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Decolorization of Reactive Black 5 by *Yarrowia lipolytica* NBRC 1658

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Abstract *Yarrowia lipolytica* NBRC 1658 could decolorize Reactive Black 5 effectively through biodegradation. The optimum conditions such as initial pH, glucose concentration, nitrogen concentration and initial dye concentration were determined. Correlations between decolorization and laccase and manganese peroxidase activities were investigated. Neither laccase nor manganese peroxidase (MnP) activities were determined in culture mediums. *Y. lipolytica* could decolorize 97% percentage of 50mg l^{-1} RB5 dye within 24 hours and could tolerate up to 300 mg l^{-1} of dye. It was found that decolorization occurred during the exponential growth phase. Aerobic batch conditions with 5g l^{-1} glucose and 1g l^{-1} ammonium sulphate at pH 7 were considered to be the optimum decolorizing conditions.

Keywords: *biodegradation, decolorization, Azo dyes, Reactive Black 5, Yarrowia lipolytica*

1. Introduction

Due to high amount of water use in dyeing process, textile industry is one of the most important sources of pollutants in liquid form [1]. Approximately 70% of all the dyes used in industry are azo dyes [2]. Azo dyes are used in paper, food, leather, pharmaceutical and cosmetic industries, as well [3]. The reactive dye Reactive Black 5 is widely used for textile dyeing process which is resistant to biodegradation [4].

Azo dyes are characterized by the presence of one or more azo groups in their aromatic rings. Due to their complex chemical structure, these dyes are resistant to biological activity, light, ozone and other degradative environmental conditions. Thus conventional waste water treatment remains ineffective [5]. Disposal of untreated effluents in to the environment leads to many adverse effects since colored water bodies create aesthetic problems, dyes and their breakdown products cause toxic effects and affect photosynthetic activity of aquatic systems by reducing light penetration [6]

Various physicochemical methods have been used to achieve decolorization of dyes from waste water effluents. These methods include membrane filtration, coagulation, flocculation and activated carbon adsorption. Physicochemical methods have some disadvantages, they are economically unfeasible and create secondary waste pollution problem [4]. However, microbiological methods are more eco-friendly and economically feasible than physicochemical methods [7]. Many researchers studied on decolorization and biodegradation of industrial dyes using white rot fungi strains and the enzymes of these microorganisms which are responsible for degradation of lignin [8-14]. Certain researchers have reported that yeasts decolorize industrial dyes efficiently [3,15,16].

Yarrowia lipolytica is a non-pathogenic aerobic yeast species widely used in industrial applications such as citric acid production, peach flavor production and enzyme production (protease, RNase, lipase). Many researchers reported that *Yarrowia lipolytica* could degrade nitraromatic compound TNT (*2,4,6*-Trinitrotoluene), halogenated alkanes, triglycerides, aliphatic and aromatic hydrocarbons [17-23].

The principal aim of this study is to demonstrate decolorization ability of *Yarrowia lipolytica* and the effects of several parameters (such as initial pH, glucose concentration, nitrogen concentration and initial dye concentration) which are thought to affect decolorization of RB5 by *Yarrowia lipolytica*.

2. Materials and Methods

2.1. Chemicals

RB5, 2,6-Dimethoxyphenol and ABTS (2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt) were obtained from Sigma-Aldrich.

2.2. Microorganism

Yarrowia lipolytica NBRC 1658 strain was obtained from Hacettepe University's Department of Food Engineering. Isolates were kept as slopes on potato dextrose agar (Fluka) for up to 20 days at 4 ℃.

2.3. Yeast growth and decolorization of RB5

The culture medium which was used for decolorization experiments composed of (per liter) : 5g glucose, 1g

ammonium sulfate, 1g KH_2PO_4, 0.5g $MgSO_4.7H_2O$, 0.1g yeast extract, 0.1g $CaCl_2.2H_2O$ [15]. The yeast was cultivated at 30 ℃, 150rpm for 24 hour at pH: 7. The cells used in decolorization studies were always grown in same medium for 24 hour without dye. One milliliter yeast suspensions were adjusted to an Abs_{640nm} = 1 in aseptic conditions then this cell suspensions were inoculated 250ml Erlenmeyer flaks which were containing 100ml medium. The growth of the yeast was described as dry weight (g) of cell per liter. Dry weight was determined by filtering the content of the flasks through pre-weighed filter paper (Whatman No.1) then filter paper was left to dry at 40 ℃ for 2 days.

