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Biodegradation of Diazo Dye Reactive Black 5 with the Isolated Yeast *Candida krusei*

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The biodegradation for a diazo dye, Reactive Black 5, was investigated by the isolated yeast, *Candida krusei*. This strain showed 99.08% decolorization within 24 h in a medium containing 1% (w/v) glucose, 0.1% (w/v) ammonium sulphate, and 100 mg/L dyestuff by inoculating 20% (v/v) yeast culture solution at natural pH, and shaking at 30°C. Its decolorization maintained efficient within the range of the initial dye concentration from 100 mg/L to 500 mg/L. Furthermore, the degradation of Reactive Black 5 resulted in a significant pH decrease by this yeast. UV-Vis analysis of batch culture solutions and color observation of cell mats indicated that the decolorization proceeded dominantly by biodegradation, while biosorption happened in the incomplete dye degradation. In addition, this yeast could also remove other dyes such as Reactive Blue 4, Mordant Blue 1, Mordant Yellow 10, and Reactive Red 24.

Key words: Biodegradation; Diazo dye; Reactive Black 5; *Candida krusei*.

It is reported that over 10,000 commercially available dyes with a production of more than 7×10^5 metric tons are annually manufactured, among which azo dyes are the most popularly used, accounting for approximately 70% of the total number of dyes [1]. Based on chemical properties, dyes are classified as anionic (direct, acid and reactive dyes), cationic (basic dyes) and nonionic (disperse dyes). The chromophores in anionic and nonionic dyes are mostly azo groups or anthraquinone types [2].

The dye wastewater is characterized by high alkalinity, biological oxidation demand (BOD), chemical oxidation demand (COD), and total dissolved solids (TDS) with the dye concentration generally below 1 g/dm^3 [3]. Biotoxicity and carcinogenicity of dyes have been extensively proved [4–7], and hence, numerous biological and physicochemical methods have been developed to efficiently decolorize and detoxify the dye-contaminated effluents [8–11]. However, implementation of chemical/physical methods has the inherent drawbacks of being economically unfeasible (as they require more energy and chemicals). They are unable to completely remove the recalcitrant azo dyes and/or their organic metabolites may cause secondary pollution problems, involving complicated procedures [11]. In comparison, bioremediation is definitely an attractive tool. It is currently of interest to overcome the problems arising from the industrial wastewater, because it's eco-friendly and cost-effective.

So far, vast amounts of reports have clearly demonstrated the effectiveness of biodegradation and dye removal mediated by bacteria and white-rot fungus [3,12–17]. Unfortunately, during the degradation process, anaerobic bacteria often generate aryl amines that can be transformed to highly reactive electrophiles and form covalent

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adducts with DNA, thereby posing a health risk [6]. The application of white-rot fungi for dye wastewater treatment has been retarded by the rigorous conditions for enzyme production, as well as the risk of contamination by bacteria under non-sterile conditions [18].

Interestingly, yeast, combining the merits and discarding the defects of both bacteria and white-rot fungus, has been successfully exploited to treat industrial effluents such as food, molasses, and oil manufacturing wastewater, reported by Japanese scientists [19]. However, the reports on biodegradation by yeast are still limited [20].

In the present study, a yeast strain YG-1 was isolated, identified, and applied to decolorization of five dyes. Its decolorization conditions and mechanism were investigated based on batch decolorization of Reactive Black 5, a widely used and biotoxic diazo dye. In addition, the decolorization of other four dyes was evaluated with this yeast.

MATERIALS AND METHODS

Dyes

The dyes used in this study included Reactive Black 5, Reactive Red 24, Reactive Blue 4, Mordant Blue 1, and Mordant Yellow 10, supplied by Tianjin Xinmei Dyestuff Chemical Co., Ltd (China). The chemical structures of these dyes are shown in Table 1. All of the dyes were used without prior purification. The stock solution (1 g/L) was prepared by fully dissolving the dye in deionized water and being diluted before use.

Strain and culture maintenance medium

The yeast strain YG-1 was isolated from the soil samples collected from organic-rich sites near the north fourth ring in Beijing, China. Approximately 1 g wet soil samples were added into 3 mL acclimated medium (AM) for enrichment culture, and the pH is adjusted to 5.0-6.0. AM was composed of 1% (w/v) glucose, 0.1% (w/v) KH_2PO_4, 0.1% (w/v) $(NH_4)_2SO_4$, 0.05% (w/v) $MgSO_4$, and 0.02% (w/v) yeast extract [21]. Isolated strains were purified by plate streaking technique. Based on AM, the decolorization medium (DM) was inoculated with stock solution in different concentrations but without yeast extract.

