

Screening, identification and optimization of a yeast strain, *Candida palmioleophila* JKS4, capable of azo dye decolorization

Narjes Jafari, Rouha Kasra-Kermanshahi*, Mohammad Reaz Soudi

Department of Biology, Faculty of Science, Alzahra University, Tehran, Iran.

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ABSTRACT

Background and Objectives: Synthetic dyes are recalcitrant to degradation and toxic to different organisms. Decolorization of textile wastewaters is one of the major concerns since last decades. Physical-chemical treatments are very expensive and frequently producing large amounts of toxic wastes. Biological treatments can be more convenient. In the present study, an attempt has been made for decolorization of azo dyes using microbial process.

Material and Methods: Screening of microorganisms capable of azo dye decolorization was performed from activated sludge. The decolorization of various dyes (Reactive Black 5, Reactive Orange 16, Reactive Red 198, Direct Blue 71, Direct Yellow 12 and Direct Black 22) was determined by measuring the absorbance of culture supernatant at their λ_{max} . Culture supernatants were also analyzed for UV-Vis absorption between 200-800 nm. The effect of aeration, temperature, different concentrations of glucose and NaCl was studied with an aim to determine the optimal conditions required for maximum decolorization.

Results: The yeast (strain JKS4) which had high ability to decolorize different azo dyes was isolated. Under aerobic condition, the yeast strain showed 85.7% of decolorization at 200 mg/l Reactive Black 5 (as a model azo dye), 1% (w/v) glucose concentration and 35°C after 24 h. All the examined dyes were extensively decolorized (53.35-97.9%) after 24 h. With elongated incubation period, complete decolorization was observed in presence of all dyes. From the physiological properties and phylogenetic analysis based on the 26S rDNA sequences, strain JKS4 was classified into *Candida palmioleophila*.

Conclusions: Because of high decolorizing activity against various azo dyes commonly used in the textile industries, it is proposed that the isolated yeast may have a practical application in the biotransformation of various dye effluents.

Keywords: Azo dyes, Decolorization, *Candida palmioleophila*, Textile Wastewater

INTRODUCTION

Azo dyes, the largest chemical class of dyes with the greatest variety of colors, have been used extensively for textile, dying and paper painting because of their ease of synthesis, versatility and cost-effectiveness (1). The term azo dye is applied to synthetic organic colorants that are characterized by one or more azo

linkages ($R_1-N=N-R_2$) and by aromatic structures. It has been reported that azo dyes strongly resist aerobic degradation (2-6). Over 7×10^5 tons of synthetic dyes are produced worldwide every year for dying and printing (1, 6-7) and out of this about 5-10% are discharged with wastewater (6). Many dyes are visible in water at concentrations as low as 1 mg/l. Textile wastewaters, typically with dye content in the range of 10-200 mg/l are therefore highly colored (8). Dye containing effluents are among the most problematic because it contains residual dyes that are difficult to remove in conventional treatment plants (2). The release of these industrial effluents into the environment plays an important role in increasing pollution (9). Furthermore, their discharge into surface

* Corresponding author: Rouha Kasra-Kermanshahi Ph.D
Address: Department of Biology, Faculty of Science, Alzahra University, Tehran, Iran.
Tel: +98-21-88044059
Fax: +98-21-88058912
E-mail: rkasra@yahoo.com

water leads to aesthetic problems and obstructs light penetration and oxygen transfer into bodies of water, hence affecting aquatic life (1, 5). Various reports have mentioned the direct and indirect toxic effects of dyes and metals in the form of tumors, cancers (1, 3, 8) and allergies besides growth inhibitions on different trophic levels like bacteria, protozoans, algae, plants and different animals including human being (8). Physical-chemical treatments such as coagulation/adsorption, electrolysis or ozonation are sometimes unsuccessful or very expensive and frequently producing large amounts of toxic wastes (3, 7).

Microbial decolorization and degradation is an environment friendly and cost-competitive alternative to chemical decomposition processes. However, these dyes are xenobiotics and their degradation in nature is rather difficult (1). Compared to bacteria and filamentous fungi, yeast exhibit attractive features. Yeasts are an inexpensive, readily available source of biomass. Though not as fast as bacteria, yeasts can grow faster than most filamentous fungi and like them, they have the ability to resist unfavorable environments. Yeasts can adapt and grow under various extreme conditions of pH, temperature and nutrient availability as well as high pollutant concentrations. (9).