50mg l^{-1} dye was used throughout the decolorization experiments. The culture medium and RB5 solutions were autoclaved separately at 110 ℃ for 25 minute. At the end of the incubation period (24 h), culture liquid was separated from biomass through filtration (Whatman No.1). The decolorization of RB5 was determined spectrophotometrically at 598nm wave length with Shimadzu UV-1700 (Japan) spectrophotometer. The percentage of decolorization was calculated following equation:

$$Decolorization(\%) = \frac{Abs_i - Abs_f}{Abs_i} \times 100$$

(Abs_i refers to the initial absorbance and Abs_f refers to the final absorbance).

The flasks, which were used in the series of experiments were prepared triplicate and control experiments were performed without inoculation of culture.

2.4. Determination of Optimum Conditions for Decolorization

In order to determine the effect of initial pH on decolorization, the media which contains 50mg l^{-1} dye, was adjusted to 3, 4, 5, 6, 7, 8, 9 using 1N HCl and 1N NaOH then incubated for 24h. Series of dye-bearing medium with different concentrations of glucose (0–7.5g l^{-1}) and ammonium sulphate (0–1.5g l^{-1}) were examined to find out the optimal initial concentration of nitrogen and carbon sources. Glucose, fructose, glycerol, sucrose and starch were used as carbon source (5g l^{-1}) to determine optimal carbon sources for decolorization of RB5.In order to determine optimal nitrogen sources for decolorization, ammonium sulfate, ammonium nitrate, sodium nitrate and peptone (1g l^{-1}) were used.

2.5. Effect of Initial dye Concentration on the Decolorization of RB5

In order to determine effect of different initial concentration of RB5 on decolorization four different RB5 concentrations were used (50, 100, 200, 300, 400mg l^{-1}). The medium which was contained dye cultivated at 30 ℃ for 24h at pH 7.

2.6. Enzyme Assays

Laccase activity was determined spectrophotometrically (Shimadzu UV- 1700) at 420nm by oxidation of ABTS [24]. Reaction mixtures contained 1mM ABTS in 100mM sodium acetate buffer, pH 4.5. One unit of enzyme activity was defined as the amount of enzyme required to oxidize 1µmol of substrate into product per minute. Manganese peroxidase activity was assayed using the method which was described by Kuwahara et al., in 1984 [25]. One activity unit was defined as the amount of enzyme that oxidized 1µmol of dimethoxyphenol per minute.

2.7. UV-vis Characterization

Scanning the UV–vis spectrum between 200–800nm was performed using Shimadzu UV-1700 UV-vis spectrophotometer.

3. Results and Discussion

Microbial decolorization of RB5 dye was achieved using both bacterial and fungal strains [9,15,26,27,28]. It was observed that *Yarrowia lipolytica* NBRC 1658 was able to decolorize RB5 dye up to 97% within 24 hours and decolorization was performed at exponential growth phase (Figure 1).

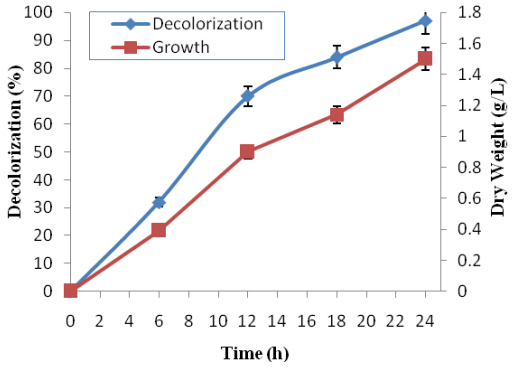

Figure 1. Effect of incubation time on decolorization of RB5 and yeast growth at 50mg l^{-1} dye concentration (pH 7, 30 ℃)

