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Identification and Characterization of a Newly Isolated Yeast *Issatchenkia orientalis* Able to Degrade Synthetic Dyes in Industrial Wastewater

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This study focuses on the decolorization of the C.I. Reactive Black 5 by a newly isolated yeast, *Issatchenkia orientalis*. The effect of varying initial dye concentration, inoculum concentration, carbon and nitrogen sources, pH, and NaCl was investigated. Maximum decolorization (99%) was achieved at initial dye concentration of 200 mg l^{-1} within 24 h of incubation at 28 °C in the medium supplemented with 10% (v/v) inoculum concentration, 0.5% (w/v) glucose, and 0.1% (w/v) $(NH_4)_2SO_4$, and a wide range of pH values 3-8. Further experiment showed that this isolate also exhibited great color removal ability on other five dyes. The slightly colored biomass which was recovered after decolorization, and UV–vis spectra of supernatant suggested that both adsorption and biodegradation mechanisms may be contributed to the decolorization of C.I. Reactive Black 5. Moreover, a significant decrease in the activity of azoreductase in the cells after decolorization presumably indicated the involvement of this enzyme in the decolorization process.

Key words: Azo dyes, Biodegradation, C.I. Reactive Black 5, Decolorization, Enzyme, Yeast.

Synthetic dyes have been widely used in a number of industries, including textile, paper printing, food processing, cosmetics and pharmaceutical[1]. About 50,000 tons of dyes are released into the environment worldwide every year[2]. The release of effluents containing dyes not only affects aesthetic appearance of natural water bodies, but also changes the absorption and reflection of sunlight on the water, negatively affecting photosynthesis[3]. Moreover, most of them are toxic, and resistant to degradation, thus threatening ecosystems and humans health[4].

Dyes in the wastewater can be treated by conventional physical and chemical procedures such as coagulation, foam flotation, electrochemical oxidation, and absorption using activated carbon etc.[5]. However, these conventional methods are often inefficient, expensive, and time-consuming[6]. Moreover, they also lead to secondary waste streams which need further treatment[7]. Compared to physicochemical treatments, the biological methods are generally considered environmentally friendly[8].

There is a wide variety of microorganisms (bacteria, fungi, yeast, etc.) that are capable of decolorizing dyes[9]. White-rot fungi and yeast are

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the major fungal strains used for decolorization purposes[10, 11]. Yeast, such as *Hansenula fabianii*, has been successfully applied to treat industrial effluents from food, molasses, and oil manufacturing wastewater[12]. Compared to bacteria and filamentous fungi, yeast exhibits attractive features. For instance, they can grow faster than most filamentous fungi. In addition, they have the ability to resist unfavorable environment conditions[13]. Yeast cells represent a promising capacity for removal of dyes from industrial effluents. However, research on the yeast strains in treating the dye wastewater has been very limited. In the present study, a newly isolated yeast *Issatchenkia orientalis* was identified, and used for degrading six commercial dyes. Optimum operating conditions for decolorizing C.I. Reactive Black 5 and the possible mechanism responsible for decolorization were investigated. Also, the decolorization of other five dyes was evaluated using this strain.

MATERIALS AND METHODS

Dyes and Chemicals

Six commercial dyes viz. C.I. Reactive Red 24, C.I. Reactive brown 2, C.I. Reactive Black 5, C.I. Reactive Orange 1, C.I. Reactive Blue 21, and C.I. Reactive Blue 19, were purchased from the Tianjin Xinmei Dye Chemical Co., Ltd. (China). They are all commercially important dyes, with a wide range of applications across the textile industry in China. Stock solutions of the dyes were prepared at 1000 mg l^{-1}, and then filter-sterilized prior to addition to the sterile culture medium. ABTS (2,2-Azino-bis-3-ethylbenzothiazoline-6-sulfonate), veratryl alcohol, DCIP (2,6-Dichlorophenolindophenol sodium salt), and NADH (Nicotinamide adenine diuncleotide hydrogen) were obtained from Sigma Chemical Company (USA), whereas tartaric acid, $MnSO_4$, hydrogen peroxide, and other chemicals were obtained from Beijing Chemical Reagent Company (China). All chemicals and reagents used were analytical reagent grade.

