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Bioremoval of an azo textile dye, Reactive Red 198, by *Rhizopus oryzae*

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ABSTRACT

Among all environmental contaminants, industrial dye is one of the major pollutants of soil, water, and air. There are different chemical, physical, and biological methods for removal of all types of synthetic media from the environment. One common method is to utilize microorganisms like yeast, fungi, or bacteria. In this study, we identified a soil-derived microorganism and evaluated its efficacy for removal of industrial dye from wastewater. First, a strain of fungus isolated from various samples of soil was defined via colonial identification and DNA sequencing. Second, the bioremoval activity of the defined fungus *(Rhizopus oryzae)* in treating the water-soluble monoazo dye, Reactive Red 198, in synthetic media was evaluated using high-performance liquid chromatography. The effects of varying factors in processing, including dye concentration, fungal volume, time, and pH were determined. Under optimal conditions, a dye removal rate of 96% was obtained.

Keywords: Bioremoval; Reactive Red 198; *Rhizopus oryzae*; High-performance liquid chromatography

1. Introduction

The azo dyes (aromatic moieties linked together by azo –N=N– chromophores) are the most important chemical class of synthetic dyes and pigments [1,2], with the greatest variety of colors. Azo dyes are classified as follows: anionic—direct, acid, and reactive dyes; cationic—basic dyes; and nonionic—disperse dyes [1]. The water-soluble reactive dyes are among the groups of dyes that are extensively used by the textile dyeing/finishing, plastic, leather, paper, food, and cosmetic industries. As a result of global industrial activities, the discharge of colored effluents has caused significant environmental problems. The colored effluents interfere with light penetration and photosynthesis, and some of the dyes are toxic, mutagenic, carcinogenic, or allergenic [3]. Physical and chemical methods used to treat colored effluents, such as ion exchange, membrane filtration, ozonation, photoelectrochemistry, and adsorption, are often highly expensive. In response to this, studies in recent years have focused on more economical biological treatments.

Many microorganisms, including bacteria, fungi, yeast, and algae—live or dead cells—can be used in the decolorization process [4]. Fungal biodegradation ability has been widely reported on. Several fungi are capable of mineralizing pollutant compounds, including *Aspergillus parasiticus* [4], *Phanerochaete chrysosporium*, *Trametes versicolor* [5], *Penicillium* sp. QQ [6], *Penicillium simplicissimum* [7], and some brown-rot fungi [8]. From a biological point of view, the objective of this study was to evaluate the potential of a fungus *(R. oryzae)*

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isolated from wastewater-saturated soil to decolorize Reactive Red 198 (RR 198) monoazo dye. The effects of dye concentration, fungal volume, time, and pH were studied. Additionally, a probable metabolic pathway was defined using product analysis.

2. Materials and methods

2.1. Dye and chemicals

The monoazo dye Remazol Red RR 198 (CI, RR 198) was obtained from a local textile company in Zanjan, Iran. (The chemical structure is illustrated in Fig. 1.) A stock solution (1 g/L) of RR 198 was prepared by dissolving a quantity of dye in distilled water, and varying concentrations were obtained by dilution. The pH solution was adjusted to the required values by 1 M HCl/1 M NaOH.

2.2. Culture conditions

The following culture media were used: Sabouraud-4% dextrose agar (SDA) medium containing 10 g peptone, 20 g dextrose, and 20 g agar in 1 L distilled water, pH 5.6 ± 0.2 and Sabouraud-2% dextrose broth (SDB) medium containing 10 g peptone and 20 g dextrose in 1 L distilled water, pH 5.6 ± 0.2.

2.3. Microorganisms

2.3.1. Isolation of dye-degrading fungus

The microorganisms were isolated from the soil around a local textile company in Zanjan, Iran. Underlying soil samples were collected from a nearby effluent disposal area and transported in sterile plastic bags and kept at 4°C. To screen dye-degrading microbial strains, serial dilutions (10^{-1} to 10^{-10}) of the soil collected were performed as follows: One gram of soil was suspended in 10 mL of distilled water (10^{-1}). Other suspensions of soil were obtained in dilutions ranging from 10^{-2} to 10^{-10}. The suspensions were inoculated into the SDA medium using the spread plate technique. Plates were incubated at 37°C until colonies developed. Fungi corresponding to different colony morph types were selected and maintained on SDA slants [9].

2.3.2. Identification of isolated fungi

Slide culture technique and microscopic morphological studies were used to analyze the isolated fungi by staining the slides with lactophenol blue [10]. The fungus *R. oryzae* was selected from several fungi tested because it produced maximum dye degradation. Further identification studies were carried out using the method described by Kachuei et al. [11], as described in the following section.

