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ORIGINAL ARTICLE

Biodecolorization of textile azo dyes by isolated yeast from activated sludge: *Issatchenkia orientalis* JKS6

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Abstract An ascomycetous yeast strain isolated from activated sludge could decolorize Reactive Black 5 azo dye at 200 mg l^{-1} up to 90 % within 12–18 h under agitated condition. Yeast decolorization ability was investigated at different RB5 concentrations and, at higher dye concentration, 500 mg l^{-1}, the decolorization was found to be 98 % after 36 h incubation time. Extensive decolorization (95–99 %) was obtained in presence of five other azo dyes, Reactive Orange 16, Reactive Red 198, Direct Blue 71, Direct Yellow 12, and Direct Black 22, by isolated yeast. HPLC analysis, UV–vis spectra and colorless biomass obtained after complete decolorization showed that the decolorization occured through a biodegradation mechanism. Decolorization was occurred during the exponential growth phase which is associated to primary metabolism. Laccase production by the yeast cells was not detected. The isolated yeast was characterized according to phenotypical and molecular procedures and was closely related (99 % identity) to *Issatchenkia orientalis*.

Keywords Azo dyes · Decolorization · *Issatchenkia orientalis* · Textile wastewater

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Introduction

Azo dyes, characterized by the presence of one or more azo groups (−N=N−), are the most commonly used dyes in textile, paper, printing and cosmetic industries. They are considered as xenobiotic compounds and highly recalcitrant against biodegradative processes (Pajot et al. 2007). The annual world production of dyestuff amounts to more than 7×10^5 tons. It is estimated that 10–15 % of the total production of colorants is lost during synthesis and dyeing processes (Vitor and Corso 2008). In fact, as much as 90 % reactive dyes could remain unaffected after activated sludge treatment (Lucas et al. 2006). Effluents of dyeing industries are markedly colored and the disposal of these wastes into waters causes environmental damage. Since color reduces light penetration, photosynthetic activity in aquatic life may be affected. In addition, due to their own toxicity as well as because of the presence of metals, chlorides, etc., they are harmful to aquatic life and also to living organisms drinking from these waters (Pajot et al. 2007).

Fungal biodecolorization ability has been widely reported and is commonly associated to the ligninolytic enzyme system (lignin-proxidase (LiP), manganese peroxidase (MnP) and laccase). The non-specific nature of these enzymes makes them able to transform, and eventually mineralize, a variety of persistent environmental pollutants including dyes (Yu and Wen 2005; Pajot et al. 2007). However, the requirement for low pH for optimum enzyme activity and the long hydraulic retention time for complete decolorization are major disadvantages in using fungi (Yu and Wen 2005). In addition, growth of filamentous fungi is slow compared with most single-cell microorganisms. In fact, they are poorly adapted to wastewater treatments because an exuberant mycelium growth generally occurs (Yu and Wen 2005; Lucas et al. 2006; Pajot et al. 2007).

Compared to bacteria and filamentous fungi, yeasts exhibit attractive features. Though not as fast as bacteria, yeasts can grow faster than most filamentous fungi, and, like them, have the ability to resist unfavorable environments (Yu and Wen 2005; Pajot et al. 2007). It has been observed that a few ascomycetous yeast species such as *Candida zeylanoides* (Martins et al. 1999; Ramalho et al. 2002), *Candida tropicalis*, *Debaryomyces polymorphus* (Yang et al. 2005), *Issatchenkia occidentalis* (Ramalho et al. 2004), *Candida oleophila* (Lucas et al. 2006), *Galactomyces geotrichum* (Jadhav et al. 2008) and *Candida albicans* (Vitor and Corso 2008) are able to biodegrade and decolorize azo dyes.

In the present study, screening and identification of newly isolated yeast capable of decolorizing six azo dyes are reported. The mechanism of the decolorization and the effect of different parameters on decolorization efficiency were studied.