Microorganism and medium

This yeast strain was isolated from the soil in Beijing International Sculpture Park, China (around 116°23' E, 39°90' N), by using the spread-plate method carried out on YM agar (Yeast/malt extract agar) containing 10 g l^{-1} glucose, 5 g l^{-1} peptone, 3 g l^{-1} malt extract, 3 g l^{-1} yeast extract, and 20 g l^{-1} agar. Purified yeast isolates were first screened for their ability to grow in the presence of the dye C.I. Reactive Black 5. The yeast was maintained on YEPD (Yeast extract, peptone, and dextrose) agar slants containing glucose 20 g l^{-1}, peptone 20 g l^{-1}, and yeast extract 10 g l^{-1} at 4°C. The normal decolorization medium consisted of glucose 10 g l^{-1}, KH_2PO_4 1 g l^{-1}, NH_4SO_4 1 g l^{-1}, $MgSO_4 \cdot 7H_2O$ 0.5 g l^{-1}, yeast extract 0.2 g l^{-1}, and pH 5.0-6.0[11]. In addition, this yeast strain was stored in CGMCC (China general microbiological culture collection).

Yeast identification

The phenotype was determined according to the procedures as described by Tondee *et al*.[14]. Morphology of colony was observed on the agar plate containing various kinds of media including Malt extract agar, and Corn meal agar under light microscope. Sugar fermentation and assimilation of the strain were also observed with the media containing various sugar sources. In addition, the genotype was identified using 26S rRNA gene sequences by the colony PCR method. Primer pairs NL1 (5'GCATATCAATAAGCGGAGGAAAAG3') and NL4 (5'GGTCCGTGTTTCAAGACGG3') were used to amplify the D1/D2 region of the 26S rRNA gene[15]. A single colony approximately 1 mm in diameter was picked up with a micropipette tip, and transferred directly to the PCR tube[16]. Single strand sequencing of the PCR product was performed with an ABI 3730XL DNA sequencer. Sequence comparisons were performed using the Blast program within the NCBI (National center for biotechnology information). The phylogenetic relationship was determined by using the Mega 5.0 program. Numbers on the branches indicated bootstrap values (1000 replicates).

Decolorization culture

For decolorization tests, a full loop of culture from a slant was used to inoculate a 250 ml Erlenmeyer flask containing 100 ml normal decolorization medium at 28 °C with shaking (200 rpm) for 24 h.Decolorization studies were carried out in 250 ml flasks containing normal decolorization media inoculated with an overnight grown culture. Control experiments were performed using the same medium without microorganisms or dyes. Growth and decolorization were monitored at various initial dye concentrations, inoculum

concentration, carbon source and nitrogen source, pH values, and salinity.

Decolorization analysis

The biomass was removed by centrifugation at 10,000 rpm for 15 min, and the dye concentration was measured using a UV-visual spectrophotometer (Unic UV-4802H, USA). The decolorization efficiency was determined by measuring the absorbance of decolorization medium at 598 nm for C.I. Reactive Black 5 and percentage decolorization was calculated as follows[17]:

Decolorization (%) = (Initial absorbance – Observed absorbance) / Initial absorbance×100.

Enzyme assays

To determine the production of three oxidation enzymes (laccase, lignin peroxidase, and manganese peroxidase) and two reductase enzymes (NADH-DCIP reductase and azoreductase), the yeast strain was cultured in normal decolorization medium at 28 °C for 24 h supplemented with 200 mg l^{-1} C.I. Reactive Black 5. Cells grown in the nutrient broth were collected by centrifugation at 10,000 rpm for 15 min. These cells were suspended in potassium phosphate buffer (50 mM, pH 7.4) for sonication, keeping the sonifier output at 40 amp and giving 8 strokes each of 40 s with a 2 min interval at 4 °C[18]. This extract was used directly as the source of enzyme without centrifugation.