In this study, decolorization of six azo dyes was carried out under aerobic condition using a yeast strain, *Candida palmioleophila* JKS4, isolated from activated sludge. The effect of various external parameters like temperature, glucose concentration and initial concentration of NaCl was studied.

MATERIALS AND METHODS

Chemicals and dyes. 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 Company, Tehran, Iran). Analytical grade chemicals were purchased from Sigma and Merck. RB5, a commonly used commercial azo dye, was used as a model dye for all optimization experiments. The chemical structure of RB 5 is shown in Fig. 1.

Screening of dye decolorizing microorganism. Activated sludge samples from several municipal wastewater treatment plants were used for screening of dye-decolorizing microorganisms. Five present

activated sludge samples were inoculated into 250-ml flasks containing 100 ml of medium with RB5 (200 mg/l). The composition of the medium used in the present study was as follows (g/l): glucose, 10; yeast extract, 0.34; NH_4Cl , 0.84; KH_2PO_4 , 0.134; K_2HPO_4 , 0.234 and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.084 (10). The pH of the solution was adjusted to 7 ± 0.2 before being autoclaved. The flasks were incubated in shaking incubator at 150 rpm and 32°C for 3 days. Flasks showing decolorization were selected for the isolation of microorganisms capable of azo dye decolorization. The isolate was purified and preserved on YMA (Yeast Malt Agar containing (g/l), yeast extract, 3; malt extract, 3; glucose, 10; peptone, 5 and agar, 15) slant at 4°C .

Identification of isolated yeast strain JKS4.

Identification of the isolated strain was performed by 26S rDNA gene sequence. Genomic DNA was extracted using an existing protocol (11) with some modifications. The 26S rDNA D1/D2 domain was amplified by PCR using forward primer NL1 (5'-GCA TAT CAA TAA GCG GAG GAAAAG-3') and reverse primer NL4 (5'-GGT CCG TGT TTC AAG ACG G-3'). The PCR products were sequenced. The 26S rDNA gene sequence was input into 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 molecular identification.

Decolorization assay. The liquid medium for the decolorization experiments was described above. The 250-ml flasks contained 100 ml of medium was 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 12000 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 (12):

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

Where A_i and A_t were the absorbance of the

Table 1. Biochemical and physiological characterizations of strain *C.palmioleophila* JKS4.

Character	Isolated yeast JKS4
Fermentation of	-
Glucose	-
Galactose	-/+
Sucrose	-
Lactose	-
Maltose	-
Trehalose	-
Diazonium blue B (DBB) test	-
Hyphae production in Corn Meal Agar	-
Acid production in Custer s medium	-

+, positive reaction; -, negative reaction

dye solution initially and at cultivation time (t), respectively.

Culture supernatants were analyzed for UV-Vis absorption between 200-800 nm using a spectrophotometer (shimadzu, TCC-240 A).

Effect of external 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, 10 % w/v) into the medium.

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 concentration) were added into the medium. Decolorization was determined by measuring the absorbance of culture supernatant at their λ_{max} .

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 (13) and were studied for the formation of positive halos for three weeks (reddish-brown and dark-brown halos in presence of guaiacol and tannic acid, respectively (14).

RESULTS

Strain isolation and identification. An azo dye degrading yeast (strain JKS4) which decolorized different azo dyes was isolated from activated sludge. From the phylogenetic analysis based on the 26S rDNA sequences, strain JKS4 showed 99% similarity to *Candida palmioleophila*. Fig. 2 shows the

Table 2. Decolorization of different azo dyes (200 mg/l) by *C. palmioleophila* JKS4 after 12 h and 24 h incubation at 32°C and 150 rpm

Dye	Decolorization (%) after exposure for	
	h 24	h 12
Reactive Black 5	30.95±3.64	82.45±6.159
Reactive Orange 16	10.7±3.394	86.8±2.545
Reactive Red 198	0	53.35±7.566
Direct Yellow 12	51±2.969	89.6±0.707
Direct Blue 71	82.5±1.5	96.55±0.353
Direct Black 22	0	97.95±0.07

