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Aerobic decolorization and degradation of Acid Red B by a newly isolated *Pichia* sp. TCL

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ABSTRACT

A yeast strain with strong abilities to decolorize various azo dyes aerobically was isolated from the sea mud. The strain designated as TCL was identified as *Pichia* sp. on the basis of 18S rDNA analysis. More than 90% of Acid Red B (100 mg/L) was decolorized within 10 h in the Martin Broth at 30 °C and 150 r/min, and strain TCL could tolerate up to 1000 mg/L of the dye. Meantime, the effects of different physicochemical parameters (media, concentrations of glucose, NH_4Cl, initial dye and NaCl) were investigated to improve the removal efficiency. The significant biodegradation process of Acid Red B rather than inactive surface adsorption was confirmed by UV–vis, HPLC analysis and colorless microbial cells. In addition, the metabolic products and partial degradation pathway were proposed with the help of HPLC-MS analysis. To the best of our knowledge, it is the first time that a yeast strain of *Pichia* sp. has been reported with the excellent decolorizing ability against azo dyes under shaking conditions. This work conferred the utilization possibility of strain TCL in the biological treatment of dyeing wastewater.

1. Introduction

Nowadays, pollution of wastewater contaminated with dyestuffs has been increasingly serious. It is estimated that nearly 300,000 t of textile dyes are discharged into the nature environment every year, among which azo dyes characterized by one or more azo groups (—N=N—) account for the majority [1–3]. Such effluent containing azo dyes will cause aesthetically unpleasant. Furthermore, most azo dyes and their metabolic intermediates are toxic, carcinogenic and mutagenic to the living organisms [4]. Therefore, various technologies such as physical, chemical and biological methods have been developed to treat this kind of industrial effluents. By contrast, microbial removal processes have become the dominant technology with the merits of environment benign and cost competitive [5,6].

Conventionally, the decolorization of azo dyes should be performed by biosorption and anaerobic degradation process. Under the anaerobic condition, the azo bonds were firstly cleaved by the azo reductase to form the corresponding aromatic amines, which should possess stronger toxicities than the parent compounds. This anaerobic decolorization is thought to be a simple, easy and non-specific process, which has been concerned many times in the literatures [7,8]. By contrast, there are relatively a few reports on decolorization of azo dyes under aerobic condition. During the process of aerobic decolorization, both monooxygenase and dioxygenase could catalyze the incorporation of the oxygen atoms from O_2 into the aromatic ring of organic compounds prior to ring fission [9]. Meanwhile, the oxygen insensitive reductase was also reported to act on azo groups under aerobic condition [10].

As is well known, the effectiveness of microbial decolorization depends on the adaptability and activity of the selected microorganisms. Consequently, various species have been tested for the decolorization and degradation of different dyes in recent years. Most existing reports about microbial decolorization and degradation of azo dyes have been focused on fungi, bacteria, actinomycetes and algae [11–14]. However, very little work has been done to explore the decolorization abilities of yeasts, which could resist unfavorable environments in textile wastewater, such as low pH and high salt concentration. More recently, some studies have shown that yeast species acted as a promising dye adsorbent are able to uptake higher dye concentration, such as *Galactomyces geotrichum*, *Saccharomyces cerevisiae* and *Trichosporon beigelii* [15–17]. However, there has still no information on decolorization of azo dyes by *Pichia* sp. strain. Due to the complex and excellent enzyme systems of yeast, it is necessary to investigate the degradation abilities of any new isolated

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yeast strain, which should provide useful information on azo dyes decolorization.

In the present study, we report the isolation and characterization of a yeast strain, *Pichia* sp. TCL, which harbors the outstanding decolorizing ability for several azo dyes under the aerobic condition. Effects of different factors on the aerobic decolorization by growing cells were investigated. In addition, the metabolites and possible pathway of the degradation process were identified and proposed. To the best of our knowledge, it is the first report of utilizing a *Pichia* sp. strain to decolorize and detoxify azo dyes efficiently.

2. Materials and methods

2.1. Dyes and chemicals

The dyes used in this study (Table 1) were kindly presented by Dye Synthesis Laboratory in Dalian University of Technology. All the other chemicals used in this study were analytical-grade purity or above.

2.2. Culture media

Five types of media were used for the culture of microorganism: (1) Czapek's medium, which contained (g/L): $NaNO_3$ 3.0, K_2HPO_4 1.0, KCl 0.5, $MgSO_4$ 0.5, $FeSO_4$ 0.5 and glucose 30.0; (2) Martin Broth, which contained (g/L): $(NH_4)_2SO_4$ 1.0, KH_2PO_4 1.0, $MgSO_4{\cdot}5H_2O$ 0.5 and glucose 10.0; (3) Mineral salt medium, which contained (g/L): $(NH_4)_2SO_4$ 1.0, KH_2PO_4 2.0, Na_2HPO_4 1.3, $FeCl_3$ 0.00025 and glucose 10.0; (4) M9 medium, which contained (g/L): NH_4Cl 1.0, KH_2PO_4 3.0, $Na_2HPO_4{\cdot}7H_2O$ 12.8, NaCl 0.5, $MgSO_4$ 0.24 and glucose 10.0; (5) Sabourand medium, which contained (g/L): peptone 10.0 and glucose 40.0.