3.1. Effect of pH on Decolorization of RB5

Decolorization of 50 mg l^{-1} RB5 was carried out in the 5 g l^{-1} glucose and 1 g l^{-1} $(NH_4)_2SO_4$ bearing medium at 30 ℃ for 24 h at varying initial pH (3, 4, 5, 6, 7, 8 and 9). Maximum decolorization of RB5 were obtained at pH 6-7 range and significant pH decrease was observed in decolorization mediums which are at pH 3-8 range (Table 1), due to SO_3 groups which were released from RB5 during decolorization process [3]. Fungal dye decolorization commonly performs at acidic pH range [5], although in our study we observed that maximum decolorization efficiency (97%) occurred at pH 7. Yang et al., in 2005, found a similar result, where suitable pH ranged from 5 to 7 when a strain of *Debaryomyces polymorphus* was employed to decolorization [3]. High pH level of textile effluents is one of the problems in their biological treatment [26]. In our study, we observed that decolorization of RB5 was performed up to at pH 8 (77%).

Table 1. Effect of initial pH value on decolorization of RB5 after 24 h incubation period

	İnitial pH						
	3	4	5	6	7	8	9
Decolorization (%)	70	72	77	90	97	77	3
Final pH	2,3	2,5	2,5	2,7	2,6	6,5	8,5

3.2. Effect of Carbon and Nitrogen Sources on Decolorization of RB5

The fungi needs readily usable carbon source to grow and then produce secondary metabolites and extracellular enzymes for biodegradation [29]. Glucose is the most readily usable carbon source for most fungi. However, glucose is not commonly used in waste water treatment since it is an expensive carbon source. Therefore, various other inexpensive carbon sources such as starch, molasses and fructose have been applied in decolorization [15]. The effect of different carbon sources on decolorization of RB5 dye by *Yarrowia lipolytica* is shown in Figure 2. The best decolorizations were achieved in glucose and fructose bearing mediums. We observed that 5g l^{-1} glucose showed best results in decolorization of RB5 (97%) followed by 5g l^{-1} fructose (94%) and glycerol (85%) in 24h. There was no significant removal of dye observed in starch and sucrose bearing mediums (4% and 5% in 24h).

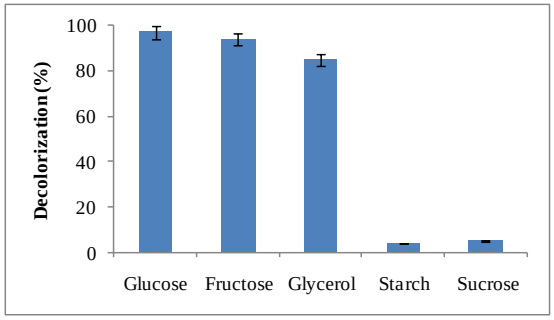

Figure 2. Effect of various kinds of carbon sources on decolorization of RB5 at the 50 mg l^{-1} initial dye concentration after 24h incubation period (pH 7, 30 ℃)

In order to determine efficiencies of different nitrogen sources on decolorization; mediums with ammonium sulfate, ammonium nitrate, sodium nitrate and pepton were tested. The most effective decolorization occurred in ammonium sulfate (97%) and ammonium nitrate (93%) bearing mediums whereas no significant removal of dye observed in sodium nitrate bearing medium (Figure 3). Yeast cells have to convert nitrogen into the glutamate and glutamine amino acids. These two amino acids serve as nitrogen donors for all other nitrogen containing compounds in the cell. Both glutamate and glutamine can be synthesized directly using ammonia [30].