2.3.3. Decolorization studies in batch liquid medium

A suspension of 3 mL of the fungus *R. oryzae* on an SDA slant was transferred to a 500 mL Erlenmeyer flask containing 200 mL of autoclaved SDB. The flask was incubated at 30°C in an orbital shaker (130 rpm) for 2 d. Following this, 5 mL of the fungal growth on SDB medium (wet fungal cells) was inoculated in a 500 mL Erlenmeyer flask containing 200 mL of

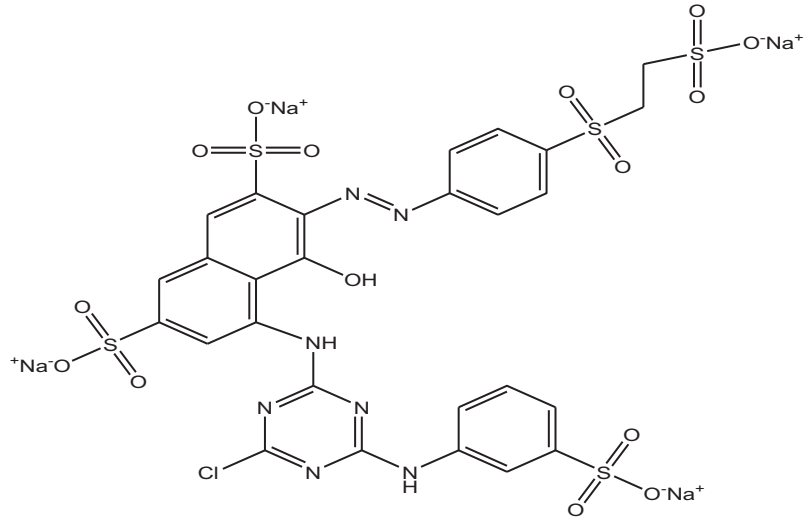

Fig. 1. Chemical structure of RR 198 (λ_{max} = 518 nm).

autoclaved solution of RR 198 (mg/L). The flask containing dye and mycelia was incubated at 30°C in an orbital shaker (130 rpm) for 2 d to ensure equilibrium was reached. Mycelia were collected by filtration, and the filtrate centrifuged for 10 min and measured spectrophotometrically using a UV–vis spectrophotometer (Varian). For RR 198, the absorbance was observed at 518 nm [12]. Decolorization efficiency (DE) was calculated as follows:

$$\mathrm{DE} = \frac{A_0 - A_t}{A_0} \times 100 \quad (1)$$

where A_0 and A_t are the absorbance value of the initial and final dye concentrations, respectively. Biomass production was evaluated by determining the dye mass of the mycelia. Mycelia were harvested from the cultivation liquid medium by filtration using a piece of filter paper, dried at 50°C overnight until reaching a constant weight, and then weighed [13]. All assays were performed in triplicate, and results are average values.

2.4. Mass balance in the process

Fig. 2 shows a model of the process used for decolorization of the azo-dye RR 198 by *R. oryzae*. The most widely used isotherm equation for modeling equilibrium is the Langmuir equation. It is assumed that there is a finite number of binding sites that are homogeneously shared over the adsorbent surface. These binding sites have the same adsorption as a single molecular layer, and there is no interaction among adsorbed molecules. The saturated monolayer isotherm can be represented as:

$$q_e = \frac{bq_{max}C_e}{1 + bC_e} \quad (2)$$

where q_e is metal ions adsorbed (mg/g), C_e is the equilibrium concentration (mg/L), q_{max} is the maximum adsorption capacity, and b is an affinity constant; q_{max} represents a practical limiting adsorption capacity when the surface is fully covered with metal ions and assists in the comparison of adsorption performance, in cases where the sorbent did not reach its full saturation in experiments, and b is constantly related to the affinity of the binding sites [14], which satisfies the mass balance equation.

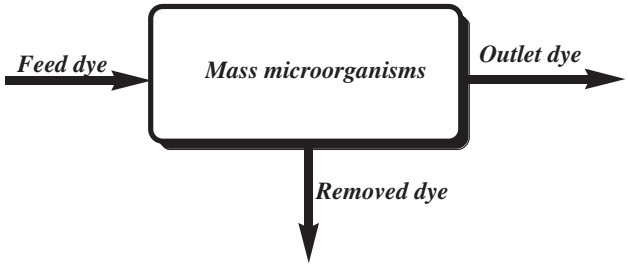

Fig. 2. Decolorization process model.

$$\frac{dC}{dt} = C_i - C_o - r_i - r_o \quad (3)$$

where C_i is all moles in, C_o is all moles out; r_i is creation moles, and r_o is destruction moles. In this equation, $r_i - r_o = 0$; so in the process, creation moles and destruction moles become equal.