2.3. Isolation and identification of the dye-decolorizing yeast

The yeast strain, named TCL, was isolated from the sea mud collected in Heishijiao Beach Park (Dalian, China) by enrichment culture in Martin broth medium containing 50 mg/L of different dyes. It was incubated at 30 °C for 12 h under aerobic condition. Subsequently, the culture was further plated on agar plates and incubated for 48 h. After about one month screening and enriching, the fastest-growing colony was selected for further characterization. Genomic DNA of strain TCL was extracted by grinding the mycelium in liquid nitrogen. Purified DNA was used as the template to amplify the 18S rDNA gene of strain TCL by polymerase chain reaction (PCR). Then the PCR product was sequenced by TaKaRa Co. Ltd. (Dalian, China), and the sequence was analyzed using BLAST program. The 18S rDNA sequence of strain TCL and related sequences obtained from GenBank were aligned by Clustal X (1.8). The aligned data were used to construct a phylogenetic tree using Neighbor-joining method by MEGA (Version 4.1) with 1000 bootstrap replicates [18].

2.4. Decolorization experiments

Strain TCL with 5% inoculums (OD_{600} = 3.5) was inoculated in 250 mL Erlenmeyer flask with 100 mL Martin Broth containing Acid Red B and incubated at 30 °C for 12 h with shaking. The effects of different parameters on decolorization were investigated, including medium (referred to Section 2.2), glucose concentration (2, 5, 10, 15 and 20 g/L in Martin Broth), NH_4Cl concentration (0.2, 0.5, 1, 2 and 5 g/L in Martin Broth), initial dye concentration (20, 40, 60, 80 and 100 mg/L in Martin Broth) and NaCl concentration (2, 4, 6, 8, 10 and 15 g/L in Martin Broth).

2.5. Assays

The decolorization process was monitored by UV–vis spectrophotometer (JASCO V-560, Japan) at different time intervals. Aliquots (3 mL) of the culture were withdrawn and centrifuged at 11,000 × *g* for 5 min. Dye concentration was determined by measuring the absorbance of the supernatant at 516 nm. The decolorization rate was calculated using the following equation:

$$\text{Decolorization } (\%) = \frac{A_0 - A_1}{A_0} \times 100 \quad (1)$$

where A_0 and A_1 represented the initial and final absorbance of the dye, respectively [11]. All the decolorization experiments were performed in triplicate and the average values were used in calculations.

In order to identify the metabolites during the decolorization of Acid Red B by strain TCL, the HPLC-MS analysis was performed. HPLC analysis was carried out with a RX-C_{18} column (150 mm × 2.1 mm Agilent, CA, USA). The eluents A (ultrapure water containing 1% acetic acid) and B (methanol) served as mobile phase in a gradient mode (5–40% B at 0–45 min, 40–100% B at 45–60 min). The samples were eluted at a flow of 1 mL/min and monitored at 254 nm. MS analysis was performed on a Finnigan TSQ 7000 triple quadrupole mass spectrometer equipped with an atmospheric pressure chemical ionization (APCI) source in negative ion mode. N_2 was used as a sheath gas and vaporizer temperature was set to 500 °C.

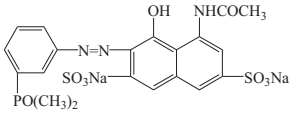
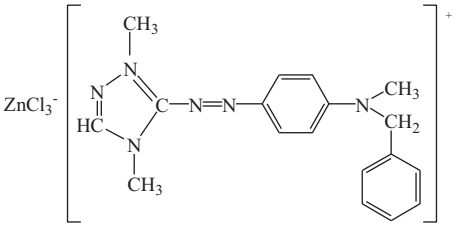
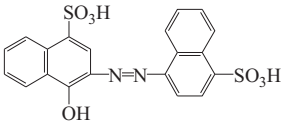
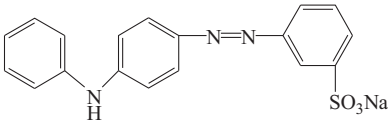
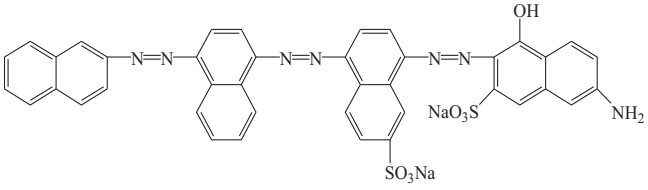
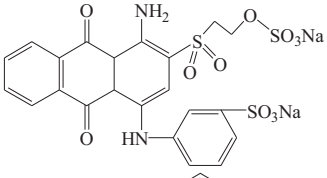
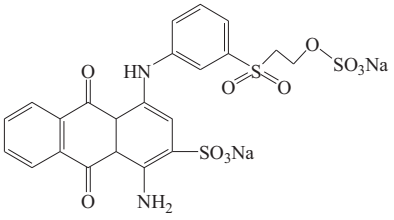
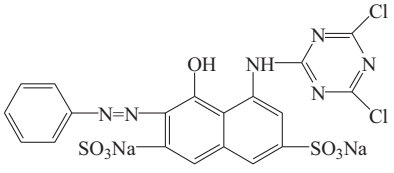
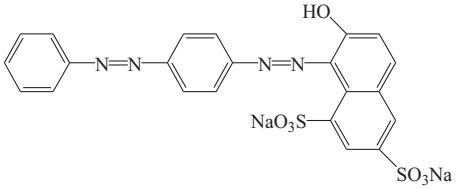
3. Results and discussion