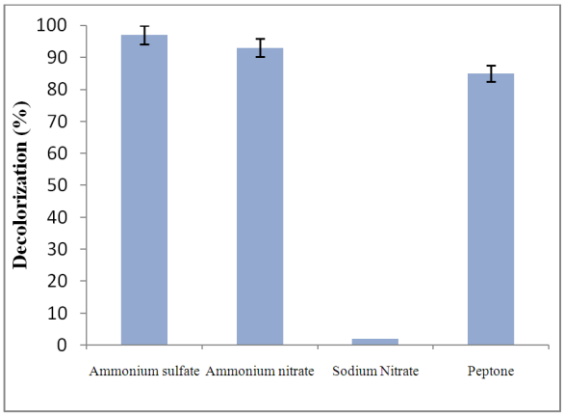

Figure 3. Effect of various kinds of nitrogen sources on decolorization of RB5 at the 50mg l^{-1} initial dye concentration after 24h incubation period (pH 7, 30 ℃)

3.3. Effect of Carbon and Nitrogen Source Concentration

The highest decolorization efficiency (97-98%) was observed at 5-7.5g l^{-1} glucose and 1-1.5g l^{-1} ammonium sulfate concentrations (Figure 4). When there is a lack of carbon and nitrogen sources in culture medium, only 6% decolorization efficiency was observed after 24h incubation, *Yarrowia lipolytica* could not use RB5 as nitrogen and carbon sources. The amount of nitrogen present in the media effects dye decolorization by altering the enzyme production by fungi [5]. For several fungal species the ligninolytic enzyme activity is suppressed rather than stimulated by high nitrogen concentrations (25–60mM). Similarly to the ligninolytic enzyme system, the degradation of Congo Red by *Phanerochaete chrysosporium* is also inhibited by a high concentration of nitrogen (12mM) [31]. Khelifi et al, in 2009, reported that low nitrogen concentration was stimulated decolorization of Congo red when *Aspergillus alliaceus* was used [29]. In contrast, we observed that decolorization activity of *Yarrowia lipolytica* strongly depended on sufficient nitrogen concentration. Yang et al., in 2005 found similar results and reported that decolorization efficiency of *Debaryomyces polymorphus* depended on a sufficient nitrogen source [3]. Also, glucose concentration of medium was important parameter for decolorization of RB5 *by Yarrowia lipolytica*. Addition of glucose was stimulated decolorization of RB5 (Figure 4). Khelifi et al., in 2009, found similar results and reported that the decolorization activity of *Aspergillus alliaceus* was stimulated by all high nutrient C-concentrations [29]. Another study which was performed by Yang et al., in 2008, showed that increase of glucose concentration from 5 to 10g l^{-1}, increased decolorization activity of *Candida tropicalis* and *Debaryomyces polymorphus* yeasts, although the production of MnP exhibited reverse trends [27]. Our studies indicated that 5g l^{-1} glucose and 1g l^{-1} ammonium sulfate were enough for effective decolorization of RB5 by *Yarrowia lipolytica* shown in Figure 4.

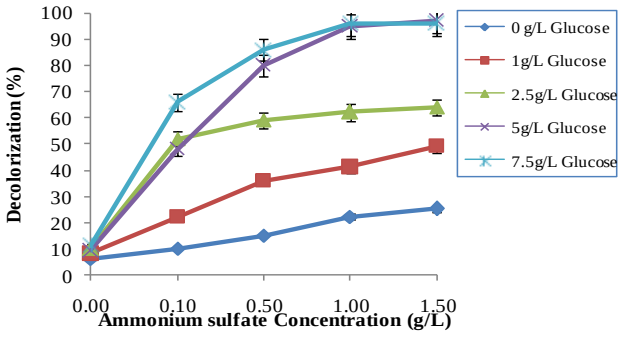

Figure 4. Effect of different nitrogen and carbon concentrations on decolorization at the 50mg l^{-1} initial dye concentration after 24h incubation period (pH 7)

3.4. Effect of Initial Dye Concentration

The growth of the fungi may be effected by the presence of dyes at toxic concentrations. This also has an effect on dye decolorization efficiency of the fungi [5].In our study, the decolorization activity decreased with increase of the initial dye concentration. The most

effective decolorization of RB5 was observed at 50mg l^{-1} (97%) between 100mg l^{-1} (93%) initial dye concentrations. When the concentration of dye above 100mg l^{-1} decolorization activity decreased dramatically, only 59% decolorization of RB5 was observed at 200mg l^{-1} dye concentration. *Yarrowia lipolytica* could tolerate dye up to 300mg l^{-1} concentration (Figure 5) and *Yarrowia lipolytica* could not grow in culture medium with 400 mg l^{-1} RB5 (data not shown). In contrast, Lucas et al., in 2006, achieved completely decolorize up to 200mg l^{-1} RB5 using *Candida oleophila* within 24h [15]. Also, similar results were found by Yang et al., in 2005 [3].