Identification of the isolates

An integrated approach including phenotypic (morphological, biochemical, and physiological characterization) and genotypic (sequencing of D1/D2 domain of 26S rDNA encoding gene) tests were performed to identify the yeast strain YG-1.

Universal fungal 18S rDNA gene primers (ITS5 and LR6) were used for polymerase chain reaction (PCR) amplification [22]. The 26S rDNA D1/D2 domain sequence was amplified by PCR with primers NL1 (5'-GCA TAT CAA TAA GCG GAG GAA AAG-3') and NL4 (5'-GGT CCG TGT TTC AAG ACG G-3') [23]. The partial sequences of 26S DNA gene were used in a similar search by means of the Basic Local Alignment Search Tool (BLAST) from GenBank database (National Center for Biotechnology Information, NCBI). The strain is stored at the China General Microbiological Culture Collection Center (CGMCC). Morphological and physiological examinations were conducted by the Yeast Identification PC program according to the methods described by Barnett *et al*.[24].

Decolorization assay

Culture supernatants were obtained by centrifugation at 9,000 rpm for 10 min. The optical density (OD) of each dye was measured at the maximum adsorption wavelength λ_{max} of the dye. The maximum adsorption wavelengths of the five dyes are shown in Table 1. Uninoculated culture medium with and without adding dyestuff were used as negative controls. The decolorization efficiency was expressed as

$$Decolorization\ (\%) = (A - B) / A \times 100\%,$$

where A is the initial absorbance and B is the absorbance of medium after decolorization at (nm).

Determination of the optimum conditions for RB5 decolorization

For any microbial degradation process, media components such as carbon source, nitrogen source, dye concentration, inoculum size, pH, and temperature are the most important parameters which affect the process [25]. Hence, it's necessary to investigate the influence of these factors on the degradation process according to color removal efficiency.

Batch cultures for decolorization experiments with the isolate YG-1 were conducted in 10 mL medium with shaking at 200 rpm, 28°C for 24h. Each factor was performed under one control and duplicate groups. Decolorization effects of different

initial dye concentrations (100-1000 mg/L) were evaluated in the medium, containing 1% (w/v) glucose and 0.1% (w/v) ammonium sulphate, by inoculating 10% (v/v) yeast culture solution at natural pH. To find a suitable amount of inoculum for the effective dye decolorization by yeast YG-1, experiments were developed with different inoculum sizes (1, 3, 5, 10, 15, 20, and 30% yeast culture solution, v/v). To detect the decolorization effect of pH, the medium was adjusted to pH 3-11 before 10% (v/v) yeast culture solution was inoculated. The optimal carbon and nitrogen sources for decolorization were tested in a series of dye-bearing medium with different concentrations of glucose (0-1.0%, w/v) and ammonium sulphate (0-1.0%, w/v).

Other analytical procedures

The decolorization process of Reactive Black 5 was conducted in 50 mL medium (pH 7) which contained the following (w/v): 1% glucose, 0.1% KH_2PO_4, 0.3% $(NH_4)_2SO_4$, 0.05% $MgSO_4$, 20% (v/v) yeast culture solution, and 100 mg/L dye concentration. The media and dyestuff solutions were separately autoclaved at 121°C for 20 min. The acclimated yeast suspension prepared from AM without dye was used to inoculate a 250 mL Erlenmeyer flasks containing 50 mL medium. Incubations were carried out on an orbital shaker set at 220 rpm, 30°C for 48h. Triplicate determinations were performed at 0, 4, 8, 12, 16, 24, 28, 32, and 48 h with 4 mL culture supernatants, respectively. The color of cell mats and decolorization culture solutions were observed, and the absorbance of each culture solution was measured at each stage.

An UV-Vis spectrophotometer (Unic UV-4802H, USA) equipped with a quartz cell of 1.0 cm path length was used to analyze the absorbance of culture supernatants from 400 to 800 nm.

All the values presented are the means of at least triplicate independent assays unless otherwise stated.