All enzyme activities were measured spectrophotometrically in the crude cell extract. Laccase peroxidase activity was determined in a reaction mixture of 2 ml containing 10% ABTS in 0.1 M acetate buffer (pH 5.0), 1 ml enzyme solution and the increase in absorbance at 420 nm was measured using a modified method of Jadhav and Govindwar[18]. Lignin peroxidase activity was determined by using procedures reported earlier. The mixture was composed of 50 mM sodium tartrate buffer, 2 mM veratryl alcohol, 0.5 mM hydrogen peroxide, and 1 ml enzyme solution. The reaction was started by the addition of hydrogen peroxide, and the appearance of veratraldehyde was determined at 310 nm[19]. Manganese peroxidase activity was measured at 468 nm by monitoring the oxidation of 1 mM $MnSO_4$ in 50 mM malonate buffer (pH 4.5) in the presence of 0.1 mM H_2O_2[20]. One unit of enzyme activity was defined as a change in absorbance unit min^{-1} ml^{-1} of the enzyme. NADH-DCIP reductase activity was assayed spectrophotometrically as described by Kalyani *et al*.[17]. The assay mixture contained 50 µM DCIP, 50 µM NADH in 50 mM potassium phosphate buffer (pH 7.4), and 1 mL of enzyme solution in a total volume of 2 ml. Azoreductase activity was estimated by monitoring the decrease in the Methyl red concentration at 440 nm in a reaction mixture of 2 ml containing 152 µM Methyl red, 50 mM sodium phosphate buffer (pH 5.5), 20 µM NADH, and 1 ml enzyme solution according to the method of Saratale *et al*.[21]. One unit of enzyme activity was defined as a microgram of substrate reduced per min per mg of protein.

All enzyme assays were carried out at 30 °C, where reference blanks contained all components except dyes. Data presented were the average of triplicate measurements.

Statistical analysis

Data were analyzed by one-way analysis of variance (ANOVA) with Tukey-Kramer multiple comparisons test. A *P* value of 0.05 or less was taken as a criterion for a statistically significant difference.

RESULTS AND DISCUSSION

Properties and identification of a dye-decolorizing yeast strain

The yeast, with strong decolorizing ability on C.I. Reactive Black 5 was isolated. The colony of this yeast cultured on Malt extract agar medium for one month at 25 °C was circular, smooth, and white. In addition, this yeast grew with pseudohyphae on the corn meal agar medium at 37 °C. Sequences analysis of 26S rRNA gene showed that the yeast had highest similarity (99%) with the *Issatchenkia orientalis* strain QD2 (accession number: EU585754). Fig. 1 shows the phylogenetic relationship between this strain and other related microorganisms found in the GenBank database. Based on the phenotypic characteristics and phylogenetic analysis, the yeast was identified as *Issatchenkia orientalis*. The 26S rRNA gene sequence has been deposited in GenBank with the accession number KC454395.

Effect of initial dye concentration and inoculum volume

Concentrations of 50, 100, 200, 400, 600, 800, and 1000 mg l^{-1} were used to determine the

Table 1. Percent decolorization and wavelengths of maximum absorption of different dyes by *Issatchenkia orientalis*

Dye	Type	λ_{max} (nm)[a]	Decolorization (%)	Strain color
C.I. Reactive Red 24	Azo	534	99	Pink
C.I. Reactive Brown 2	Azo	479	95	Brown
C.I. Reactive Black 5	Azo	598	99	Gray
C.I. Reactive Orange 1	Azo	372	93	Orange
C.I. Reactive Blue 21	Anthraquinone	339	53	Green
C.I. Reactive Blue 19	Anthraquinone	592	75	Blue

[a] Wavelengths of maximum absorption of different dyes

Table 2. Enzyme activities in control (0 h) and induced state (24 h decoloriztion) of *Issatchenkia orientalis*