3.1. Identification and decolorization characteristics of strain TCL

A yeast strain, designated as TCL, was successfully isolated from the sea mud sample (Dalian, China). It was aerobic, oval-shaped, and could efficiently remove Acid Red B when growing on the petri plate (Fig. 1A). To taxonomically identify the strain TCL, the 18S rDNA genes were amplified. The sequence of partial 18S rDNA (508 bp) from strain TCL was deposited in GenBank database under the accession number JN695916. Based on the analysis of sequence similarity, strain TCL exhibited 100% homology to *Pichia jadinii* isolate M9 (accession number FJ865435.1). Therefore, strain TCL was identified as *Pichia* sp. strain. The phylogenic tree demonstrating the relationship between the isolated strain TCL and other *Pichia* sp. strains was shown in Fig. 1B.

Decolorization of various dyes by the growing cells of strain TCL under aerobic condition was shown in Fig. 2 and Table 1. Strain TCL possessed the remarkable abilities to remove both azo and anthraquinone dyes with relatively high removal efficiency (86–95%), of which the highest decolorization efficiency was 95% for Acid Red B within 12 h (Table 1). It was obvious that all the tested dyes (9 kinds) could be significantly decolorized by strain TCL, and the supernatant of each dye became colorless after centrifugation, which was shown in tube 3 (Fig. 2A). Among these dyes, the decolorization processes of Cation Red and Acid Red B should be different from others judging by the colorless pellets, whereas, the pellets changed into different color after centrifugation (Fig. 2B). The results indicated that the decolorization of Cation Red and Acid Red B might be due to the cleavage of azo bonds by strain TCL, while the decolorization of the other dyes were probably owing to the biosorption processes. Taking the high efficiency and different mechanism for considerations, Acid Red B was chosen for further study. It needed to point out that no decolorization of any dye was observed in the static conditions by strain TCL (data not shown). Ramalho et al. reported that *Candida zeylanoides* could effectively decolorize four model azo dyes but with

Table 1
Decolorization of dyes by growing cells of strain TCL under aerobic conditions.

Azo dye	Molecular structure	λ_{max} (nm)	Maximum decolorization (%)	Time (h)
Reactive Red		541	94	18
Cation Red		530	90	22
Acid Red B		516	95	12
Acid Orange G		438	94	16
Direct Fast Blue B 2R L		585	86	12
Reactive Brilliant Blue K-GR		611	94	18
Reactive Black 5		598	93	18
Reactive Red X-3B		536	93	18
Great Red GR		509	94	18

relatively long time (40–60 h) [19]. A number of simple azo dyes were degraded by yeast *C. zeylanoides* after 22 h with color removal of 46–67% [20]. Saratale et al. also reported that *T. beigelii* NCIM-3326 could decolorize various azo dyes such as Navy blue HER (100%), Red HE7B (85%), Golden yellow 4BD (60%), Green HE4BD (70%) and Orange HE2R (50%), among which the decolorization rates of some dyes were not desirable [17]. Therefore, the high decolorization efficiency and comparatively short removal time