2.5. DNA extraction

A single-spore isolate was transferred onto potato dextrose broth. Fungal DNA was extracted using the chloroform method. Briefly, the harvested mycelia mass was frozen in liquid nitrogen and ground to a fine powder. The mycelia powder was suspended in a DNA extraction buffer containing 50 mM tris-HCl (pH 8), 50 mM EDTA, 3% sodium dodecyl sulfate, and 50 μL of proteinase K (20 mg m/L). The suspension was incubated at 65°C for 1 h, and the cellular debris was removed by centrifugation at 3,000 rpm for 5 min. The suspension was extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform:isoamyl alcohol (24:1). The DNA was precipitated by addition of an equal volume of isopropanol and sodium acetate (3 M), followed by centrifugation at 12,000 rpm for 10 min, frozen at −20°C for 1 d, and then centrifuged at 12,000 rpm for 30 min. Finally, the DNA pellet was rinsed with 70% ethanol and resuspended in TE buffer.

2.6. PCR and DNA sequencing

A PCR buffer was used to amplify the isolated DNA of the fungal strain. The internal transcribed spacer (ITS) region of isolate was amplified using universal primers ITS1 and ITS4.

Forward (ITS1): 5′ TCCGTAGGTGAACCTGCGG3′
Reverse (ITS4): 5′ TCCTCCGCTTATTGATATGC3′

Amplification was performed in a final volume of 25 μL. The reaction contained 2.5 μL of 10 × PCR buffer and 0.5 μL deoxynucleoside triphosphate at 0.2 mM, each forward and reverse primer at 20 pmol, 1.25 μL of template DNA, and 2.5 U of Taq DNA polymerase

(0.5 μL in 25 μL of reaction). The PCR conditions used consisted of the initial denaturation at 94˚C for 2 min, 35 cycles at 94˚C for 30 s (denaturation), 58˚C for 45 s (annealing), 72˚C for 1 min (extension time), and a final extension at 72˚C for 7 min. A temperature of 58˚C was selected as suitable for the PCR process. Approximately 600 bp fragments of the PCR product were obtained and sequenced using 18s rRNA gene-based analysis, performed by Faza Biotech Research. The result of the sequence analysis indicated that the isolated fungal strain was the zygomycete *R. oryzae*.

3. Results and discussion

3.1. Isolation and identification of dye-degrading fungi

Dyes are stable and soluble with high rates of molar extinction and low toxicity. The use of dyes and colored indicators that enable visual detection of lignolytic activities is a simple method of screening as no measurement is required [15]. The present work aimed to study the potential decolorizing ability of RR 198 in a synthetic medium. The fungus was isolated from soil collected from an effluent disposal area near a textile company. Effective identification of the isolated fungal strains was performed by 18s rRNA gene sequence analysis. Bioremoval of 96.53% of RR 198 by *R. oryzae* was achieved. Final dye concentration, pH, and equilibrium time were investigated. Slide culture and microscopic morphological studies were done by staining the slide with lactophenol cotton blue wet mount preparation [10,16], which revealed conidiophores, specifically basipetal conidia (Fig. 3). Colonies were again isolated on SDA medium, and grew rapidly with green colored spores.

3.2. Effect of incubation time on the process

The DE of fungi can be due to the presence of chitin with hydroxyl and amino groups in their cell walls, which make them an efficient adsorbent of dye effluent. Differences in the capacity of dye decolorization among different fungi have been related to inter- and intraspecific variations, the molecular complexity of the dye, and culture conditions. Therefore, some parameters that influence the decolorization of dyes were studied using this dye with both a pure and a mixed culture [17]. To ensure adequate time to reach equilibrium, color removal of RR 198 (25 mg/L) was investigated over a period of 40 h. As can be seen in Fig. 4 and Table 1, the decolorization process can be divided into two stages: a rapid degradation stage within the first 17 h and a slow decolorization stage thereafter, with 99.04% of the dye removed in 26 h. In a recent study, maximum visible decolorization for Reactive Red 195 (RR 195) and Reactive Green 11 (RG 11) by *Aspergillus niger* was observed after 72 h [10]. Another study investigated decolorization of materials containing Reactive Red 141 (Red HE7B) and Reactive Yellow 206 (Yellow FN2R) dyes using various fungal species, achieving the highest success with *A. niger* (94%) and *Mucor racemosus* (92%) after 5 d [18]. In other studies, Ranocid Fast Blue and Acid Black 210 were degraded within 48 and 96 h by *Phanerochaete chrysosporium* and *Pleurotus ostreatus* [19]. Methyl Violet was degraded by more than 95% after 24 h by *Aspergillus* spp. [11,20,21], and RR 198 decolorized after 7 d with *P. simplicissimum* [7]. Waste material containing RR 195 showed 99.5% decolorization within 90 min using *Enterococcus faecalis* strain YZ66 [22]. In our own research with *Aspergillus flavus*, rapid degradation of over 90% occurred during the first 4 h, with color removal continuing for 24 h and ultimately reaching around 99%.