Enzymes	Before dye addition	After dye decolorization
Lignin peroxidase[a]	0.028±0.007	0.034±0.009
Manganese peroxidase[a]	0.084±0.016	0.074±0.012
Laccase[a]	0.043±0.002	0.049±0.001
NADH-DCIP reductase[b]	0.276±0.069	0.460±0.076[**d]
Azoreductase[c]	4.968±0.054	1.921±0.054 [***d]

[a] Enzyme activity-units ml^{-1} min^{-1}
[b] µg DCIP reduced mg^{-1} min^{-1}
[c] µg Methyl red reduced mg^{-1} min^{-1}
[d] Values are mean of three experiments ± SEM. Significantly different from control cells at $^{*}P<0.05$, $^{**}P < 0.01$, $^{***}P<0.001$ by One-way analysis of variance (ANOVA) with Tukey-Kramer comparison test

effect of initial dye concentration on decolorization. As seen in the Fig. 2, approximately 97% decolorization was observed within 12 h when the initial dye concentration increased from 50 to 200 mg l^{-1}, followed by 85%, 38%, 21%, and 9.6% at 400, 600, 800, and 1000 mg l^{-1} dye, respectively. Complete decolorization (99%) of 50, 100, 200, 400 mg l^{-1} dye was achieved in 24 h. In addition, at initial dye concentrations exceeding 400 mg l^{-1}, increasing the initial dye concentration resulted in decreasing the color removal efficiency. The decolorization efficiency was 60%, 38%, and 23% for the dye concentrations ranged from 600 to 1000 mg l^{-1}, respectively. Daneshvar *et al.*[22] suggested that initial dye concentration provided an important driving force to overcome all mass transfer resistances of the dye between the aqueous and solid phases. However, saturation of the sorption sites on the biomass occurred as the concentration of the dye increased[23]. Moreover, high dye concentrations may have toxic effect on the strains[24-26].

The inoculum concentration is also an important parameter influencing the decolorization. To examine its effect, different inoculums (3, 5, 10, 20, and 30% (v/v)) were added into the medium containg 200 mg l^{-1} dye. The decolorization of C.I. Reactive Black 5 (200 mg l^{-1}) increased with rising biomass amounts (3-10% (v/v)), achieving up to 99% within 12 h (data not shown), which may be explained by the greater surface area and the large number of vacant biosorption sites[27]. However, further increase in inoculum volume of 10-30% (v/v) did not improve the decolorization. Similar results were reported by Radha *et al.*[28] that an inoculum size of 10% was sufficient for the delocorization of the textile wastewater. Therefore, the optimum dosage of initial inoculums was 10% (v/v), and the same concentration (200 mg l^{-1}) was used for further studies.

Effect of carbon and nitrogen sources

Effect of carbon source concentration was studied by adding glucose of 0, 0.05, 0.10, 0.30, 0.50, 0.70, and 1% (w/v) into the medium (Fig.

3). The results indicated that the strains could still decolorize without the glucose, but the decolorization efficiency only achieved 51%. The higher the concentration of carbon source within 0.50% was, the more efficient the decolorization was. A possible explanation was that an additional carbon source could fuel the decolorization process[29, 30]. Another previous study suggested that glucose promoted microbial proliferation, thus producing more biomass capable of removing dyes[31].

According to Tondee *et al*.[14], nitrogen source had large effects on the yeast growth. An optimum concentration of nitrogen for yeast decolorization was studied using $(NH_4)_2SO_4$ (0-1.0% (w/v)) (Fig. 3). Even in the absence of nitrogen source, partial decolorization still took place (82%). Addition of nitrogen source enhanced the decolorization efficiency. This is consistent with a previous study that decolorization was stimulated by higher nitrogen nutrition[20]. However, the critical nitrogen concentration was about 0.1% (w/v). Further increase in the nitrogen concentration had no noticeable effect on dye decolorization.