RESULTS

Isolation and identification

Numerous yeast strains were isolated after one week incubation. Primary morphological identification was performed to differentiate the isolated strains. Based on further decolorization tests, one of colonies YG-1, performing well at high dye concentration up to 100 mg/L, was selected from white colonies for further study. Its morphology is ovate with size of (2.0-5.5) μm × (4.5-15.5) μm, and the colony is grey-white, matte, with waviness edge, and no feigned hypha generated. The results of biochemical and physiological characteristics were also evaluated by the Yeast Identification PC program. It was identified as *Candida krusei*. Based on the sequencing of 26S rDNA D1/D2 domain, the strain YG-1 was finally confirmed as *C. krusei*, matching 100% homology of its GenBank database.

Optimum conditions for RB5 decolorization

The optimum decolorization conditions were investigated based on the parameters of initial dye concentration of Reactive Black 5, glucose concentration, ammonium sulphate concentration, inoculum size, and pH (Fig.1)

Initial dye concentrations

To determine the maximum Reactive Black 5 tolerated by *C. krusei*, experiments with different initial dye concentrations (100-1000 mg/L) were performed (Fig. 1(A)). The decolorization efficiency was above 96% for initial dye concentration less than 500 mg/L after 24 h cultivation, which means an acceptable high color removal could be achieved in an extensive range of azo dye concentrations by the strain *C. krusei*. However, a substrate inhibition effect was observed at dye concentration higher than 500 mg/L. Moreover, further cultivation indicated that higher dye concentration (> 400 mg/L) required more time for complete decolorization. Similar results were observed using Reactive Black 5 by Saratale et al. [26]. In the present study, *C. krusei* showed a good performance on the decolorization of different concentrations of Reactive Black 5.

Furthermore, the color of cell mats and decolorization culture solutions were observed based on different initial dye concentrations of Reactive Black 5. Within the range of 100-300 mg/L of this dye, the cell mats were yellow-white and the decolorization culture solutions were colorless, while the cell mats become blue and the solutions become colorless or light blue in 400-600 mg/L. In the higher dye concentration of 700-1000 mg/L, the cell mats and batch solutions were all blue.

Inoculum sizes

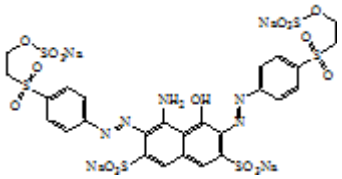
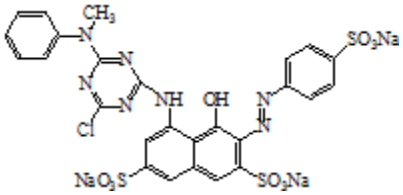
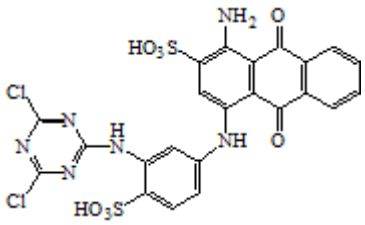
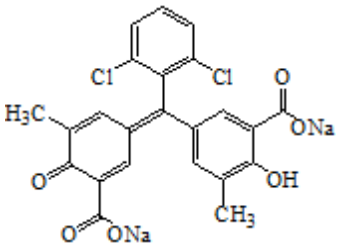
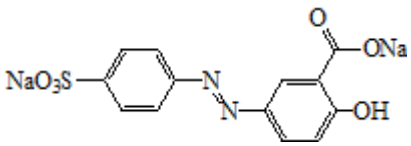
Complete color removal was obtained within 24 h cultivation under different inoculum sizes (1, 3, 5, 10, 15, 20, and 30% yeast culture solution, v/v) (Fig.1 (B)). It could infer that the

amount of inoculum had little effect on the decolorization rate. Obviously, the decolorization all ran in high efficiency, above 98.5%. The inoculum size was set at 20% (v/v) in the following experiments with the highest decolorization efficiency, 99.08%.

Carbon and nitrogen sources

Different initial concentrations of glucose (0-1.0 %, w/v) in medium were used to determine their effects on decolorization rate of Reactive Black 5 (100 mg/L). The results present in Fig. 1(C) indicated that glucose was essential for decolorization of Reactive Black 5. In the absence of glucose, only less than 10% decolorization of the added dye was achieved in 24 h, which means that the dyestuff couldn't be as sole carbon source for the growth of *C. krusei*. However, when glucose supplement increased in medium, decolorization

Table 1. Chemical structures and maximum absorbance wavelengths of the dyes

Dye	Structure	Chemical class	*λmax (nm)
Reactive Black 5		Diazo	598
Reactive Red 24		Azo	535
Reactive Blue 4		Anthraquinone	595
Mordant Blue 1		Triphenylmethane	490
Mordant Yellow 10		Azo	362

*The maximum absorbance wavelength of each dye

efficiency of *C. krusei* was increased obviously at low glucose concentration (< 0.2 %). Further increase had little effect on decolorization rate, similar profile was reported by Khehra et al. [14]. The maximum decolorization efficiency (99.24%) was obtained at 1% glucose in 24 h.