Material and methods

Chemicals and dyestuff

The azo dyes, Reactive Black 5 (RB5), Reactive Orange 16 (RO16), Reactive Red 198 (RR198), Direct Blue 71 (DB71), Direct Yellow 12 (DY12), and Direct Black 22 (DB22), were obtained from a local company (Alvan Sabet, Tehran, Iran) and used as received. These dyes were chosen due to their extended use in Iran. The structures of the dyes are shown in Fig. 1. All other analytical grade reagents were purchased from Sigma and Merck. RB5, a commonly used commercial azo dye, was used as a model dye for all optimization experiments. A 10-g l^{-1} stock solution of each dye was prepared in distilled water and used for further studies by diluting as required.

Screening and identification of dye decolorizing microorganism

Activated sludge samples were collected from several municipal wastewater treatment plants. Five percent of activated sludge samples were inoculated into 250-ml flasks containing 100 ml of medium with RB5. The composition of the medium used in the present study was as follows (g l^{-1}): glucose, 10; yeast extract, 0.34; NH_4Cl, 0.84; KH_2PO_4, 0.134; K_2HPO_4, 0.234; $MgCl_2 \cdot 6H_2O$, 0.084 (Kumar et al. 2009), and 200 mg RB5 l^{-1}. The pH of the solution was adjusted to 7±0.2 before being autoclaved. The flasks were incubated in a shaking incubator at 150 rpm and 32 °C for 3 days. Screening of the decolorizing microorganisms was also performed using the same medium without glucose and containing RB5 as sole source of carbon and energy. When decolorization in the medium was observed, the cultures were serially diluted and 100-μl aliquots of each dilution were spread onto nutrient agar plates. After 48 h incubation at 32 °C, morphologically different colonies were selected to be cultivated again in the liquid medium with RB5 to check their decolorizing ability. The pure culture stock of the isolate was maintained on YMA (Yeast Malt Agar containing (g

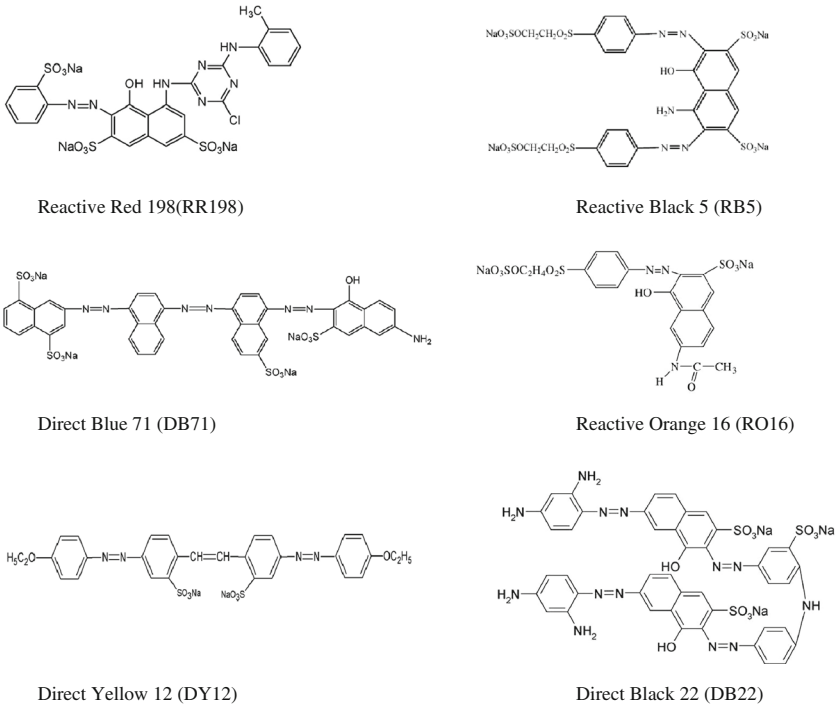

Fig. 1 Chemical structure of the dyes used in experiments

l^{-1}): yeast extract, 3; malt extract, 3; glucose, 10; peptone, 5 and agar, 15) slants at 4 °C.