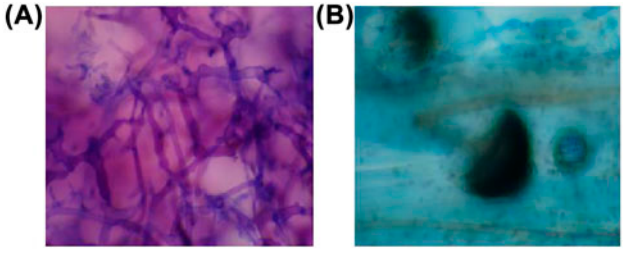

Fig. 3 *Rhizopus* fungus (A) before bioremoval of dye and (B) after bioremoval of dye.

3.3. Effect of initial dye concentration on the process

The concentration of dye contained in effluents varies between 10 and 200 mg/ml, depending on the dyeing process and type of treatment method employed. Many dyes and pigments are hazardous and toxic for humans as well as for aquatic life in the concentrations at which they are being discharged to receiving water bodies [5]. High concentration of dyes leads to ulceration of the skin and mucous membrane, dermatitis, perforation of the nasal septum, and severe respiratory tract irritation, and on ingestion may cause vomiting, hemorrhage, and diarrhea [17]. The color removal of RR 198 over 26 h at four different initial dye concentrations (25, 50, 75, and 100 mg/L) was evaluated. As shown in Fig. 5 and Table 2, for RR 198 with a concentration of mg/L, 99% of the dye was removed in 26 h. The dye decolorization was strongly inhibited at dye concentrations above 50 mg/L. Xinjiao and Wenhai [20] used *Aspergillus ficuum* for

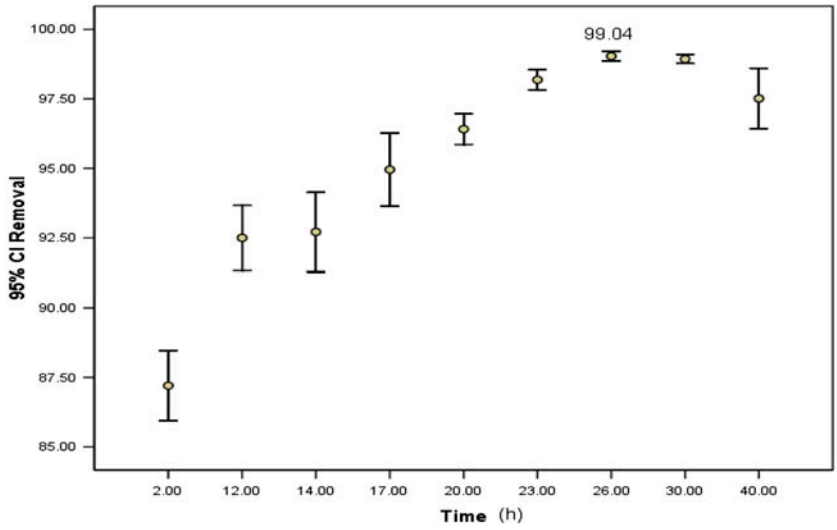

Fig. 4. Decolorization of 200 mL RR 198 solution (25 mg/L) by 5 mL wet fungal cells, 130 rpm, 30˚C at different time intervals.

Table 1
Time: ANOVA variance statistical analysis; dye solution (25 ppm), 15 mL wet fungal cells 24 h

	Sum of squares	df	Mean square	*F*	Sig.
Between groups	608.993	8	76.124	126.052	0.000
Within groups	21.741	36	0.604		
Total	630.734	44			