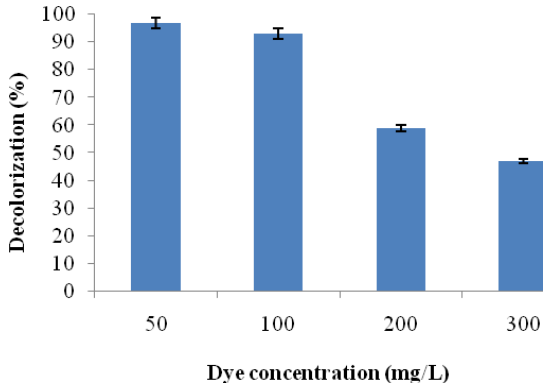

Figure 5. Effect of initial dye concentrations on decolorization of RB5 after 24h incubation period (30 ℃, pH 7)

3.5. Decolorization Mechanism

Yarrowia lipolytica was able to decolorize RB5 without visible signs of dyestuff adsorption to cell surface effectively (Figure 6). RB5 shows two main absorption peaks, one in visible region (598nm) and others in UV region (311 and 259nm). The absorption peak in the visible region shifted from 598 nm to 515 nm and peak in UV region shifted from 311 to 261 (Figure 7). It was indicated new metabolites formed from RB5 degradation. The UV-vis analysis demonstrated changes in absorption pattern, occurring both in UV and visible spectra, thus *Yarrowia lipolytica* cells decolorized RB5 through biodegradation.

Figure 6. Fungal biomass on filter paper after incubated 24h with 50 mg l^{-1} RB5

"The yeast-mediated enzymatic biodegradation of azo dyes can be accomplished either by reductive reactions or by oxidative reactions. The oxidative cleavage of azo dyes can be achieved by the action of the so-called ligninolytic enzymes laccase, manganese-dependent peroxidase, and lignin peroxidase'' [32]. However, we could not determine any laccase nor manganese peroxidase activity. Also, we have not been able to detect any decolorizing activity in culture supernatant and heat killed cells. It thus appears that decolorization of RB5 dye depended on intact living yeast cells. The azoreductase is responsible for azo bond cleavage in some yeast [15,33]. We suggest that RB5 decolorization could be depends on azoreductase activity.

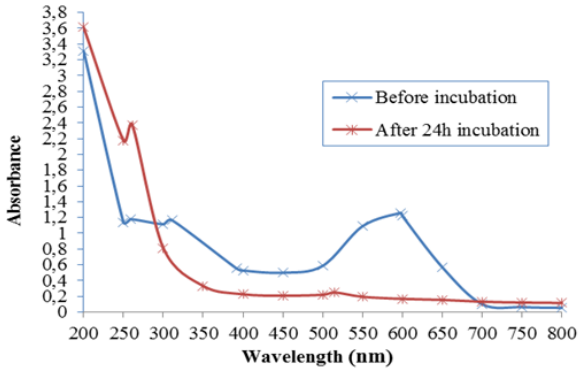

Figure 7. Variation in the UV-vis spectra of RB5 before and after 24h incubation

4. Conclusion

Yarrowia lipolytica NBRC 1658 could decolorize RB5 dye up to 97% within 24 hours in optimized conditions, through biodegradation rather than adsorption. Decolorization occurred at exponential growth phase of the yeast. Decolorization activity of the yeast was greatly affected by addition of different carbon sources. The best decolorization was observed in glucose bearing medium (97%) followed by fructose bearing medium (94%). *Yarrowia lipolytica* could not use RB5 as nitrogen and carbon sources. Decolorization ability of yeast depended on the presence of sufficient nitrogen and carbon sources. Optimum pH for decolorization was found 7. Any laccase and manganese peroxidase activity was not determined during studies. Further studies are needed to clarify mechanism decolorization of RB5 by *Yarrowia lipolytica*.

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