Ammonium sulphate was selected as the nitrogen source for the growth of yeast. The results are shown in Fig. 1(D). The yeast could decolorize 46% Reactive Black 5 even without Ammonium sulphate. The maximum color removal (94%) was achieved at the concentration 0.1%. The decolorization efficiency of the dye was improved by increasing the ammonium sulphate concentration within 0-0.1%, w/v, but increased slightly when the concentration was over 0.1% , and even decreased at higher concentration of 1%. It indicated that low ammonium sulphate concentration benefited the growth of yeast, but the high leaded to opposite results.

pH

Fig. 1(E) shows that the yeast strain could decolorize the diazo dye of Reactive Black 5 in a wide range of pH. An optimal pH for decolorization

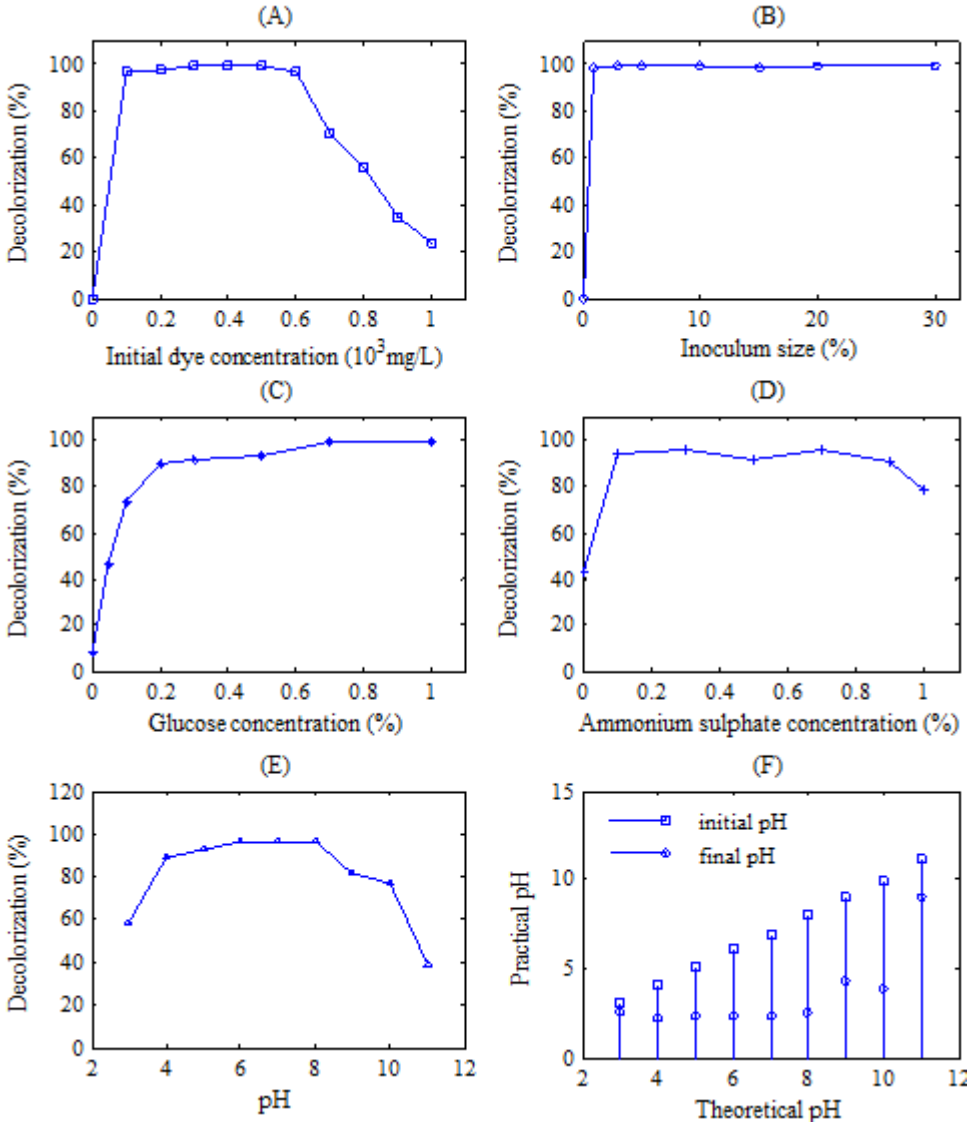


Fig. 1. Decolorization of Reactive Black 5 by *C. krusei* under different culture conditions: (A) initial dye concentration, (B) inoculum size, (C) glucose concentration, (D) ammonium sulphate concentration, and (E) pH. (F) The relationship between the initial pH and the final pH after 24 h cultivation