Effect of pH

Solution pH is an important factor for microbial decolorization performance. To find a more suitable pH value for effective decolorization by this yeast, experiments were conducted at different initial pH values (3-10) (Fig. 4). The reaction mixture was adjusted to the desired pH by the addition of sodium hydroxide or sulfuric acid

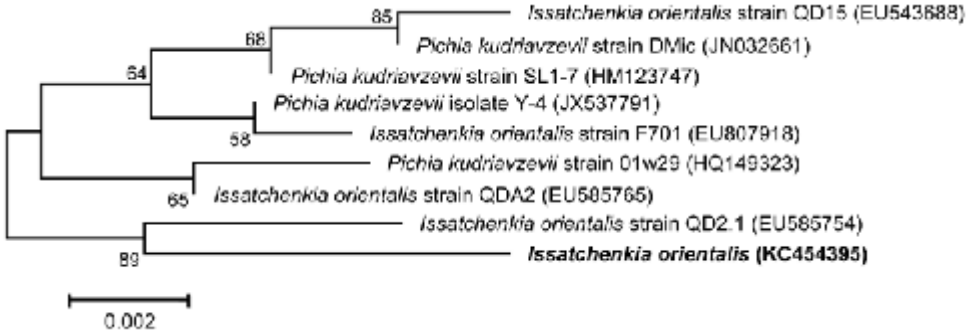

Fig. 1. Phylogenetic tree of *Issatchenkia orientalis* based on 26S rRNA D1/D2 domain sequence comparison

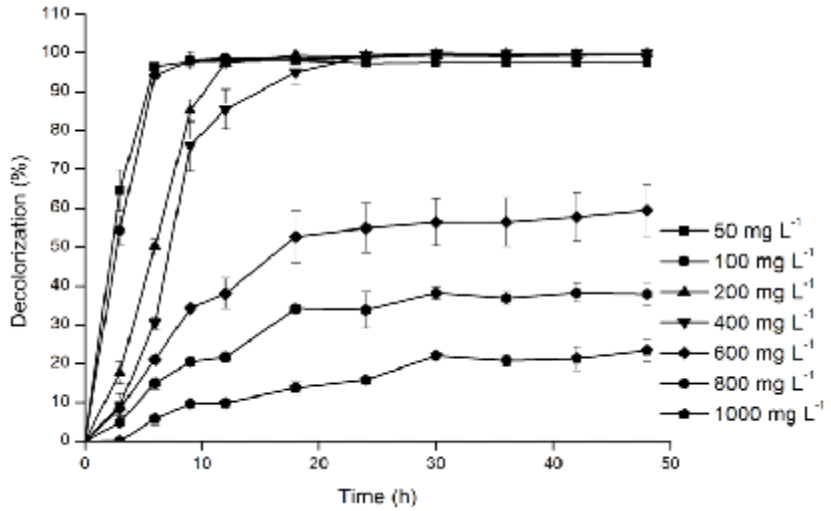

Fig. 2. Effect of different initial dye concentrations on decolorization of C.I. Reactive Black 5 by *Issatchenkia orientalis*

solution. Results indicated that initial pH values in the wide range of 3-8 supported good color removal efficiency, which was a desirable feature for various industrial applications. However, the decolorization efficiencies decreased rapidly at pH values 9 and 10. This may be due to osmotic charges and hydrolyzing effect[32, 33]. Solution pH influences both the cell surface dye binding sites such as chitin, acidic polysaccharides and the dye chemistry in water[23].

Effect of salinity

Effect of different salt concentrations on the decolorization was studied by using different NaCl concentrations 0-15% (w/v) (Fig. 4). Above 90% decolorization efficiency was achieved when the salt concentration ranged from 0-4%. However, further increase of NaCl concentration resulted in obvious inhibition effects on decolorization. Only 17% decolorization efficiency was observed with NaCl concentration up to 15%. Inhibition to microorganisms by high salt concentration may be explained by plasmolysis or loss of normal metabolic activity of cells[34]. Similarly, it was reported that high NaCl concentrations had adverse effect on decolorization[3, 35]. The results indicated that this strain exhibited good decolorization capacity under low NaCl concentrations (<4%), and thus could be widely used in treating low-salt wastewaters.