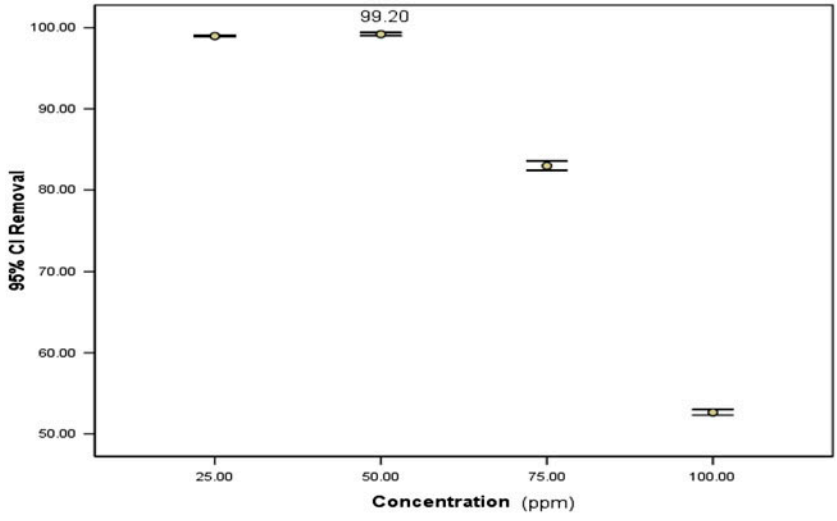

Fig. 5. Decolorization of dye concentrations (25, 50, 75, and 100 mg/L) of 200 mL RR 198 solution by 5 mL wet fungal cells, 130 rpm, 30˚C, 26 h.

Table 2
Dye concentration: ANOVA variance statistical analysis; 15 mL wet fungal cells, pH 2, 24 h

	Sum of squares	df	Mean square	*F*	Sig.
Between groups	84.686	3	2394.895	29829.923	0.000
Within groups	1.285	16	0.080		
Total	7185.971	19			

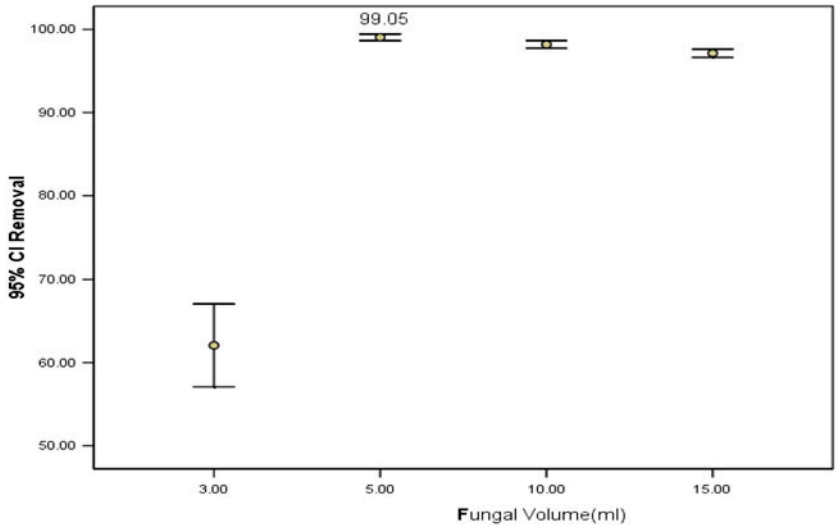

Fig. 6. Decolorization of 200 mL RR 198 dye solution (50 mg/L) by 5 mL wet fungal cells, 130 rpm, 30°C, 26 h, at different volumes of fungi.

decolorization of the anthraquinone dye Reactive Brilliant Blue KN-R in concentrations of 50 mg/L.

3.4. Effect of inoculated fungal volume on the process

A concentration of 50 mg/L of RR 198 was used throughout the decolorization experiments. To find a suitable amount of inoculum for effective dye biodegradation by *R. oryzae*, experiments were performed in 200 mL dye solution inoculated with four different volumes of wet fungal cells (3, 5, 10, and 15 mL). Results showed that dye removal increased with increasing inoculated fungal volume (Fig. 6 and Table 3). A high volume of fungal cells (5 mL) removed a high volume of dye. Similar results were obtained earlier for other dyes. Bioremoval of RR 198 by *R. oryzae* reached over 96.53%, a higher percentage than that achieved with *A. flavus*. Yang et al. [23] observed an increase in Reactive Black 5 removal by increasing the inoculum size of the *Debaryomyces polymorphus* yeast cells employed.