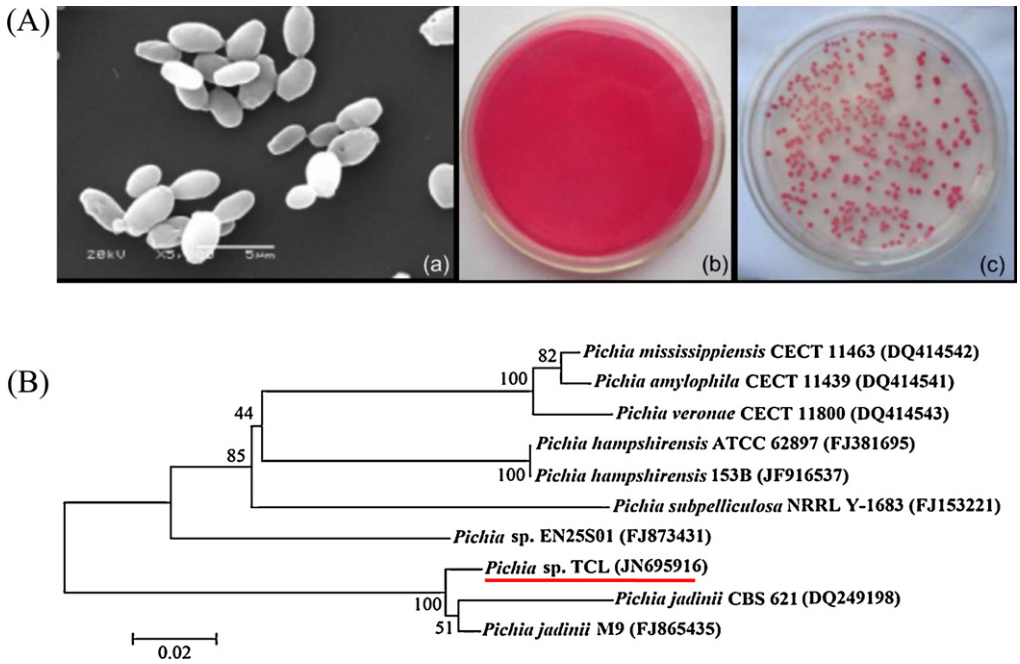


Fig. 1. (A) Morphological characteristics of strain TCL. (a) The electron microscopic micrograph of spores (5000 × folds); (b) The photo of solid culture medium with Acid Red B (50 mg/L); (c) The photo of strain TCL growing on solid culture medium with Acid Red B (50 mg/L) after 48 h. (B) Phylogenetic tree of *Pichia* sp. TCL and related species. The GenBank accession number corresponded to each microorganism was exhibited in parentheses. Bootstrap values were showed at each branch. Scale bar indicated 0.02 Jukes-cantor distances.

suggested the potential of *Pichia* sp. TCL in bioremediation of industrial effluents containing azo dyes.

3.2. Optimization of Acid Red B decolorization by strain TCL

Five different media with initial dye concentration of 20 mg/L were used to obtain the most suitable condition for the aerobic decolorization of Acid Red B. It was observed that decolorization of Acid Red B in Martin Broth and Czapek's medium was more than 85% within 10 h. By contrast, in Sabourand, Mineral Salt and M9 medium, the decolorization efficiency was only 32%, 30% and 8%, respectively (Fig. 3). The components of these five media are different with glucose as the same carbon source. Therefore, it was suggested that the carbon resource seemed no significant effects on decolorization efficiency. From the components analysis, some mineral ions were necessary for decolorization process, such as SO_4^{2-}, Fe^{3+}, and PO_4^{3-}. Therefore, the different decolorization efficiency should be due to the proportion of different cations and anions, which could make the yeast strain TCL grow well with excessive enzymes secretion. Meanwhile, the decolorization efficiency was different in MSM (30%) and Martin Broth (90%) (Fig. 3), which could be explained that iron(III) should affect enzymes