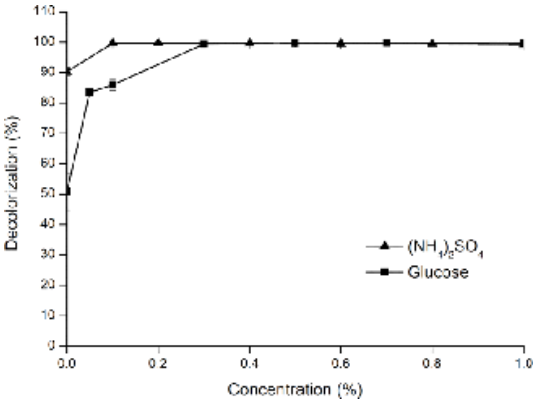

Fig. 3. Effect of carbon source and nitrogen source on decolorization of C.I. Reactive Black 5 by *Issatchenkia orientalis*

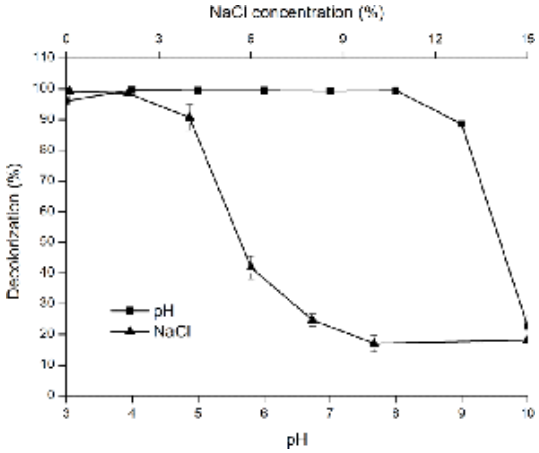

Fig. 4. Effect of pH and salinity on decolorization of C.I. Reactive Black 5 by *Issatchenkia orientalis*

Decolorization efficiencies with different dyes

Six commercial dyes were tested to demonstrate the general decolorization ability of this strain. Experiments were performed in the optimum conditions: 200 mg l^{-1} initial dye concentration, 10% (v/v) inoculum volume, 0.5% (w/v) glucose, 0.1% (w/v) $(NH_4)_2SO_4$, and without further pH adjustment and addition of salt. As can be seen in the Table 1, azo dyes were decolorized efficiently, attaining to more than 90% decolorization after 24 h. However, the decolorization efficiencies of two Anthraquinone dyes such as C.I. Reactive Blue 19 and C.I. Reactive Blue 21 were 75% and 53%, respectively. Therefore, this work demonstrated that the ascomycete yeast *Issatchenkia orientalis* displayed an effective azo dye decolorization capacity. It was obvious that cells of this strain changed into different colors after collection by centrifugation from different dye decolorization solutions (Fig. 5). The results suggested that adsorption may involve in the removal of these dyes[8]. The different decolorization of the tested dyes was affected by their molecular weights, substitution group of the dye molecules, and intramolecular hydrogen bonds[36, 37].

Decolorization mechanism

Obvious decolorization was observed on static solid culture medium with C.I. Reactive Black 5 (200 mg l^{-1}) after 24 h (Fig. 6a). Moreover, actively growing yeast cells in liquid cultures almost completely decolorized C.I. Reactive Black 5. The supernatant of decolorization medium went from blue to red and then became nearly colorless after centrifugation. In addition, the cells went from blue

to light blue and then became grey (Fig. 6b). The decolorization of dyes could be due to adsorption by the fungal biomass and biodegradation[20]. Compared to the cells before decolorization (Fig. 6c), dye adsorption can be clearly judged by inspecting the blue cells after decolorization under a light microscope (Olympus, Japan) (Fig. 6d).