3.5. Effect of initial dye pH on the process

As shown in Fig. 7 and Table 4, the optimal pH for decolorization of 50 mg/L of RR 198 was around 2, with 96.53% removal in 26 h. This indicates that an acid condition is most effective for decolorization. RR 198 is an anionic dye and thus in an acidic condition is transformed from an ionic to a non-ionic state. This

Table 3
Fungal volume: ANOVA variance statistical analysis; dye solution (ppm), pH 2, 24 h

	Sum of squares	df	Mean square	*F*	Sig.
Between groups	16828.943	4	4207.236	3456.271	0.000
Within groups	24.346	20	1.217		
Total	16853.289	24			

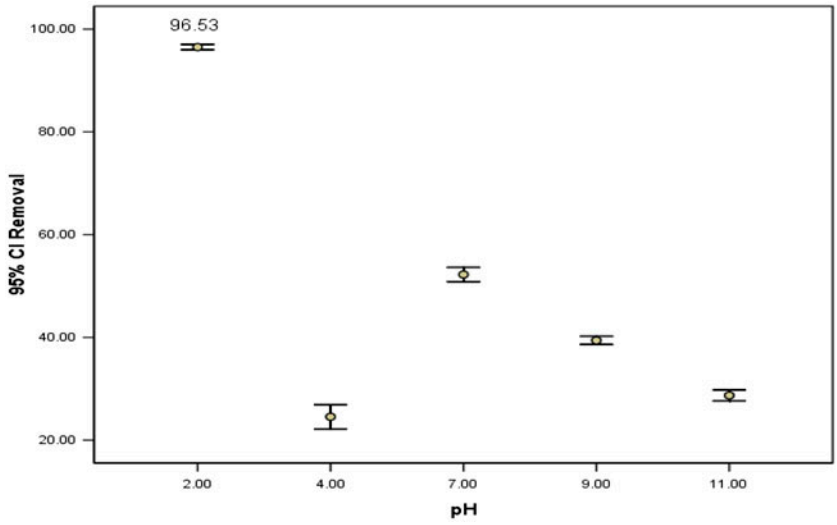

Fig. 7. Decolorization of 200 mL RR 198 dye solution (50 mg/L) by 5 mL wet fungal cells, 130 rpm, 30˚C, 26 h, at varying pH.

Table 4
pH: ANOVA variance statistical analysis; dye solution (25 ppm), 15 mL wet fungal cells, 24 h

	Sum of squares	df	Mean square	*F*	Sig.
Between groups	4884.319	3	1628.106	394.438	0.000
Within groups	66.043	16	4.128		
Total	4950.362	19			

could cause electrostatic repulsion between the dye and the fungal cells and increase the capacity for removal of the dye. Zope et al. [10] observed maximum decolorization of RR 195 and RG 11 with the *A. niger* group in a neutral medium. Akar et al. [4] observed maximum biosorption of RR 198 on *A. parasiticus* at pH 2. Yang et al. [23] observed maximum color removal of RB 5 by *D. polymorphus* in a pH range of 5–7.

The optimal pH for color removal ranges between 1.5 and 11.5 for most dyes, with an observed decrease in decolorization toward both ends of the optimal pH values. Higher uptake obtained at lower pH values may be due to the electrostatic attraction between the positively charged fungal cell surface and the dye anions [17].

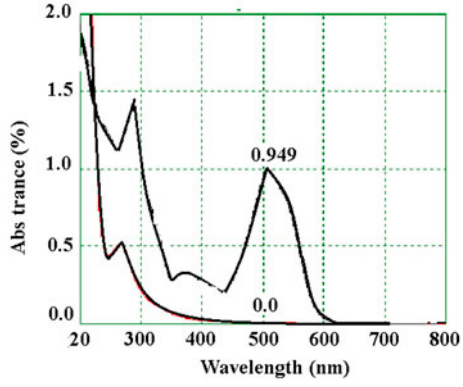

Fig. 8. Spectra analysis of RR 198 (50 mg/L) before (black line) and after (red line) dye degrading by 5 mL *Rhizopus oryzae*, 30˚C, 26 h.

3.6. Spectra analysis

Color reduction was evaluated using spectrophotometric analysis of UV–visual spectra from 200 to 800 nm (Fig. 8) and high-performance liquid chroma (HPLC). An HPLC chromatogram of RR 198 showed a major peak at 3.477 min, with 173,839 area (Fig. 9(a)). Degradation of the dye by *R. oryzae* resulted in a peak at 3.307 min with 4,198 area for the resulting metabolites (Fig. 9(B)). The rate of decolorization of dye was affected by increasing concentrations of dye from 25

