The results obtained in the present study indicate the potential of the strain KMK6 in decolorization of dye mixture.
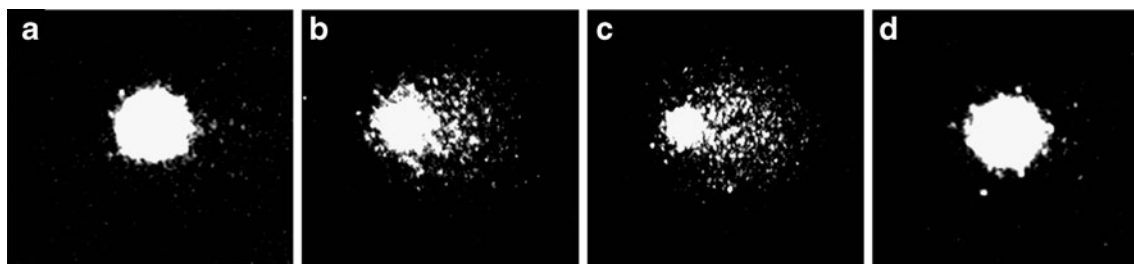
Fig. 3 Representative comet images of the coelomocytes of *D. curgensis*: control (**a**), exposed to mixture of dyes (**b–c**), and exposed to degradation products (**d**)

The decolorization potential of the strain *Alishewanella* sp. KMK6 was verified by increasing concentration of dye mixtures. In a study with mixture of four dyes, the time of decolorization was dependent on the chemical structure of dyes (Elisangela et al. 2009). Similarly, *Brevibacillus laterosporus* MTCC 2298 showed 87 % of decolorization of golden yellow HER within 48 h under static condition at the concentration 50 mg l^{-1} (Gomare et al. 2009). The strain KMK6 showed decolorization of 2 gl^{-1} of dye mixture within 8 h, highlighting the potential of the strain in biodegradation of textile dyes.

The present study revealed that *Alishewanella* sp. strain KMK6 utilized additional carbon and nitrogen sources during metabolism and degradation of dye mixture. Decolorization performance obtained with all easily available and economic sources was satisfactory and resulted in better decolorization efficiency compared to other high cost media reported (Lin et al. 2010). The alternate and supporting media as a peptone, yeast extract may help the strain KMK6 to utilize the other low cost carbon and nitrogen sources during dye decolorization. The addition of soya flour, corn, and yeast extract with peptone to the medium showed efficient dye decolorization. The replacement of yeast extract and peptone in nutrient media by corn flour and soya flour was clearly efficient in enhancing the dye decolorization. The yeast extract and peptone are expensive organic carbon and nitrogen source, its replacement by a low value compound (soya flour) can be convenient to reduce the cost of the medium. Moreover, the substitution of yeast

extract by soya flour improves the decolorization ability of the strain KMK6 by 15 %. It was reported earlier that the bacteria grown in media containing 1 % peptone, 0.5 % yeast extract and 1 % NaCl were able to decolorize 45 % of dye from industrial effluent; the substitution of one of the nutrient medium components was found to affect the decolorization ability. The decolorization is strictly dependent on bacterial growth and the effect of media was also observed during dye degradation by *Pseudomonas* sp. OX1 (Galai et al. 2010). The analysis of dye removal suggested that the maximum percentage of decolorization was observed when maximum substrate carbon conversion was achieved by the microorganism, suggesting a close coupling between bacterial metabolism and dye decolorization (Lodato et al. 2007).