Moreover, the progress in the absorbance spectrums of the dye solution was monitored during a time period of 0-24 h containing 200 mg l^{-1} C.I. Reactive Black 5 under optimized incubation conditions (Fig. 7). As the decolorization proceeded, the maximum absorbance at 598 nm decreased, while new peaks at 260 nm emerged, suggesting the degradation of the dye molecules to smaller intermediates[38]. In addition, as mentioned above, the microbial cells became grey after 24 h incubation with C.I. Reactive Black 5, which suggested that the biodegradation process occurred accompanying with decolorization[26]. According to Saratale *et al*.[21], a major mechanism behind biodegradation of synthetic dyes in microbial system was caused by the biotransformation enzyme. The most outstanding enzymes associated with degrading dyes are ligninolytic enzymes (laccase, lignin peroxidase,

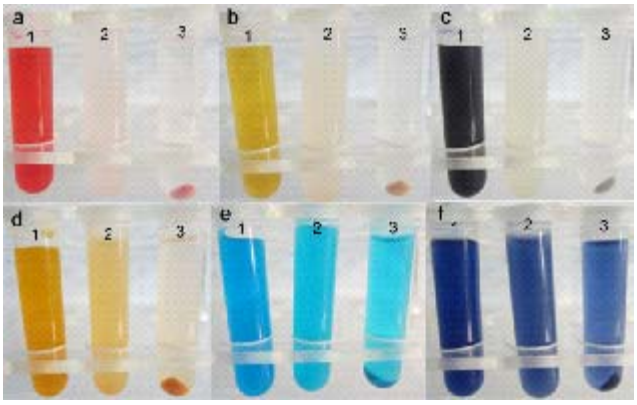

Fig. 5. Decolorization of various dyes by *Issatchenkia orientalis* for 24 h. (1) Before decolorization, (2) after decolorization without centrifugation, and (3) after decolorization with centrifugation. The dyes tested were as follows: (a) C.I. Reactive Red 24, (b) C.I. Reactive Brown 2, (c) C.I. Reactive Black 5, (d) C.I. Reactive Orange 1, (e) C.I. Reactive Blue 21, and (f) C.I. Reactive Blue 19

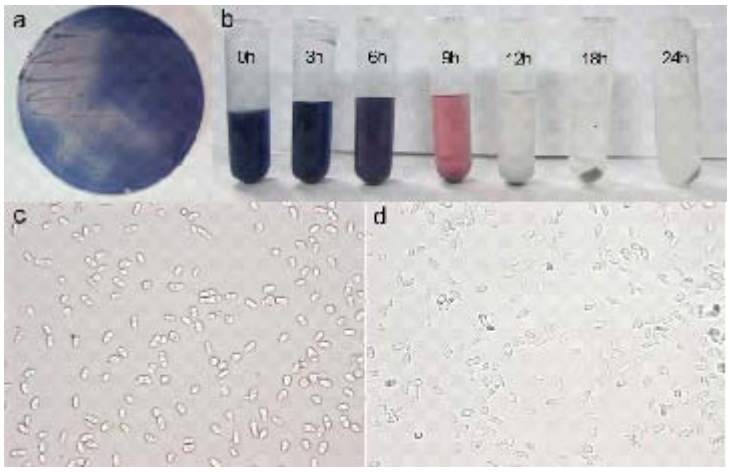

Fig. 6. Decolorization of C.I. Reactive Black 5 by *Issatchenkia orientalis*. (a) The photo of colonial morphology of *Issatchenkia orientalis* on agar plate with C.I. Reactive Black 5 (200 mg l^{-1}) after24 h, (b) the photo of decolorization medium after centrifugation at different time intervals (0, 3, 6, 9, 12, 18, 24 h), (c) the light microscope appearance of *Issatchenkia orientalis* before decolorization (40×), (d) the light microscope appearance of *Issatchenkia orientalis* after decolorization in 24 h (40×)

and manganese peroxidase), as well as reductase enzymes (NADH-DCIP reductase and azoreductase)[13, 39, 40]. In order to gain additional insights into the possible decolorization mechanism of the strain *Issatchenkia orientalis*, these enzymes mentioned above were monitored (Table 2).