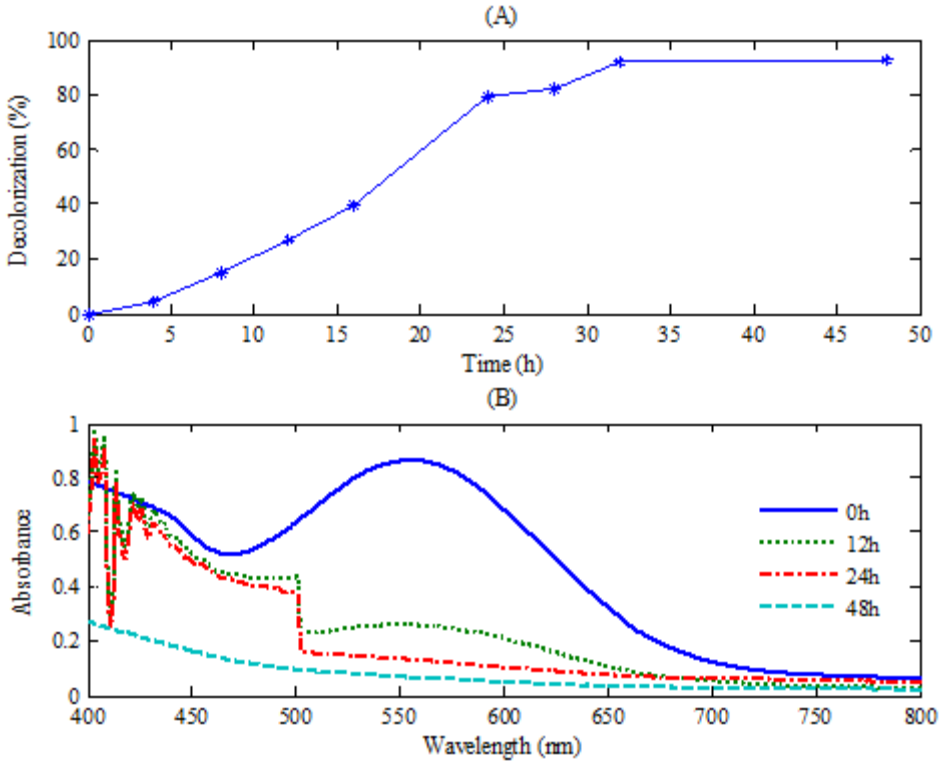


Fig. 2. The decolorization effect of Reactive Black 5 with *C. krusei* within 48 h. This experiment was in a 50 ml medium with 1% glucose, 0.1% KH_2PO_4, 0.3% $(NH_4)_2SO_4$, 0.05% $MgSO_4$, 20% (v/v) yeast culture solution, and 100 mg/L dye concentration, pH 7, shaking at 220 rpm, 30°C. (A) The trend of decolorization efficiency of Reactive Black 5 at different time intervals (0, 4, 8, 12, 16, 24, 28, 32, and 48h); (B) The variation in UV-Vis spectra of Reactive Black 5 at 0, 12, 24, and 48 h. The decolorized medium of this strain were centrifuged before drawing the UV-Vis spectra to delete the interference of cellular OD

by the isolate was confirmed within the range of 6-8 and the maximum decolorization is up to 96%. In contrast, common decolorizing bacteria have a narrow pH range [15,27]. Interestingly, a significant pH decrease could be observed for all culture solutions (Fig. 1(F)). The decolorization of Reactive Black 5 was possibly accompanied by the production of acids, suggesting that SO_3 groups were released from the structure of Reactive Black 5 during the decolorization process [19].