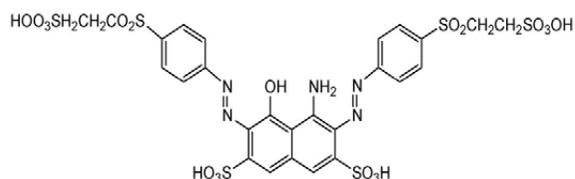


Fig. 1. Chemical structure of Reactive Black 5.

phylogenetic relationship between different members of the genus *Candida* and isolated yeast JKS4. The 26S rDNA partial sequence of the isolated yeast JKS4 was deposited in the GenBank database with the accession number JQ650231. Some of physiological properties of the strain JKS4 were showed in Table 1. The isolate yeast exhibited decolorization ability of 82.45% at 32°C after 24 h incubation under aerobic condition. With extension of incubation time, complete decolorization was observed. Lower decolorization of 10.5% was obtained after 24 h incubation under static condition.

The biodecolorization of the RB5 azo dye was monitored by UV-Vis analysis. Untreated dye solution (Fig. 3a) showed that RB5 presented two absorbance peaks at 597 and 310 nm. After aerobic treatment of the azo dye solution by *C. palmioleophila* JKS4, the

absorbance peak in the visible region disappeared indicating complete decolorization was occurred. In the UV spectra, the peak at 310 nm disappeared following by the formation of a new peak at 260 nm. This suggests that *C. palmioleophila* JKS4 transformed azo dye RB5 into colorless compounds.

Effect of different factors on the decolorization.

Fig.4 shows the effect of different glucose concentrations (0.5-2%) on decolorization of RB5 azo dye at 200 mg/l concentration after 24 h incubation. The decolorization increased from 63.45% to 82.45% with an increase in glucose concentration from 0.5 to 1%. With further increase in glucose concentration to 2%, lower decolorization (79.3%) was obtained. No decolorization was observed when the culture medium was not supplemented with glucose. Fig.5 shows decolorization of dye (at 200 mg/l concentration) at different temperatures (29, 32, 35 and 40°C) after 24 h incubation. It is clear from the figure that decolorization increased (from 78.3% to 85.7%) with an increase in temperature form 29 to 35°C. The decolorization decreased (to 40%) with further increase in temperature to 40°C. Fig. 6 shows the effect of different NaCl concentrations on

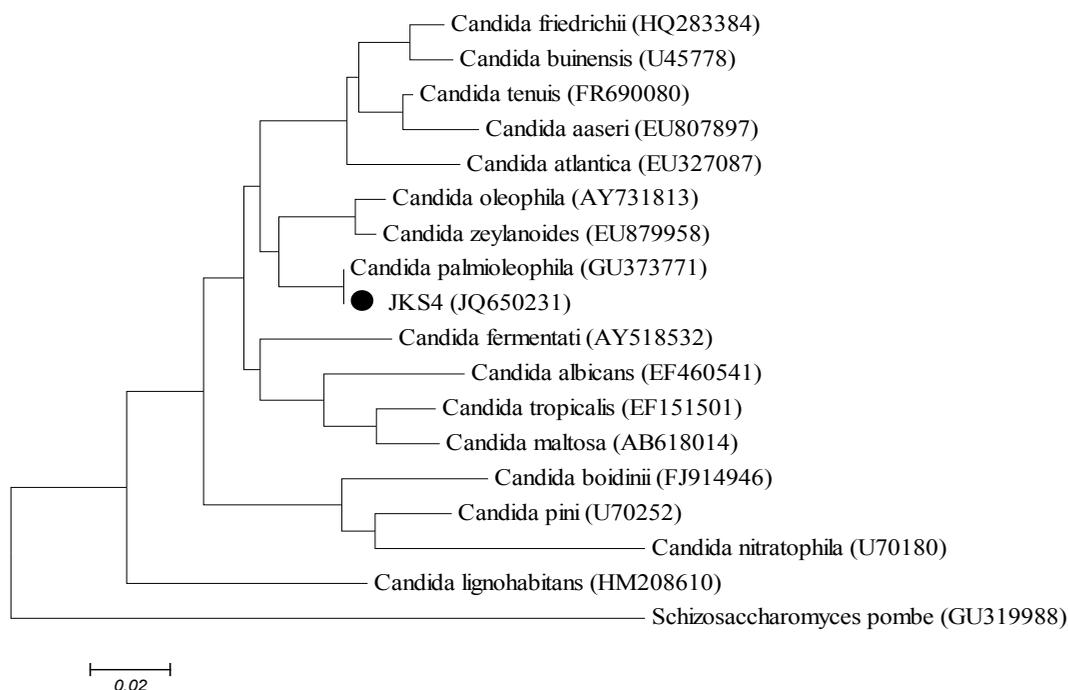


Fig. 2. The phylogenetic tree was constructed using neighbor-joining analysis of the 26S rDNA D1/D2 domain. The ascomycetous yeast *Schizosaccharomyces pombe* was used as an out group. Gene bank accession numbers of references sequences are given in parenthesis.