The induction in biodegradation with peptone was reported for reactive brilliant blue by *Rhodocyclus gelatinosus* XL-1 (Dong et al. 2003). *Staphylococcus arlettae* decolorized four dyes (CI Reactive Yellow107, CI Reactive Red198, CI Reactive Black 5, and CI Direct Blue 71) in mixture effectively when the medium was supplemented with yeast extract (1 %) as the source of the electron donors, NAD and NADH. In the absence of yeast extract, partial decolorization (<50 %) was achieved after 168 h for Reactive Yellow 107 and Reactive Red 198, but there was no decolorization for any of the dyes when the yeast extract and glucose were substituted by sodium pyruvate. There was obligatory requirement of yeast extract as redox mediator to attain efficient dye decolorization by *S. arlettae* (Elisangela et al. 2009). Kalme et al. (2010) showed that *Pseudomonas desmolyticum* NCIM 2112 utilized 20 gl^{-1} of peptone to accelerate the decolorization up to 100 % in 48–72 h, although ability of the strain to decolorize dye (100 mg l^{-1}) was very slow, typically required several hours and was costly. Ultimately, the biodegradation process carried out in the present study is significant when related with time required and the concentration of dye.

The comparison of UV–visible absorption spectra (200–800 nm) of the dye mixture and degradation product indicates complete disappearance of peaks corresponding to λ_{max} of the dye mixture in 8 h, which is in good agreement with similar observations reported earlier (Anastasi et al. 2010; Kalme et al. 2010). These results indicate the removal and degradation of dyes in mixture.

Table 1 Assessment of genotoxicity of dye mixture and its biodegraded product on earthworm coelomocytes using alkaline comet assay

	Tail length (µm)	%DNA in tail	Olive tail moment
Control	1.28±0.48	4.98±4.05	0.89±0.62
Dye mixture	14.26±4.39*	38.23±9.88*	12.25±5.38*
Degraded product	1.43±0.98	6.57±3.24	1.08±0.92
H$_2$O$_2$ (70.4 µM)	17.21±2.24**	56.68±13.21**	24.85±7.98**

Values are mean of three experiments ± SEM

*$P<0.001$; **$P<0.0001$ (significantly different from control cells by one-way ANOVA analysis with Tukey–Kramer comparison test)

Table 2 Enzyme activities of *Alishewanella* sp. strain KMK6 exposed to dyes

Enzyme	Before dye degradation	After dye degradation
Azoreductase (nmol min^{-1} mg^{-1} protein)	0.61±0.01	3.73±0.07***
DCIP reductase (µmol min^{-1} mg^{-1} protein)	1.19±0.11	5.30±0.29***

Values are mean of three experiments ± SEM

***$P<0.0001$ (significantly different from control (before dye degradation) by parametric *t*-test for twin samples)

The COD removal of green HE4B was reduced by 66 % at static and 33 % at shaking conditions, less reduction in COD under shaking conditions, resulted in slow degradation (Kalme et al. 2010). The strain *Pseudomonas* sp. SU-EBT showed more than 90 % decolorization of congo red (100 mg l^{-1}) and textile industry effluent, whereas, only 50 % reduction in COD was reported (Telke et al. 2010). In present study, the COD removal was 28 % immediately after decolorization of dyes which increased to 66 % and 90 % on further incubation for 24 h at static anoxic and aerobic conditions, respectively, indicating the suitability of sequential static anoxic and aerobic conditions in dye degradation. The reduction in COD under aerobic conditions may be due to further degradation of dye intermediates. These observations are in good agreement with the previous results (Jang et al. 2007) and suggest that strain KMK6 is a potential strain for the degradation of textile dyes and significantly reducing the COD. The increase in % COD removal indicates degradation of organics (Gopinath et al. 2009), and based on these results, it can be confirmed that the strain KMK6 in this investigation was efficient in dye degradation.