Decolorization mechanism

According to the reports [11,28,29], some yeast species could decolorize the dyes due to their enzymatic biodegradation, and some yeasts could act as a promising dye adsorbent which could uptake higher dye concentration. In the process of dye adsorption, the absorption spectrum could reveal that all peaks decrease approximately in

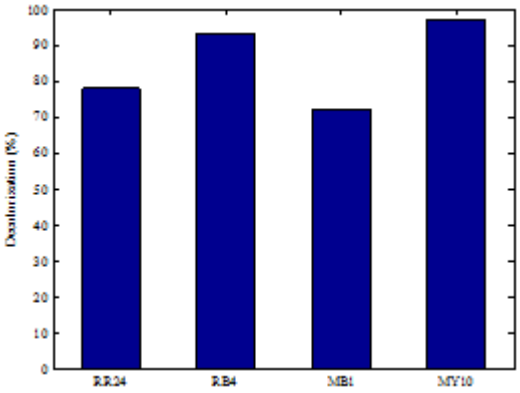


Fig. 3. Degradation of different dyes with *C. krusei*. All the initial dye concentrations were 50 mg/L, cultivated for 24 h. RR24 – Reactive Red 24; RB4 – Reactive Blue 4; MB1 – Mordant Blue 1; MY10 – Mordant Yellow 10

proportion to each other. However, once the dye removal is attributed to biodegradation, the major visible light absorbance peak will completely disappear or a new peak will appear[30]. Dye adsorption could also be judged clearly by inspecting the cell mats. Cell mats become deeply colored because of adsorbing dyes, whereas those retaining their natural colors are often accompanied by the occurrence of biodegradation [15].

The decolorization efficiency of Reactive Black 5 was recorded at different time intervals (0, 4, 8, 12, 16, 24, 28, 32, and 48 h) (Fig.2 (A)). Obviously, the decolorization efficiency of *C. krusei* had a sharp increase with the extending of cultivation time before 24 h, and increased slightly thereafter, even remained constant (92%) at the last 4 h. Hence, it could be concluded that the decolorization process of the yeast could be divided into two stages: a rapid degradation stage within the first 24 h and a slow decolorization stage thereafter. In addition, throughout the whole decolorization process, the cell mats remained their natural color and their corresponding supernatant went through dark-purple, red-purple, light-purple, light-yellow, and colorless. This phenomenon revealed that the yeast *C. krusei* decolorizing Reactive Black 5 was governed by biodegradation. However, the reason for the constant color removal after 24 h might be others but not the adsorption of the toxic azo dye, yet for further study.

UV-Vis analysis results (Fig.2 (B)) indicated that the maximum absorption peak of Reactive Black 5 in the visible region shifted gradually from 557 to 550.5 nm before 12 h, and diminished after 24 h. It clearly suggested that the decolorization of the diazo dye Reactive Black 5 by *C. krusei* was caused by biodegradation, which was consistent with the color observation of the cell mats. The details in the mechanism of diazo dye decolorization by the yeast will be studied in the future.

Decolorization of other dyes

The strain *C. krusei* was not only able to decolorize diazo dye Reactive Black 5, but also other dyes. Fig.3 reveals the decolorization of Reactive Red 24, Reactive Blue 4, Mordant Blue 1, and Mordant Yellow 10 with the yeast strain *C. krusei* had a satisfying decolorization rates, ranging from 72% to 97%. The colors of cell mats in each dye degradation experiment were white, blue, brown, and light yellow, by the order of Reactive Red 24, Reactive Blue 4, Mordant Blue 1, and Mordant Yellow 10.

DISCUSSION

The foregoing results confirmed that the yeast strain YG-1 (*C. krusei*) could effectively decolorize diazo dye Reactive Black 5, which indicated the natural adaptation of this yeast to survive in the presence of toxic azo dyes. Ayed et al.[1] reported isolation of microorganisms adapted to high dye concentration from textile effluent treatment plant and dyeing industry.

In this study, the colors of cell mats and batch culture solutions revealed that *C. krusei* decolorized this diazo dye not just by biodegradation but also through biosorption when the dye was degraded incompletely. However, the toxicity to the yeast increased with the adsorption of the diazo dye and inhibited the growth of yeast, contributing to the reduction of decolorization efficiency at high initial dye concentration. The effects of initial dye concentration on dye decolorization have been widely reported [31–33]. Reduction in decolorization efficiency and cell growth might result from the toxicity of dyes through the inhibition of metabolic activities [34]. Azo dyes generally contain one, or more sulphonic-acid groups on the aromatic rings, which might act as detergents, thereby inhibiting the growth of the microorganisms [35]. It could be speculated that growth and decolorization are shown to be greatly inhibited at high initial dye concentrations and usually require longer incubation times.