Identification of decolorizing microorganism

Isolated yeast was identified using the 26S rRNA gene sequence. Total DNA was extracted from the isolate using an existing protocol (Sampaio et al. 2001). Amplification of 26S rDNA D1/D2 domain was performed using forward primer NL1 (5′-GCA TAT CAA TAA GCG GAG GAA AAG-3′) and reverse primer NL4 (5′-GGT CCG TGT TTC AAG ACG G-3′). The PCR products were sequenced. The 26S rRNA gene sequence was input into the National Center for Biotechnology Information (NCBI) to identify it by Basic Local Alignment Search Tool (BLAST). Some biochemical tests were also performed for confirmation of the molecular identification.

Decolorization assay

The liquid medium for the decolorization experiments was described above. The 250-ml flasks contained 100 ml of medium were inoculated with 5 % (v/v) of the yeast suspensions in 0.85 % (w/v) saline and incubated at 150 rpm and 32 °C. The control flasks containing the same medium without inoculums were also kept to observe the abiotic decolorization. All the experiments were performed at minimum in duplicate.

At regular time intervals, an aliquot (2 ml) of the culture media was withdrawn from the flasks and centrifuged at 12,000 rpm for 10 min and the supernatant was analyzed for remaining dye content. The decolorization of dyes was determined by measuring the absorbance of culture supernatant at their λ_{max}. The percentage of decolorization was calculated according to the following equation (Jadhav et al. 2008):

$$\text{Percentage of decolorization}: \quad (A_i - A_t)/A_i \times 100$$

Where A_i and A_t were the absorbance of the dye solution initially and at cultivation time (t), respectively. Additionally, turbidity (a measure of the yeast growth) was assessed by determining the difference between the absorbance of culture samples before and after centrifugation at 600 nm (Asad et al. 2007) using the following equation;

$$\text{Turbidity}: \quad \text{OD (before centrifugation)} - \text{OD (after centrifugation)}$$

Effect of different factors on the decolorization

Decolorization of RB5 was studied in two culture condition, namely static (in the incubator) and agitated (in the shaking incubator) at 32 °C and the effect of aeration was examined. The various concentrations (0, 0.5, 1, and 2 % w/v) of glucose were used to determine the effect of glucose concentration on the decolorization. The effect of temperature was also investigated by incubation at 29, 32, 35, and 40 °C. The effect of salt concentration was evaluated by addition of various NaCl concentrations (0, 3, 5, and 10 % w/v) into the medium. Additionally, decolorization of different initial dye concentrations (50–1,000 mg l^{-1}) was also studied. The results were analyzed statistically by one-way analysis of variance and Tukey test with 95 % confidence level using Minitab (v.15.2) software.

Decolorization of other azo dyes

The ability of isolated yeast in the decolorization of other azo dyes, namely Reactive Orange 16 (RO16), Reactive Red 198 (RR198), Direct Blue 71 (DB71), Direct Yellow 12 (DY12), and Direct Black 22 (DB22), was examined. These dyes (200 mg l^{-1} concentration) were added into the medium. Decolorization was determined by measuring the absorbance of culture supernatant at their λ_{max}.

UV–vis analysis

Culture supernatants were subjected to spectral scanning between 200 and 800 nm using a UV–vis spectrophotometer (Shimadzu, TCC-240 A) and changes in the absorption spectra were recorded in order to analyze dye degradation.

HPLC analysis

After complete decolorization of culture contained 200 mg RB5 l^{-1}, centrifugation was performed at 12,000 rpm for 30 min. The supernatant of the decolorized culture was extracted with an equal volume of ethyl acetate. The concentrated extract in a rotary evaporator were dissolved in a small volume (2 ml) of HPLC-grade methanol (Jadhav et al. 2007) and the samples were used for analysis by HPLC (Knaver, C-18 column with 4.6 mm diameter and 25 cm length). The mobile phase used was methanol:water:H_3PO_4 in a proportion of 50:49.7:0.3 (Asad et al. 2007) with flow rate of 0.6 ml min^{-1}. The products were monitored by their absorbance at 254 nm with a UV detector.