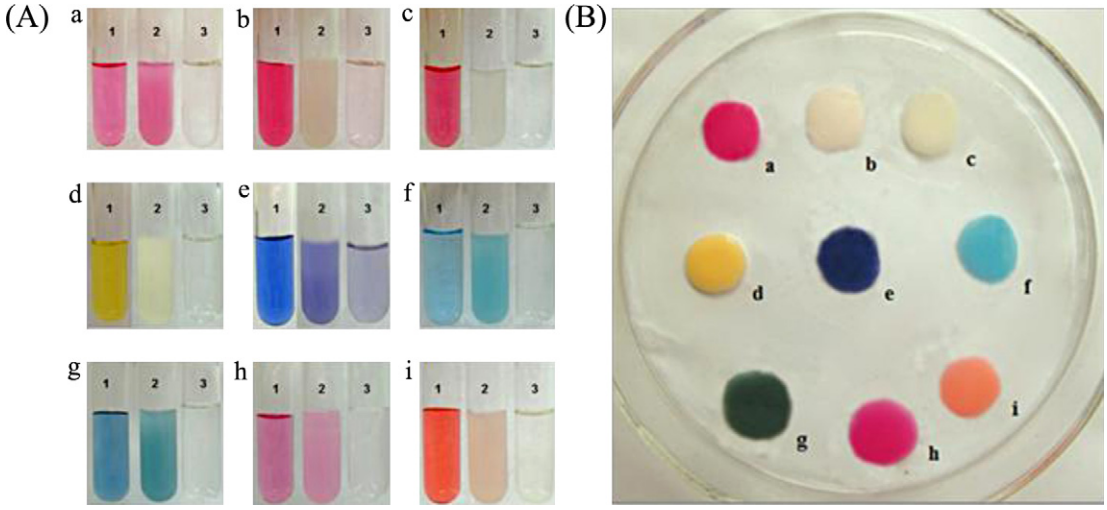


Fig. 2. Decolorization of various dyes by strain TCL under aerobic condition. (A) The pictures of reaction mixtures (1) before decolorization, (2) after decolorization and (3) the supernatant after centrifugation. (B) The colonial morphology of strain TCL on agar plate incubated with various dyes. The dyes tested in this study were as follows: (a) Reactive Red, (b) Cation Red, (c) Acid Red B, (d) Acid Orange G, (e) Direct Fast Blue B 2R L, (f) Reactive Brilliant Blue K-GR, (g) Reactive Black KN-B, (h) Reactive Red X-3B and (i) Great Red GR.

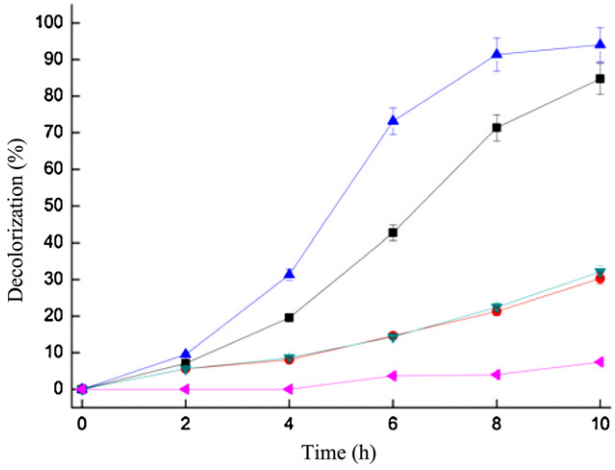


Fig. 3. Effects of medium on aerobic decolorization of Acid Red B (20 mg/L) by strain TCL. (■) Czapek's medium; (●) Mineral salt medium; (▲) Martin Broth; (▼) Sabourand medium; (◀) M9 medium. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

secretion of the strain TCL. According to the previous study, the biodecolorization of azo dyes by yeast strain was implemented by azo reductase and ferric reductase, and 1.0 mM iron(III) would delay the decolorization process from 8.5 to 50 h [16]. Thus, Martin Broth was the best candidate to investigate the aerobic decolorization in the following experiments.

Fig. 4A showed the effects of glucose concentration (2, 5, 10, 15 and 20 g/L) on decolorization with 80 mg/L of initial Acid Red B and 5% of inoculum at 30 °C, 150 r/min. It was clearly indicated that the maximal removal (90%) was achieved after 10 h incubation with 5 g/L and 10 g/L glucose. When the glucose concentration was above 15 g/L, the decolorization process was inhibited. This observation could be explained that glucose acted as a co-substrate and electron donor, which favored the yeast growth and Acid Red B decolorization within a certain range [21,22]. When the concentration was increased above 15 g/L, the cells preferred to using glucose rather than the dye molecule as the carbon source, thus the removal efficiency was decreased [17,23].

Ammonium salts such as NH_4Cl are widely adopted as nitrogen sources for their effectiveness and low costs [24]. To investigate the effects of nitrogen source concentration on the aerobic decolorization, different concentrations of NH_4Cl were assigned from 0.2 g/L to 5 g/L. The optimal NH_4Cl concentration was 0.5 g/L and 1 g/L (Fig. 4B). Further increase of NH_4Cl concentration up to 5 g/L would cause decrease in the maximal decolorization efficiency, which was in accordance with the observation by Srinivasan et al. [25]. In a word, the growth and azo dye decolorization efficiency of strain TCL was directly correlated to the NH_4Cl concentration, and maximal decolorization efficiency of 96% was observed in the medium with 1 g/L NH_4Cl.