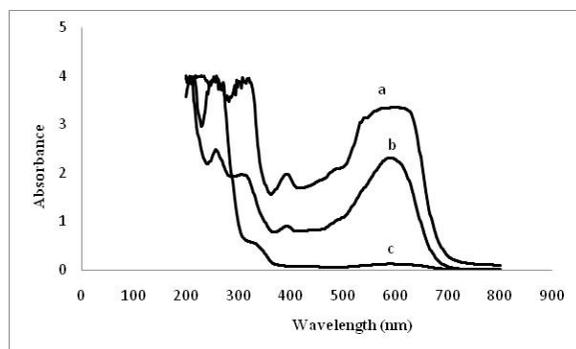


Fig. 3. UV-Vis spectra (200-800 nm) of the treated medium by *C. palmioleophila* JKS4, a: control; b,c: after 12 h and 72 h incubation at 32°C.

decolorization. After 24 h, the decolorization of dye was found to be 82.45, 67.75, 51.9 and 5.5% at NaCl concentrations 0, 3, 5 and 10% respectively.

Decolorization of other azo dyes: The ability of *C. palmioleophila* JKS4 to decolorize five other azo dyes including RO16, RR198, DB71, DY12 and DB22 was examined. The results (Table. 2) showed that *C. palmioleophila* JKS4 could effectively decolorize (53.35-97.9%) all tested azo dyes.

DISCUSSION

Azo dyes represent a major group of dyes causing environmental concern because of their color, biorecalcitrance, potential toxicity and carcinogenicity to animals and human (1). Biological process could be a more economical and effective method for treatment of colored effluents contained these pollutants.

Reductive azo dye decolorization by microorganisms usually starts with the cleavage reduction of the azo bond under anaerobic or microaerophilic conditions, and leads to the accumulation of toxic aromatic amines

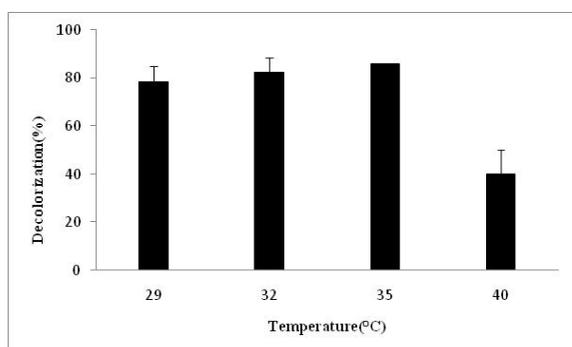


Fig. 5. Effect of different temperatures on decolorization of RB5 (at 200 mg/l) by *C. palmioleophila* JKS4.

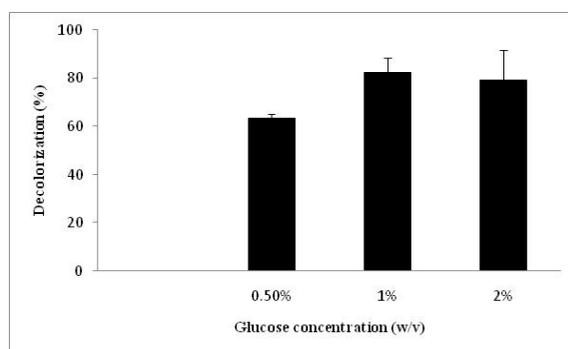


Fig. 4. Effect of various glucose concentrations on decolorization of RB5 (at 200 mg/l) by *C. palmioleophila* JKS4.