Laccase production

The isolate was cultured on PDA plates containing a laccase (a ligninolytic extracellular enzyme) indicator (0.01 % guaiacol or 0.5 % tannic acid). Guaiacol was added to the media before autoclaving. Tannic acid was autoclaved separately before addition to the media. Plates were incubated at 30 °C (Kiiskinen et al. 2004) and were studied for the

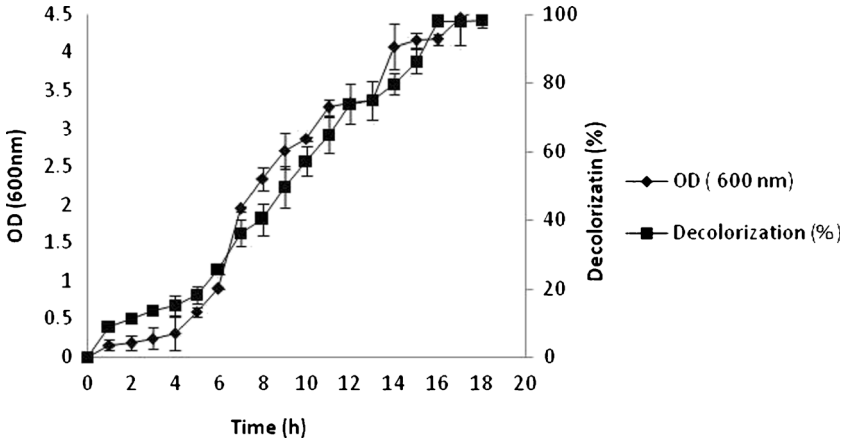

Fig. 2 Growth of strain JKS6 culture (OD at 600 nm) and decolorization in medium containing RB5 (200 mg l^{-1}) at 32 °C and 150 rpm

formation of positive halos for 3 weeks; reddish-brown and dark-brown halos in the presence of guaiacol and tannic acid, respectively (Forootanfar et al. 2011).

Results and discussion

Screening and identification of decolorizing yeast

Yeast strain JKS6, which had high decolorization ability against RB5 in liquid medium, was isolated. Strain JKS6 could decolorize 200 mg RB5 l^{-1} up to 90 % within 12–18 h under agitated condition (at 150 rpm). Lower decolorization was observed under static condition in which only 15.6 % of decolorization occurred after 12 h incubation. Similar decolorization efficiency in yeasts has been previously reported. *Candida oleophila* could completely decolorize RB5 within 24 h (Lucas et al. 2006). and complete decolorization of 200 mg RB5 l^{-1} also occurred by *Debaryomyces polymorphus* within 24 h (Yang et al. 2005). In contrast, decolorization of azo dyes by filamentous fungi especially the white-rot type required long incubation periods. According to Chagas and Durrant (2001), 20–98.5 % of color removal was achieved using *Phanerochaete chrysosporium* and *Pleurotus sajorcaju* on the 8th day of growth for different azo dyes. As previously reported (Martins et al. 1999; Ramalho et al. 2004; Lucas et al. 2006; Yang et al. 2008) and also observed in the present study (Fig. 2), decolorization occurred during the exponential growth phase by yeasts and is associated with primary metabolism, but filamentous fungi decolorized azo dyes during secondary metabolism which required long incubation times (Lucas et al. 2006). Laccase production by the isolate was not detected in plates containing guaiacol or tannic acid. It was reported that azo-reductases play a key role during aerobic azo bond cleavage (Lucas et al. 2006). Azo bond cleavage by aerobic azo-reductase in the yeast species *Candida oleophila* and *C. zelanoides* was noted in previous studies (Ramalho et al. 2002; Lucas et al. 2006). However, Ramalho et al. (2004) reported the azo-reductase activity of the NADH: ubiquinone oxidoreductase in *Issatchenkia occidentalis* that acted in microaerophilic conditions. A further study is needed in this context. The sequence analysis of 26S rDNA D1/D2 domain showed a 99 % homology to the *Issatchenchia orientalis* sequences available on GenBank database. Figure 3 showed the phylogenetic relationship between the strain JKS6 and other related microorganisms found in the GenBank database. The 26S rRNA partial sequence of the strain JKS6 was deposited in the GenBank database with the accession number JQ650233. The result of biochemical and physiological characterizations of strain JKS6 was as follows: fermentation of glucose, +; galactose, −; sucrose, +/−; lactose, −; maltose, +/−; trehalose, −;