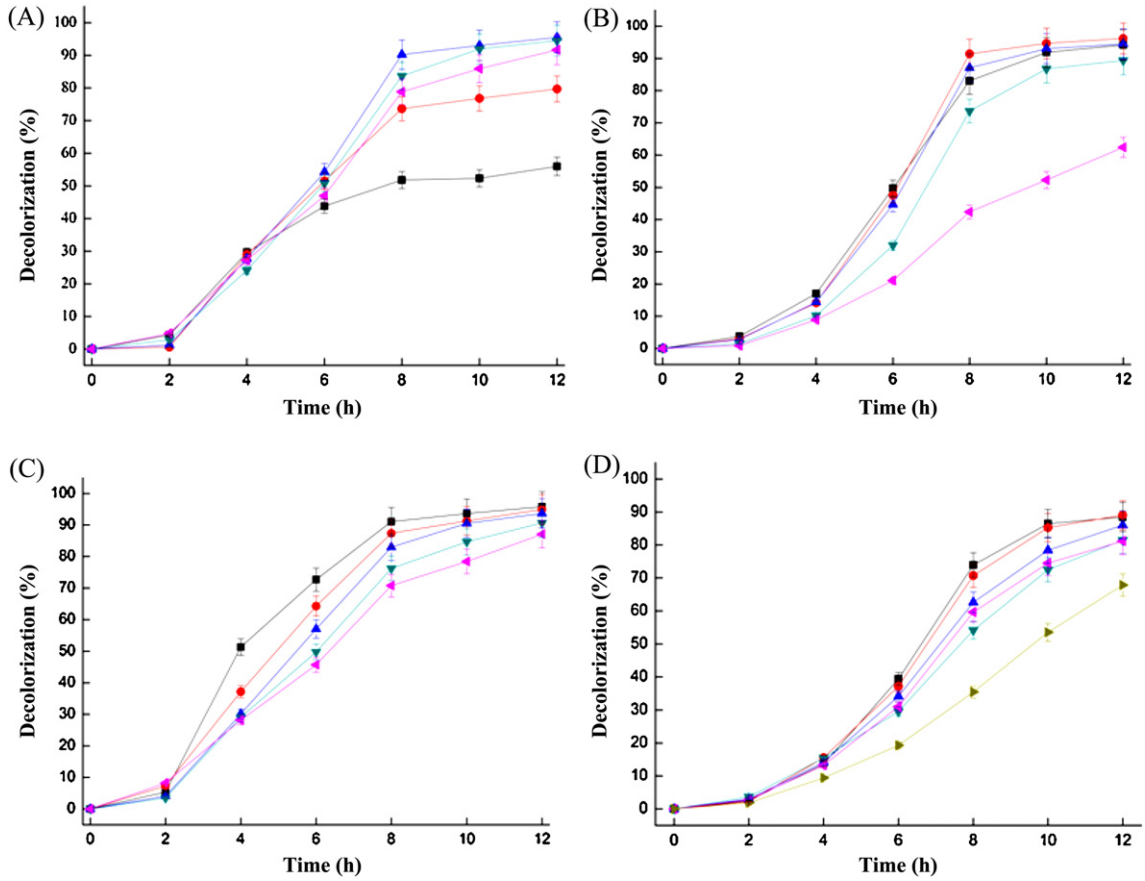


Fig. 4. (A) Effects of glucose concentration on aerobic decolorization of Acid Red B (80 mg/L) by strain TCL, (■) 2 g/L; (●) 5 g/L; (▲) 10 g/L; (▼) 15 g/L; (◀) 20 g/L; (B) effects of NH_4Cl concentration on aerobic decolorization of Acid Red B (80 mg/L) by strain TCL, (■) 0.2 g/L; (●) 0.5 g/L; (▲) 1 g/L; (▼) 2 g/L; (◀) 5 g/L; (C) effects of initial dye concentration on aerobic decolorization of Acid Red B by strain TCL, (■) 20 mg/L; (●) 40 mg/L; (▲) 60 mg/L; (▼) 80 mg/L; (◀) 100 mg/L; (D) effects of NaCl concentration on aerobic decolorization of Acid Red B (80 mg/L) by strain TCL, (■) 2 g/L; (●) 4 g/L; (▲) 6 g/L; (▼) 8 g/L; (◀) 10 g/L; (▶) 15 g/L. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Nearly 90% of Acid Red B was removed within 10 h when the dye concentration was between 20 and 60 mg/L, whereas, it should take more than 12 h to decolorize higher concentration, i.e. 80 and 100 mg/L (Fig. 4C), due to the toxic effects of the dye on the microorganisms, which was the same with other studies [26,27]. Furthermore, TCL could tolerate Acid Red B up to 1000 mg/L, nearly 50–60% of Acid Red B (300–500 mg/L) could be removed within 4 days. Compared with the related literature, strain TCL displayed better decolorization performances such as excellent tolerability of Acid Red B and quick decolorizing capacity with relative high dye concentration [28,29]. The ability of strain TCL to decolorize Acid Red B in the presence of different salt concentrations (2, 4, 6, 8, 10 and 15 g/L) was also tested. It was observed that the removal efficiency was about 81–89% within 12 h with 2–10 g/L salt concentration (Fig. 4D), which showed that the decolorization process was slightly dependent on salt concentration. However, significant decrease of dye decolorization efficiency (68%) was observed in the presence of 15 g/L salt concentration (Fig. 4D).