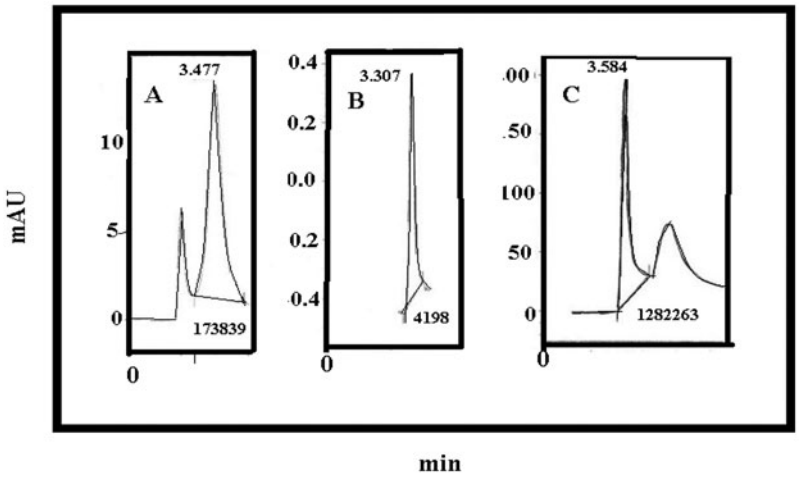

Fig. 9. HPLC elution profile of (A) RR 198, (B) its degradation metabolites, and (C) addition of standard RR 198 dye powder to degraded sample.

up to 100 mg/L. The graphs show a marked decrease in intensity of the peak, confirming the degradation of RR 198 by *R. oryzae*. Addition of standard dye powder to the sample made the peak sharper (Fig. 9(C)).

4. Discussion

Our results suggest that RR 198 is not toxic to *R. oryzae*, and that this fungus, isolated from soil, has the ability to remove RR 198. Researchers have investigated varying vehicles for bioremoval of dye, including fungi, PVA-immobilized microorganisms, and plant roots [24]. Others have previously reported on gel-immobilized biosorbent systems in which a significantly lower rate of dye and metal uptake occurred in comparison with that achieved using free fungal biomass [25]. Verma and Madamwar [19] accomplished complete decolorization of Ranocid Fast Blue and Acid Black 210 (mg/L) over 48 h and 96 h using *P. chrysosporium* and *P. ostreatus*.

Fig. 5 shows color removal of RR 198 over 26 h at four different initial dye concentrations. The dye decolorization activity was almost completely inhibited at *dye concentrations* around 99%. Fig. 6 and Table 3 show the effect of varying volumes of wet fungal cells, from 3 to 15 mL. With increasing volume of biomass from 3 to 5 mL of fungal cells, removal of the dye increased initially from 64.81 to 99.05%, and then decreased to 96.53% with greater biomass volumes. The best removal was achieved using a volume of 5 mL. In other studies of biodegradation of organic compounds with *A. niger* [26] and *Pseudomonas aeruginosa* [27], lesser reactions were produced. We also examined the influence of pH as a factor in removal of this dye. Fig. 7 and Table 4 show pH 2 to be optimal for decolorization of RR 198, with 96.53% removal accomplished in 26 h. These results indicate that an acid condition is most effective for decolorization. This could cause electrostatic repulsion between the dye and the fungal cells and increase the capacity for removal of the dye. Similar results were obtained earlier for other dyes [4]. In contrast, maximum decolorization of RR 195 and RG11 with *A. niger* was observed in a neutral medium [28].

Spectral analysis of RR 198 before degrading presented two absorbance peaks, at 287 and 518 nm (Fig. 8: black line). After dye degrading by fungus, the absorbance peaks in the visible region disappeared, indicating their decolorization, and showing strong $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions involving –C=C–, –C=O, and –S=O (Fig. 8: red line). The intermediates were generally suggested to be aromatic amines, which could be interpreted as 5-(4-(3-sulfophenylamino)-6-chloro-1,3,5-triazin-2-ylamino)-3-amino-4-hydroxynaphthalene-2,7- disulfonic acid (SCTYAHDA), 3-(4-chloro-1,3,5-triazin-2-ylamino) benzenesulfonic acid (CTYBA), 3,5-diamino-4-hydroxynaphthalene-2,7-disulfonic acid (DHDA), and 2-(4-aminophenylsulfonyl)ethanol (p-Base). RR 198 presented characteristic adsorption peaks at 518 and 287 nm, which could be ascribed to the presence of chromophoric azo bonds and aryl and naphthalene-like moieties [29]. The result indicates possible mineralization of partial aromatic amines during the extended period. This finding is of great importance since aromatic amines produced during azo dye reduction were always supposed to be recalcitrant under anaerobic conditions. Previous studies, therefore, pointed toward anaerobic reduction of azo dyes as the first step of the