In the present study, a slight increase in the activities of laccase, and lignin peroxidase was found from the yeast cells after decolorization. In addition, a significant increase in the enzyme activity of NADH-DCIP reductase was observed, which was supported by an earlier study[18]. Jadhav *et al*. reported that a significant increase in the activity of NADH-DCIP reductase appeared during the decolorization of malachite green by *Saccharomyces cerevisiae*. The induction of these enzymes by dyes in the culture media suggested their prominent role in the decolorization process. Additionally, the activity of manganese peroxidase was slightly decreased as compared to control cells. Similar results were reported by Pajot *et al*.[40] and Yang *et al*.[12] that manganese peroxidase could play an important role in the degradation of dyes by *Trichosporon akiyoshidainum* and *Debaryomyces polymorphus*, respectively. Moreover, a significant decrease in the activity of azoreductase was observed. According to Kalyani *et al*.[17], the relative contributions of ligninolytic enzymes and reductase to decolorization of dyes may be different for each microorganism. In this study, it could be assumed that the major mechanism of decolorization was mostly caused by azoreductase. Previous studies on biological decolorization of the azo dyes mainly focused on the azoreductase present in microorganisms since they could catalyse reductive cleavages of the azo groups primarily responsible for the biodegradation[41, 42]. In addition, Lucas *et al*.[43] also showed that an azoreductase catalyzed the degradation of azo dye C.I. Reactive Black 5 by a yeast isolate *Candida oleophila*.

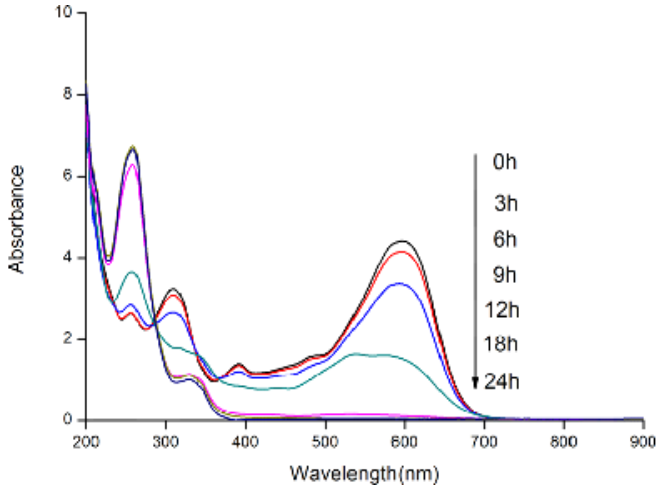

Fig. 7. UV-vis spectra of culture supernatants during the decolorization of C.I. Reactive Black 5 by *Issatchenkia orientalis* within 24 h

CONCLUSIONS

The study presented the decolorization ability of a newly isolated yeast, *Issatchenkia orientalis*. It was found that this yeast strain was a valuable fungus to decolorize the azo dyes. The effect of various operational parameters on color removal efficiency was investigated and optimized. Results showed that color removal efficiency decreased when the initial dye concentrations were more than 400 mg l^{-1}. The inoculum concentration within 10% (v/v) increased the decolorization rate rapidly. Increasing the carbon sources within 0.5% (w/v) and nitrogen sources within 0.1% (w/v) enhanced the decolorization efficiency. This yeast strain also exhibited decolorization ability within a wide range of pH (3-8) and could tolerate low salt concentration (<4%, w/v). Varying colored biomass

and UV-vis analyses suggested that decolorization was caused by both surface adsorption and biodegradation. Furthermore, enzymatic studies indicated the involvement of azoreductase as prominent enzymes for biodegradation under the optimized conditions.

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