3.3. Possible degradation pathway of Acid Red B by strain TCL

As mentioned above, the microbial cells became colorless after 24-h incubation with some dyes, which suggested that the biodegradation process occurred accompanying with the decorlorization. To further investigate the possible mechanism of the dye decolorization, the products of decolorization were analyzed by UV–vis spectra and HPLC-MS analysis. UV–vis spectra (200–800 nm) of supernatants at different time intervals were shown in Fig. 5A. It was exhibited that the intensity at 516 nm (λ_{max}) remarkably decreased and reached virtually zero after 24 h. Thus, it could be presumed that the azo bonds were cleaved with the primary chromophore being destroyed. Then, HPLC-MS was used to detect the new metabolites and confirm the mechanism of Acid Red B removal. The mass spectra of three different metabolites were shown in Fig. 5B. The formation of I (4-amino-naphthalene-1-sulfonic acid) was confirmed at the retention time of 6.876 min and *m/z* ion peak of 222.1 ([M$^-$]), and IV (naphthalene-1,2,3,4-tetraol) was concluded from the mass peak of 191.1 ([M$^-$]). According to previous literatures, the product VI with a mass peak of 194.9 ([M$^-$]) was concluded to be 3-7-dihydroxy-octahydro-naphthalene-2,6-dione [30].

It has been comprehensively studied that biological degradation of the azo dyes mainly depends on the azo reductases present in the microorganisms, which can catalyze the reductive cleavage of azo groups (—N=N—) [3,27]. This cleavage process of azo groups can be easily achieved under the microaerophilic conditions. Therefore, we still proposed that Acid Red B was firstly transformed to the corresponding amines, the intermediate I (4-amino-naphthalene-1-sulfonic acid) and the intermediate II (3-amino-4-hydroxy-naphthalene-1-sulfonic acid) (Fig. 6). In the previous studies, the yeast had been proved to possess more complex oxygenases systems, which supplied strong support to the presumption that intermediate I and II could be further oxidized to form ortho-hydroxyl compounds (Fig. 6) [17,31]. Thus, the intermediate III (3,4-dihydroxy-naphthalene-1-sulfonic acid) and IV (naphthalene-1,2,3,4-tetraol) should be formed via several steps such as the release of NH_3, the release of HSO_3^- and hydroxylation by oxygenase systems (Fig. 6). Because the intermediate VI was found, it could be proposed that the intermediate IV was subsequently cleaved into catechol (V), which then formed the intermediate VI (3-7-dihydroxy-octahydro-naphthalene-2,6-dione) (Fig. 6) [30,32]. It is doubted that the intermediate II was not found in the HPLC-MS, and the possible reason should be that most intermediate II was degraded to the small molecules, which played the key role in the degradation of Acid Red B by strain TCL.

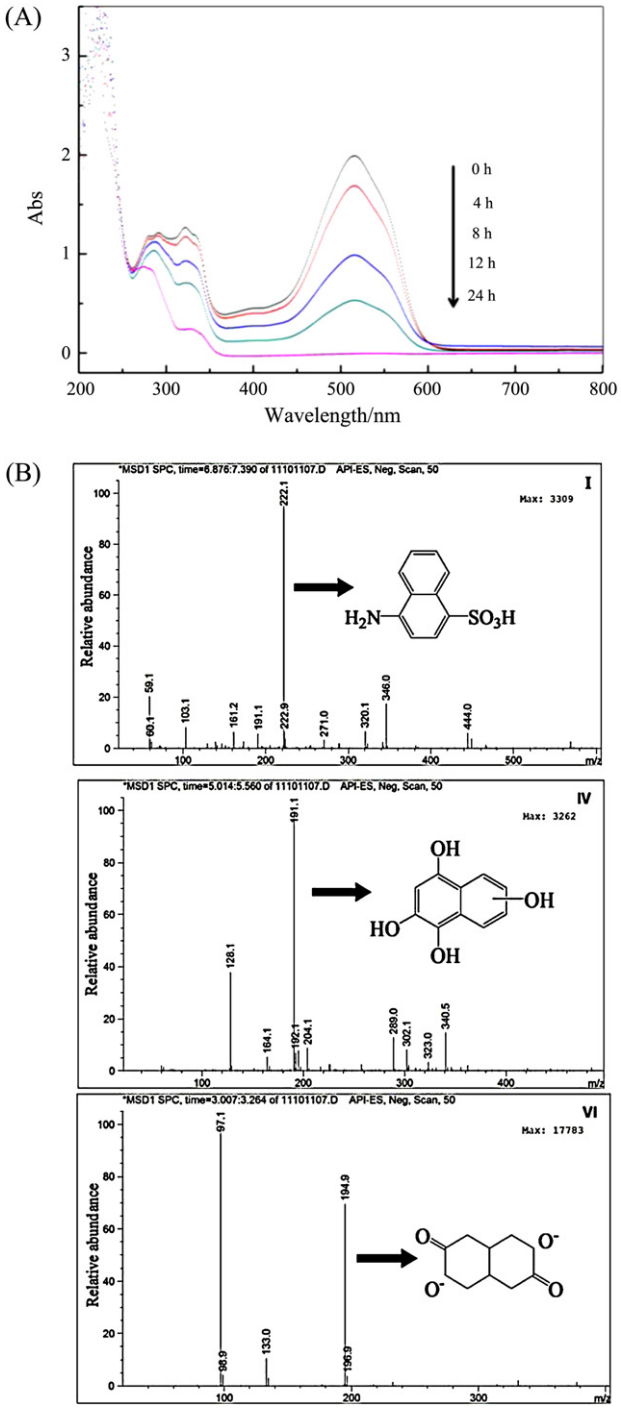


Fig. 5. (A) UV–vis spectra of Acid Red B (80 mg/L) decolorization by strain TCL; (B) the mass spectra of intermediates formed from Acid Red B removal by strain TCL, I: 4-amino-naphthalene-1-sulfonic acid; IV: naphthalene-1,2,3,4-tetraol; VI: 3-7-dihydroxy-octahydro-naphthalene-2,6-dione.