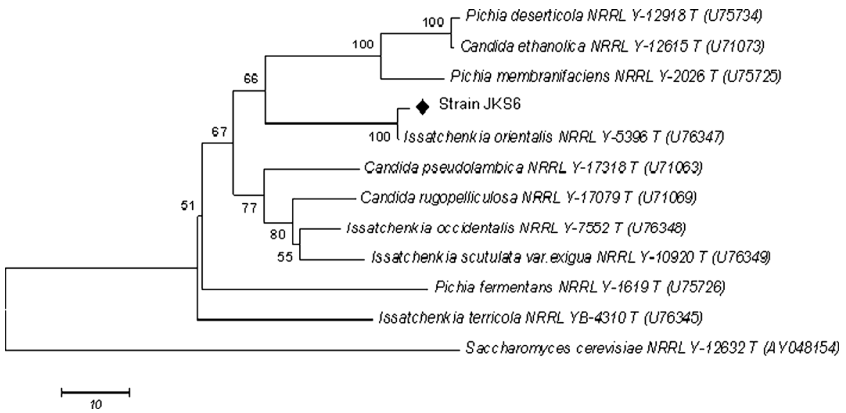

Fig. 3 The phylogenetic tree was constructed using neighbor-joining analysis of the 26S rRNA D1/D2 domain. Bootstrap values were calculated from 1,000 replications. The ascomycetous yeast *Saccharomyces cerevisiae* was used as an out group. Gene bank accession numbers of references sequences are given in *parentheses*