It's proven that medium composition greatly affects decolorization process, but the studies concerning the effects of different concentrations of carbon or nitrogen sources on dye decolorization are limited. Since azo dyes are deficient in carbon sources and the biodegradation of dyes without any supplement of carbon or nitrogen sources is vey difficult [36], the reduction of azo dyes depends on the presence and availability of co-substrate as electron donor for the azo dyes reduction [13]. In this study, *C. krusei* could not use Reactive Black 5 as a sole carbon source for the cell growth and the decolorization depended on an additional carbon source. This implied a cometabolic pathway of degradation of

aromatic compounds, which has also reported for decolorization of synthesized azo dyes by white rot fungi [17,18]. However, the decolorization process in yeast occurred during the exponential growth stage of cells and only under the conditions of enough nutrients existence, which is different from most white rot fungi occurred after enzyme production [37]. In addition, nitrogen sources are also important for microbial degradation, but most researches focus on the organic nitrogen sources and few reports are related to inorganic nitrogen sources. The present study implied that high ammonium sulphate concentration might hinder the degradation process of *C. krusei*, because of the inhibition of production of lignolytic enzymes, similar to *Phanerochaete chrysosporium* reported by Zacchi *et al*. [38]. The acquirement of reliable reasons requires further work.

Adsorption and enzymatic activity are dependent on the pH. As the extent of decolorization is influenced by the pH of medium, pH also affects the color of the solution and the solubility of the dye [39]. The foregoing results showed that the rate of color removal was higher at the optimum pH, and tended to decrease rapidly at low pH. It is thought that the effects of pH might be related to the transport of dye molecules across the cell membrane, which is considered as the rate-limiting step for the decolorization [40]. Since the decolorization of Reactive Black 5 in this study was mainly through biodegradation, the azo bond could be reduced by the yeast. This profile resulted in an increasing in the pH, due to the formation of aromatic amine metabolites which are more basic than the original azo compounds. The significant pH decreasing from the initial pH to the final pH might result from the consumption of glucose which could be decomposed to organic acids [15]. It demonstrated the importance of pH control to decolorization if some active biodegradable carbon sources were present in dye wastewater.

The effect of inoculum size on dye decolorization is rarely reported. It was reported that high decolorization rate was observed when the high inoculum size is used [25,41]. However, for bacteria, Dafale *et al*. think Reactive Black 5 decolorization rate isn't correlated with bacterial counts when the inoculum size increases [42]. The different results may be because of the inoculum size and nutrient in the medium. The number of bacteria is an important factor for the degradation of dyes. Bacteria deficiency can result in low degradation rate and prolong degradation time. On the contrary, excessive inoculum usually leads to cell waste and nutrient deficiency, which may decrease the growth and activity of bacteria [43]. In the present study, there's no significant influence on decolorization efficiency but all contributed to high color removal. Further experiments are needed to clarify the mechanism of this profile.

Fig.3 well indicates that the strain *C. krusei* has a promising degradation potential of a variety of dyestuffs and could be applied to printing and dyeing industry with great latent capacity. In this study, decolorization of these dyes with *C. krusei* might be through biodegradation according to the observation of colonies' color. More studies on metabolism of *C. krusei* should be put into effect.

CONCLUSIONS

The yeast identified as *C. krusei* had a promising decolorization potential of a variety of dyestuffs and could be applied to treat wastewater of printing and dyeing industry in the future. The results indicated that this strain achieved 99.08% decolorization rate within 24 h in DM which contains 1% (w/v) glucose, 0.1% (w/v) ammonium sulphate (natural pH), 100 mg/L dyestuff of Reactive Black 5, and 20% (v/v) yeast culture solution. The degradation with *C. krusei* could be proceeded by increasing initial dye concentration from 100 to 500 mg/L. Moreover, the degradation of Reactive Black 5 was possible through a broad pH (6-8) range and had a significant pH decrease after degradation. For decolorization mechanism of Reactive Black 5, the decolorization proceeded dominantly by biodegradation, and biosorption could happen in the incomplete dye decolorization.

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