(5). Azoreductase isolated from several bacteria have been shown to be inducible flavoproteins and to use both NADH and NADPH as electron donors. The presence of oxygen normally inhibits the azo bond reduction activity, since aerobic respiration may dominate use of the NADH; thus impeding electron transfer from NADH to the azo bonds (5). However, several studies reported decolorization and degradation of azo dyes under aerobic conditions (10, 15-17). In present study, the decolorization of RB5, as a model azo dye, by *C. palmioleophila* JKS4 was evaluated under two culture conditions, aerobic and static, that higher decolorization (82.45% after 24 h) was observed under aerobic condition as comparison to static condition (10.5%). The present decolorization result was in agreement with the findings reported by Yang et al. (2005) that complete decolorization of RB5 was obtained in treated culture by *Debaryomyces polymorphus* within 24 h (18).

The degradation products were studied by UV-Vis technique. After treatment of the RB5 by the *C. palmioleophila* JKS4, the absorbance peak in the visible region disappeared, indicating complete

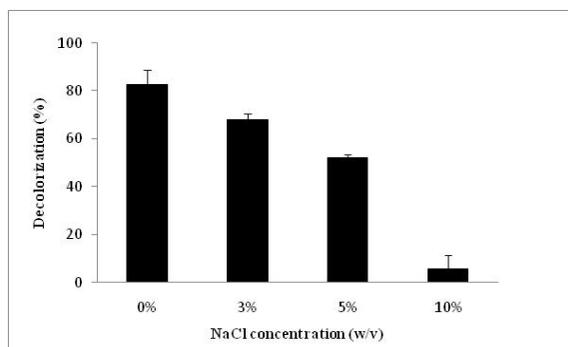


Fig. 6. Effect different NaCl concentrations on decolorization of RB5 (at 200 mg/l) by *C. palmioleophila* JKS4.

decolorization was occurred. In the UV spectra, the decrease in absorbance peak at 310 nm, related to the benzene and naphthalene rings, and the formation of a new peak at 260 nm was observed. This result suggested that *C. palmioleophila* JKS4 was able to break the conjugated azo bonds but mineralization of the dye was not occurred, consistent to previous studies on the other microorganisms (5, 19). Because of the toxic potential of many aromatic amines, further degradation of the dye compound is necessary if toxicity is to be eliminated or reduced (6).

According to the result extracted from UV-Vis spectra and also colorless yeast biomass obtained after complete decolorization of dye contained culture, biodegradation played a major role in decolorization of RB5 in comparison with biosorption.

Effect of different external parameters was studied on the decolorization. The decolorization was found to be 63.45, 82.45 and 79.3 % at glucose concentrations 0.5, 1 and 2%, respectively. The reason for lower decolorization at 0.5%, as compared to 1% glucose concentration, might be that low glucose concentrations could not meet the growth requirements of the microbes (10). When the glucose concentration was 2%, the yeast could utilize glucose preferentially, thus resulting in lower decolorization. In the present study it was observed that 1% glucose concentration is optimum for decolorization of RB5. At different temperatures tested for decolorization process, decolorization efficiency was significantly decreased at 40°C, this might be due to the loss of cell viability or deactivation of the enzymes responsible for decolorization at 40°C (10).

In the present study, *C. palmioleophila* JKS4 was tested for separately decolorize six azo dyes RB5, RO16, RR198, DB71, DY12 and DB22. After 24 h incubation, the decolorization was assessed 82.45, 86.8, 53.35, 89.6, 96.55 and 97.95%, respectively. With elongated incubation period, complete decolorization was observed in presence of all examined dyes. The chemical structures of the dyes greatly influence their decolorization rates (5). Lower decolorization for RR198 was probably due to the triazine group, whose degradation is more recalcitrant than that of the benzene and naphthalene rings (5). Results (Table 2) showed that direct dyes (DY12, DB71 and DB22) exhibited higher decolorization efficiency in comparison with reactive dyes (RB5, RO16 and RR198). It has been reported that the presence of sulfonates in reactive dye structures results in

low levels of color removal. However, this is not applicable to direct dyes that usually exhibit high levels of decolorization independent of the number of sulfonate groups in the dye structure, reinforcing the idea that steric hindrance and the number of azo bonds are responsible for the different decolorization efficiency (5).

Enzyme involved in the degradation of azo dyes are mainly peroxidases and laccases (1). In present study, laccase production by isolate was not detected in plate containing guaiacol or tannic acid. Further investigation is needed to clarify the enzymatic mechanism responsible for dye biodegradation. Because of high decolorizing activity against various azo dyes commonly used in the textile industries, it is proposed that the *C. palmioleophila* JKS4 may have a practical application in the biotransformation of various dye effluents.

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