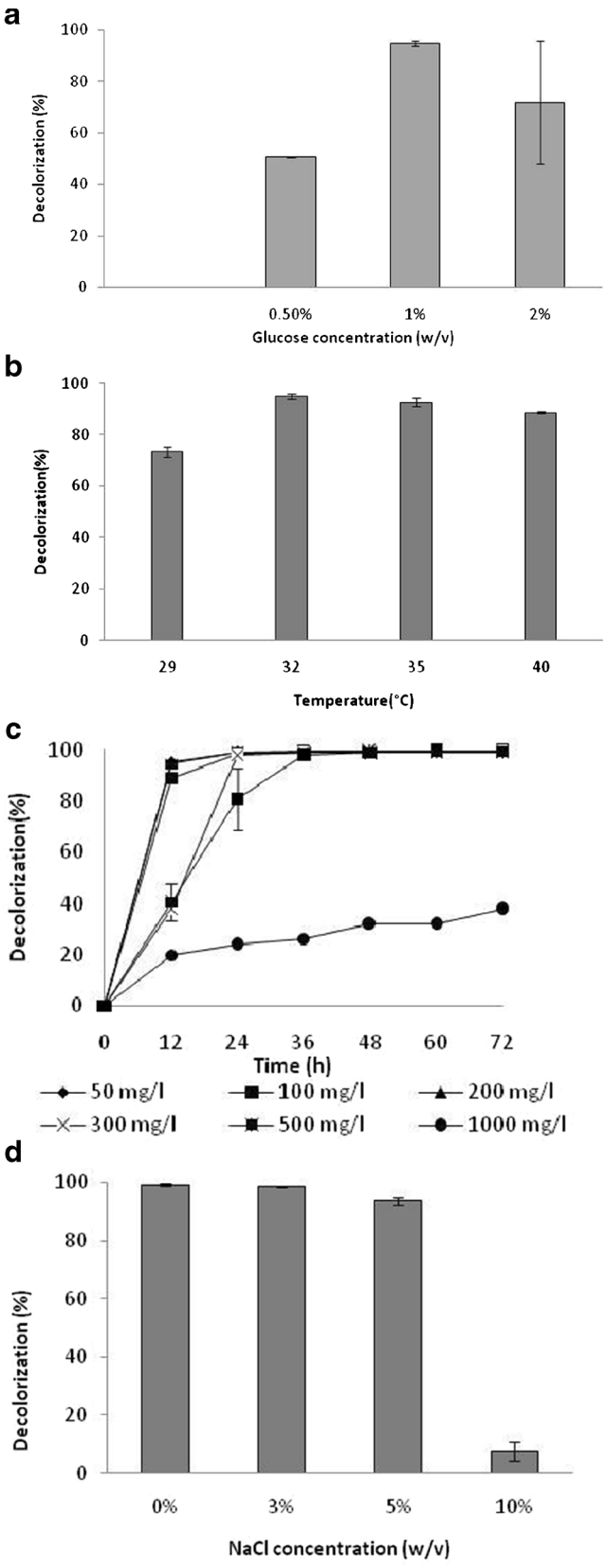

Fig. 4 Effect of various glucose concentrations (**a**), different temperatures (**b**), various dye concentrations (**c**), and different NaCl concentrations (**d**) on decolorization of RB5 by strain JKS6

diazonium blue B (DBB) test, −; hyphae production in Corn Meal Agar, +; acid production in Custer's medium, −.

Effect of different factors on the decolorization of RB5

Figure 4a shows the effect of various glucose concentrations (0.5, 1, and 2 %) on the decolorization of RB5 by strain JKS6 after 12 h. No decolorizaton was observed when the medium was not supplemented with glucose, because glucose is required for yeast growth and acted as a cometabolic substrate for dye degradation (Yang et al. 2008). In this study, strain JKS6 could not use the dye as a sole carbon source for cell growth under the experimental conditions. Similar behavior has been observed during the decolorization by other microorganisms such as *Candida oleophila* (Lucas et al. 2006) and *Debaryomyces polymorphus* (Yang et al. 2005). Probably, assimilation of a carbon source and its subsequent metabolism supplies the yeast cells with the reducing power (NADH and or/FADH2) required for the reduction of azo bonds (Lucas et al. 2006). By increasing the glucose concentration to 1 %, further decolorization efficiency (94.85 %) was achieved by strain JKS6. When the glucose concentration was higher, at 2 %, lower decolorization (71.9 %) occurred. In the temperature range tested, 32 °C was optimum for decolorization (Fig. 4b). It was observed that, with an increase in temperature from 29 to 32 °C, the decolorization efficiency increased from 73.4 to 94.85 % and, with a further increase in temperature to 40 °C, the decolorization was significantly decreased (88.75 %), which might be due to the loss of cell viability or deactivation of the enzymes responsible for the decolorization at 40 °C (Wang et al. 2009). The decolorization of RB5 at different concentrations was studied and extensive decolorization was observed by strain JKS6 at all the initial dye concentrations except at 1,000 mg l^{-1}(Fig. 4c). With an increase of the initial dye concentration, the decolorization efficiency over the same time interval decreased. After 36 h of incubation, complete decolorization was observed for all the examined concentrations except for 1,000 mg l^{-1}. The decolorization efficiency was 37.85 % at

Table 1 Decolorization of different azo dyes (200 mg l^{-1}) by strain JKS6 after 12 h and 24 h incubation at 32 °C and 150 rpm

Dye	Decolorization (%) after exposure	
	12 h	24 h
Reactive Black 5	94.85±1.06	99.30±0.417
Reactive Orange 16	97.55±2.04	99.2±0.14
Reactive Red 198	78.05±3.88	95.85±0.63
Direct Yellow 12	93.8±1.83	95.5±0.98
Direct Blue 71	87.55±4.87	95.05±2.19
Direct Black 22	5.7±8.06	97.1±0.14