The HPLC spectra of the individual dyes confirmed the decrease in their absorbance after degradation and resulted in good separation. Peaks appearing in the dye degradation products were identified by comparison of their retention times with

Table 3 Azoreductase enzyme activity using different dyes as substrates

Substrate dyes	λ_{max} nm	Enzyme activity (nmol min^{-1} mg^{-1})
Carmoisine	512	1.59±0.01
RasBery Red	515	2.02±0.01
Navy Blue HE2R	620	1.89±0.07
Chocolate Brown HT	480	0.90±0.02
Reactive blue 59	585	3.09±0.07
Golden Yellow HER	371	0.60±0.01
Navy Blue HE5R	590	0.89±0.05
Red BLI	549	1.30±0.29
Orange 2R	480	1.12±0.01
Orange M2R	490	1.86±0.01

Values are mean of three independent experiments ± SEM

the individual dyes. The chromatogram revealed that the intensity of peaks in the dye samples was reduced significantly after degradation at different retention times. As the decolorization progressed, additional peaks were emerged which may be due to degradation of parent dye red HE3B by bacterial consortium (Senan and Abraham 2004; Phugare et al. 2011). Similarly, the HPLC of golden yellow HER showed a single peak at a retention time of 1.59 min, whereas two peaks of the degradation products were observed at the retention times 2.29 and 2.45 min (Gomare et al. 2009). In the FTIR spectrum, the predicted peaks in the mixture of dyes were mostly by -NH, -OH stretching, alkynes, aromatic, S=O sulphonyl and chlorides. The peaks observed after decolorization were for -OH, C–H, C=O clearly indicating the removal of amine from the mixture of dyes and formation of alkanes and aldehydes as well. The absence of peaks at 3,500–3,304, 2,250, and 1,606–1,480 cm^{-1} indicates loss of aromaticity or benzene ring. The results indicated were similar to the earlier report (Pathak et al. 2011).

An assessment of the ecological and genetic impact of the environmental pollutants on the earthworm population is of great importance as these are soil purifier and has an important place in terrestrial ecosystem. Moreover, earthworms have been used as biosensors of genetic toxicity of the various environmental pollutants. The genotoxicity of dye mixture found to be very high as compared to the degradation products, these results are in agreement with our previous reports (Kolekar and Kodam 2011; Kolekar et al. 2012). It was also reported that the dye Red HE3B was genotoxic to *Allium cepa* while its degradation metabolites were non-toxic (Phugare et al. 2011). The strain KMK6 was found to be efficient not only for degradation of dye mixture but also for its detoxification.

Bacterial decolorization of azo dyes has been widely studied in recent years. The NAD(P)H-dependent cytosolic azoreductase catalyzing the reduction of azo bond have been characterized and classified into two families (Chen 2006). Its amino acid sequence shows 97 % identity to that of azoreductase of *Bacillus* sp. OY1-2 which is representative of flavin-dependent azoreductase family. In the present study, the azoreductase activity observed in cells exposed to dye was six times more than unexposed cells. Similarly, five-fold induction in DCIP reductase was observed in cells exposed to dye mixture. This observation strongly supports their role in

the degradation of dye mixture (Elisangela et al. 2009; Maier et al. 2004; Chen et al. 2005). Thus, it can be presumed that the major mechanism of decolorization in the cells is because of the biotransformation enzymes (Kalyani et al. 2008).

The NADH-dependent azoreductase was isolated and purified from *Alishewanella* sp. strain KMK6 and further investigated for decolorization of different dyes. It was clearly observed that the azoreductase activity was significant, even with different types of azo dyes. The azoreductase showed higher activity for reactive blue 59 as compared to golden yellow HER. The variation in azoreductase activity observed towards different dyes might be due to their complex structures (Bechtold and Turcanu 2006). Bozic et al. (2010) reported earlier that the NADH-dependent azoreductase isolated from *Bacillus subtilis* was involved in the dye degradation as it was able to use different dyes as substrate.

The strain KMK6 was able to degrade dye mixture with significant reduction in COD, which further reduced under sequential static anoxic and aerobic conditions. The strain KMK6 was able to use easy carbon and nitrogen sources and increased the efficiency of dye degradation. Partially purified azoreductase was able to utilize different azo dyes as substrate, showing its ability in biodegradation of different azo dyes in mixture. The biodegradation and detoxification potential of the bacterial strain KMK6 makes it better candidate in treatment of textile dye effluents.

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