Certainly, this proposed partial pathway only depended on HPLC-MS and previous literatures, and it still need to be investigated systematically. Meanwhile, the phytotoxicity tests were performed to assess the toxicity of the metabolites and products derived from Acid Red B decolorization process by TCL, it was showed that no more toxicity than Acid Red B itself occurred (data not shown). All in all, we just confirmed here that Acid Red B could be transformed to some compounds by yeast strain, which were easier to be degraded further.

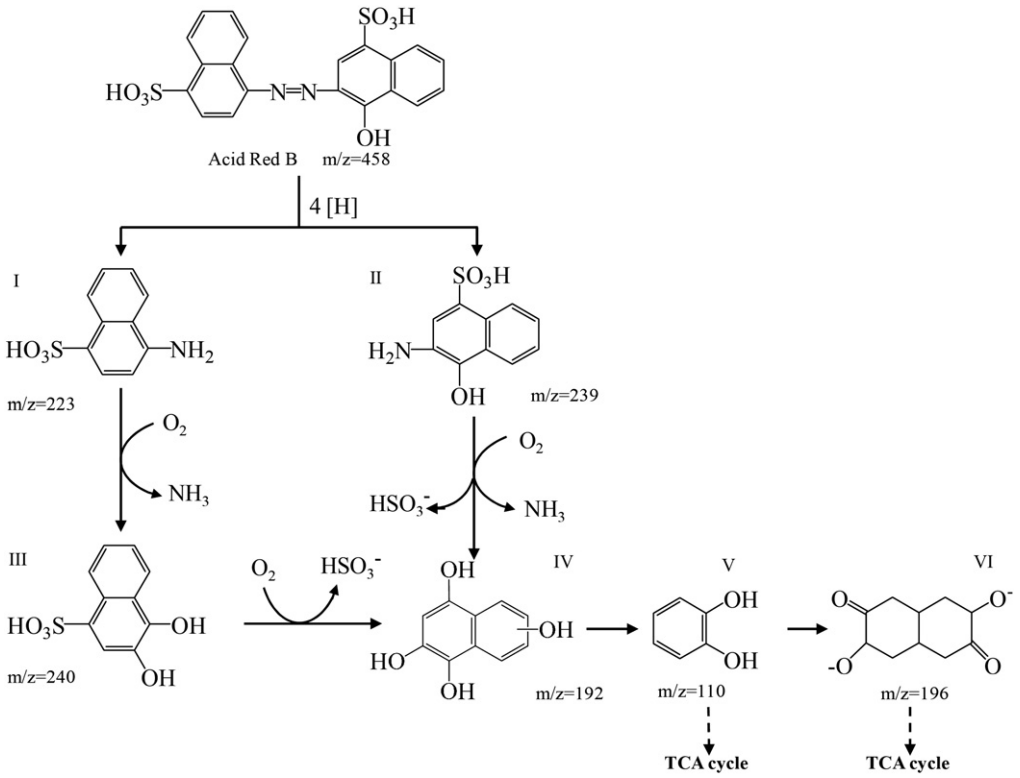


Fig. 6. Proposed partial pathways for Acid Red B decolorization by strain TCL under aerobic condition, the undetected intermediates were indicated with square brackets. I: 4-amino-naphthalene-1-sulfonic acid; II: 3-amino-4-hydroxy-naphthalene-1-sulfonic acid; III: 3,4-dihydroxy-naphthalene-1-sulfonic acid; IV: naphthalene-1,2,3,4-tetraol; V: catechol; VI: 3-7-dihydroxy-octahydro-naphthalene-2,6-dione.

4. Conclusions

This study demonstrated that *Pichia* sp. TCL was capable of decolorizing and degrading the toxic sulfonated azo dye Acid Red B under aerobic condition. The efficient decolorization of Acid Red B by *Pichia* sp. TCL was affected by various physical and chemical parameters. UV–vis spectroscopy and HPLC-MS analysis confirmed the biodegradation process occurred accompanying with decolorization of Acid Red B. A possible pathway for biodegradation of Acid Red B was proposed with the help of HPLC-MS analysis. However, further studies will be necessary to characterize the enzymatic systems involved in the azo dye aerobic degradation.

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