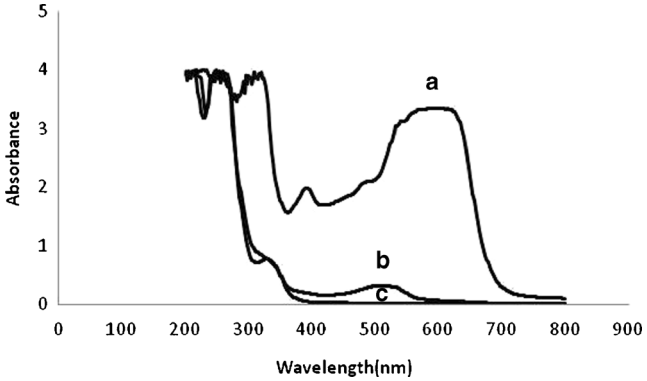

Fig. 5 UV–vis spectra (200–800 nm) of the treated medium by strain JKS6, *a* control; *b*, *c* after 12 h and 72 h incubation at 32 °C

1,000 mg RB5 l^{-1} after 72 h incubation. It has been reported that dye decolorization can be strongly inhibited when a high concentration of dye is used, because of the poisonous effect of the dye on the degrading microorganisms (Wang et al. 2009). Figure 4d shows the effect of various concentrations of NaCl on the decolorization efficiency by strain JKS6 after 24 h incubation. The strain showed lower decolorization (7.65 %) in the presence of 10 % sodium chloride.

Decolorization of other azo dyes

Textile wastewater consists of a mixture of various dyes. Strain ability was examined to decolorize different azo dyes. Table 1 shows the decolorization percentage of different azo dyes after 12 and 24 h incubation at 32 °C. Results show that the three reactive azo dyes and three direct azo dyes used in this study were efficiently decolorized (95–99 %) by strain JKS6 which suggests that the decolorization of azo dyes by isolated yeast was not a specific process. The effectiveness of the decolorization depends on the structure and complexity of each dye, and relatively small structural differences can markedly affect the decolorization. These differences are