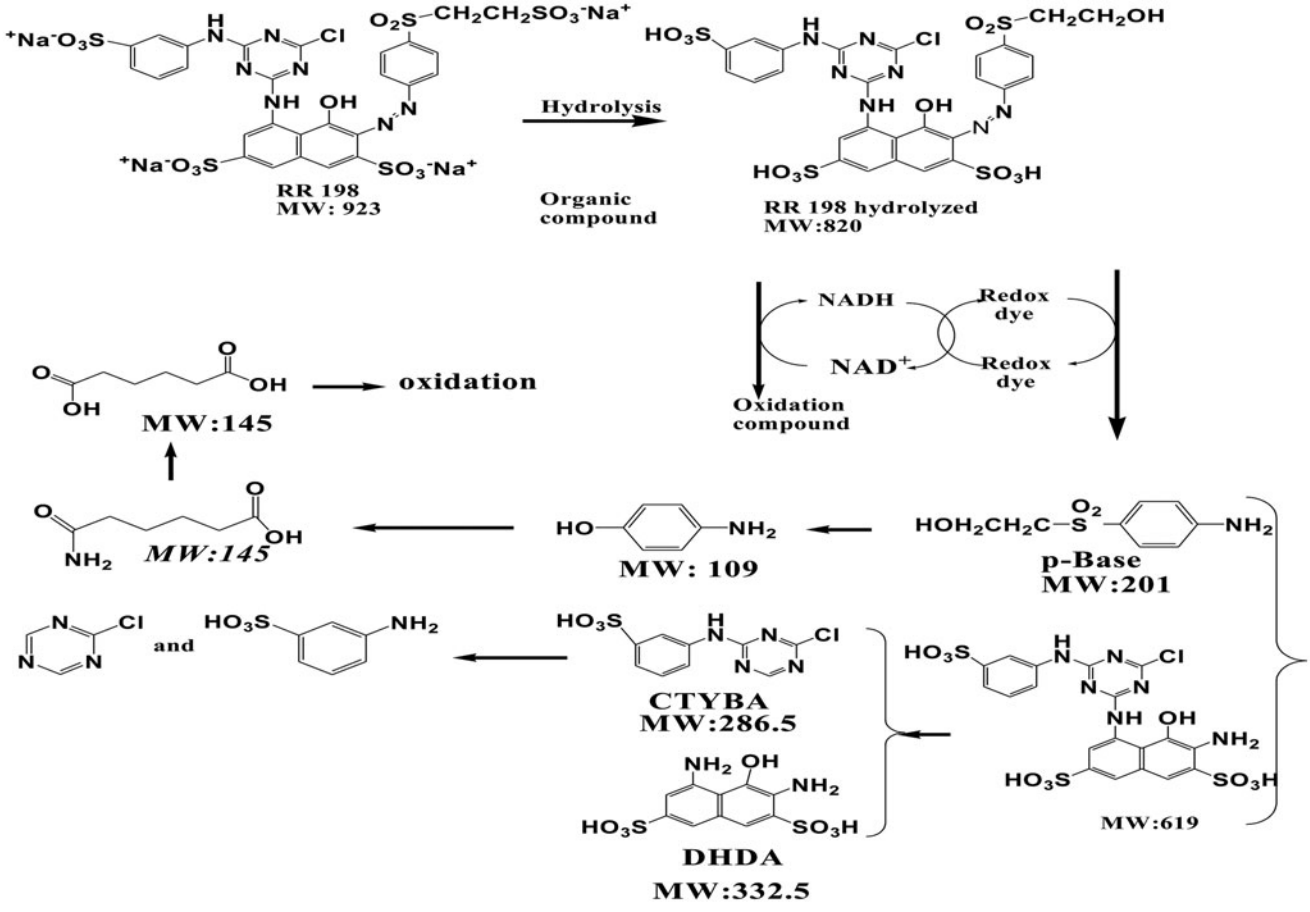

Fig. 10. Proposed metabolic pathway for the degradation of RR 198 by *R. oryzae* (MW: molecular weight).

overall textile wastewater treatment, followed by aerobic units for complete mineralization of aromatic amines [30]. The possible degradation pathway was therefore proposed as shown in Fig. 10. In the process, the creation moles and destruction moles equalize, as diagramed in Fig. 3.

5. Conclusions

In the present study, biodegradation studies of the water-soluble azo dye RR 198 were carried out by a new azo dye decolorizing fungi strain, a group of zygomycete fungi, that was isolated from soil and identified by biological tests as *R. oryzae*. Effective identification of the isolated fungal strains was performed by 18s rRNA gene sequence analysis. UV–vis spectroscopy and HPLC spectroscopy analyses confirmed the degradation of RR 198 by *R. oryzae*. The possible degradation pathways were diagramed. The biodegradation of the dye was dependent on the amount of injected fungus, dye concentration, pH, and equilibrium time. Under optimized conditions (time 26 h, pH 2, dye concentration 50 ppm, and 5 mL of injected fungus), decolorization exceeded 96%.

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