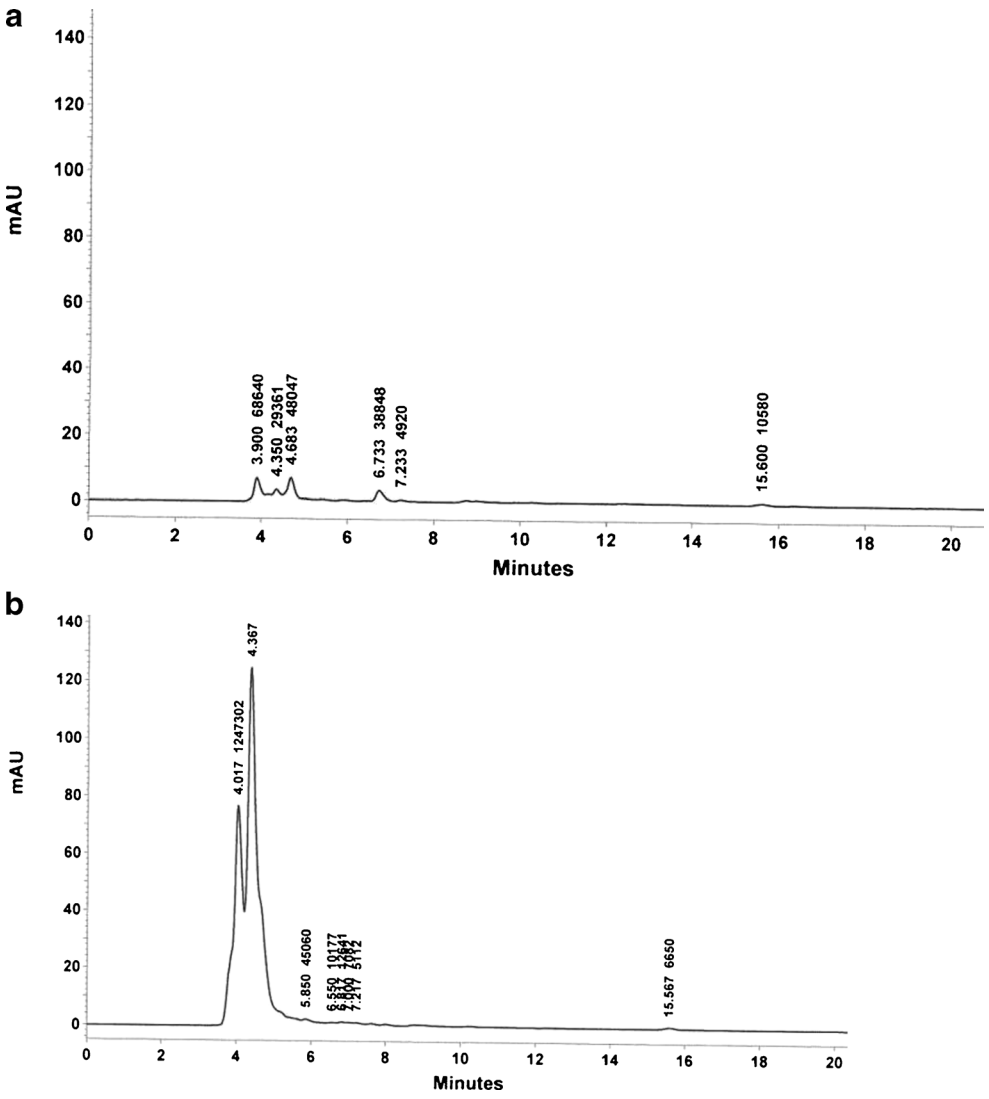

Fig. 6 HPLC analysis of the products extracted with ethyl acetate: immediately after exposure as control (**a**) and after complete decolorization of RB5 by strain JKS6 (**b**)

presumably due to electron distribution and charge density (Verma and Madamwar 2005). In this study, a latent period (12 h) was observed in the decolorization of DB22. Low decolorization efficiency (5.7 %) by strain JKS6 occurred in medium containing DB22 after 12 h incubation. This may be due to the complex structure of this tetraazo dye. The decolorization efficiency of strain JKS6 against all the azo dyes tested in this study was up to 90 % after 24 h incubation.

Mechanism of dye removal

Decolorization of dyes by microorganisms can be due to adsorption of the dyes by microbial cells or to biodegradation (Wang et al. 2009). The result (Fig. 5) obtained from UV–vis analysis (200–800 nm) shows the untreated dye solution containing RB5 presented two main absorption peaks at 597 and 310 nm, which can be ascribed to the presence of chromophoric azo bonds and both aryl and naphthalene-like moieties, respectively (Lucas et al. 2006). In the treated samples, maximum absorbance in the visible region shifted from 597 to 520 nm and finally disappeared with an extended incubation period, indicating that a complete decolorization occurred. In the UV spectra, the peak at 310 nm disappeared following by the formation of a new peak at 254 nm. The absorbance peak at UV spectra did not disappear at the end of decolorization, indicating RB5 was not completely mineralized while some new metabolites formed in the culture. A disability of other decolorizing microorganisms to mineralize textile dyes has been reported in previous studies (Ramalho et al. 2004; Lucas et al. 2006; Jadhav et al. 2007, 2008; Wang et al. 2009). In order to gain additional insight into the decolorization mechanism, HPLC analysis of supernatant samples was performed. After complete decolorization of the culture, investigation by HPLC exhibited (Fig. 6) the presence of new peaks (in 254 nm) at different retention times (4.01 and 4.36 min) when compared to a control sample. The color of the cultures containing RB5 and treated by strain JKS6 turned from dark blue to light gray and finally disappeared completely. No residual color on the biomass was observed. These observations suggest that strain JKS6 removed the color of RB5 through biodegradation.

Conclusion

Biological processes could be more economical and effective methods for the treatment of colored effluents contained azo dyes that represent a major group of dyes causing environmental concern. In the present study, an ascomycetous yeast strain isolated from activated sludge showed extensive decolorization efficiency against different azo dyes. Because of high degrading and decolorizing activity against various azo dyes commonly used in the textile industries, it is proposed that strain JKS6 has a practical application in the treatment of various dye effluents. However, the potential of the culture needs to be demonstrated for its application in the treatment of real dye wastewaters using appropriate bioreactors. Further research is needed to clarify the enzymatic mechanism of dye decolorization by the strain JKS6.

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