

# Treatment of dyeing wastewater including reactive dyes (Reactive Red RB, Reactive Black B, Remazol Blue) and Methylene Blue by fungal biomass

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## Abstract

The decolourisation potential of growing *Rhizopus arrhizus* fungal strain in liquid medium containing thiamine was investigated for the removal of anionic reactive dyes such as Reactive Red RB (RR), Reactive Black B (RBB) and Remazol Blue (RB) and a cationic basic dye Methylene Blue (MB). To determine the optimal pH value, pH 2 to 6 was examined. Fungal growth was not observed at pH 2. Maximum fungal decolourisation occurred at pH 3 for anionic reactive dyes (RR, RBB, RB) and pH 6 for cationic MB dye. The fungal dye bioremoval was associated with the surface charge of the fungus due to electrostatic interactions. Growing *R. arrhizus* strain decolourised 100% of RB in 2 days, 100% of RBB in 3 days, 71.83% of RR in 8 days at pH 3 and 92.5% of MB in 8 days at pH 6 at 100 mg/l dye concentration. Results indicate that growing *Rhizopus arrhizus* is an effective candidate for removal of different types of dyes from textile effluents.

**Keywords:** *Rhizopus arrhizus*, wastewater treatment, decolourisation, textile dye

## INTRODUCTION

Dyeing effluents are potentially problematic compounds because of their toxic and carcinogenic effects on aquatic biota and humans (Crini, 2006). It is well known that the treatment of dyeing wastewater is very difficult due to the complex aromatic structure and synthetic origin of most dyes (Hadibarata et al., 2012). Generally, synthetic dyes can be classified as anionic (direct, acid and reactive dyes), cationic (basic) and non-ionic (disperse) (Mishra and Tripathy, 1993). Reactive dyes are formed by the combination of azo-based chromophores with different types of reactive groups such as vinyl sulfone, chlorotriazine, trichloropyrimidine and difluorochloropyrimidine (Aksu, 2005). Reactive dyes are commonly used in textile industries because of their favourable characteristics of bright colour, water-fastness, and simple application techniques with low energy consumption (Aksu, 2005). Removal of reactive dyes is especially problematic because they can easily pass through conventional treatment systems and without much change (Zhao et al., 2005). Methylene Blue (MB) is a cationic basic thiazine dye extensively used in dyeing cottons (Aksu et al., 2010). MB dye can cause health problems in humans after inhalation (Cengiz and Cavas, 2008). It is important to treat dyeing wastewaters containing reactive dyes and Methylene Blue dye because of their harmful effects.

Physical and chemical methods, such as chemical coagulation/flocculation, ozonation, oxidation, ion exchange, irradiation, precipitation and adsorption are used for decolourisation of dyes (Ayed et al., 2011). These methods are not feasible techniques since they are very expensive and experience operational problems (Dönmez, 2002). Biological wastewater treatment is often the most economical and eco-friendly alternative, relative

to other physical and chemical processes (Davies et al., 2005). Microbial decolourisation methods, such as bioremoval by growing culture in medium and biosorption by (living or dead) microbial biomass, are commonly applied to the treatment of textile industry effluents because various microorganisms, such as bacteria, yeasts, algae and fungi, are able to remove different classes of dyes (Fu and Viraraghavan, 2001; Srinivasan and Viraraghavan, 2010).

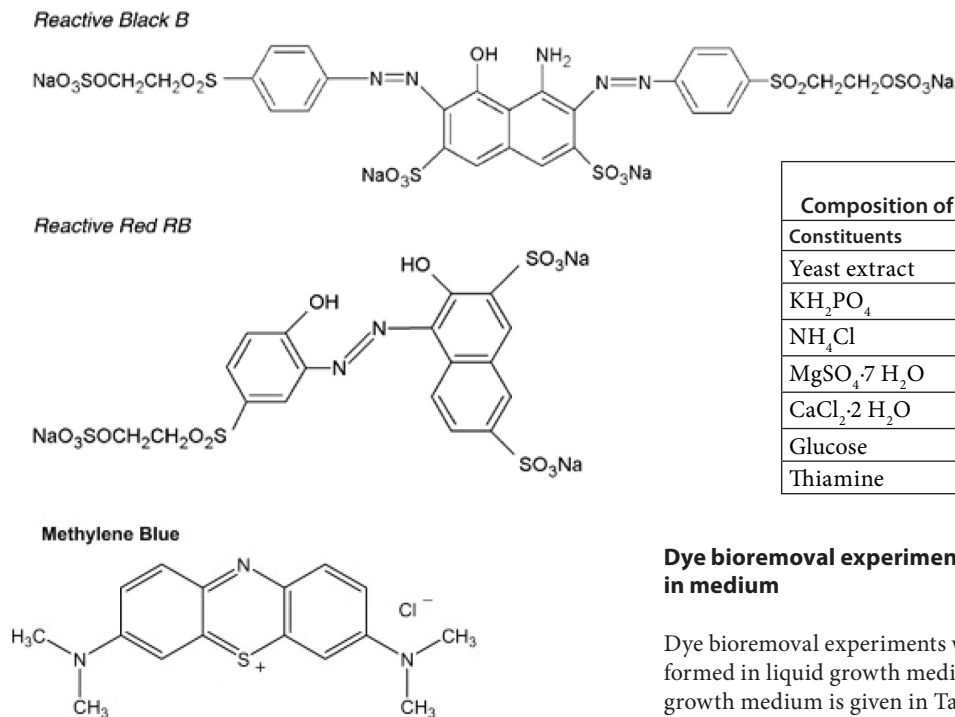
The genus *Rhizopus* comprises of filamentous fungi extensively found in soil (Cardoso et al., 2012). The filamentous fungal strains are of great interest for biotechnological applications such as wastewater treatment because polymers are present in the structure of the cell wall which are responsible for the adsorption of dyes and other pollutants (Aksu, 2001). Studies have demonstrated the effective dye removal properties of dead *R. arrhizus* biomass (Aksu and Tezer, 2000; O'Mahony et al., 2002; Aksu and Karabayır, 2008). Bioremoval of dyes using growing *R. arrhizus* cultures is one of the more frequently reported emerging methods for decolourisation of textile dyes. Use of growing cultures for dye bioremoval processes has the advantage of avoiding separate biomass production processes, such as cultivation, harvesting, drying, processing and storage prior to use (Aksu and Dönmez, 2005).

This study aims to investigate the bioremoval potential of commonly available growing *Rhizopus arrhizus* culture for textile dyes such as Remazol Blue (RB), Reactive Black B (RBB), Reactive Red RB (RR) and Methylene Blue (MB), at batch-scale level, in order to assess application of this technique in an oxidation pond. The study also aimed to investigate the potential improvement of fungal decolourisation performance by adding thiamine to the growth medium. Although *R. arrhizus* is capable of utilising a variety of nutrients, thiamine was chosen due to its positive effect on fungal growth and decolourisation (Chung and Tzeng, 2004; Kirk et al., 1978; Glenn and Gold, 1983). This is the first report to date, and to our knowledge, that investigates the use of growing *R. arrhizus* culture in treating industrial wastewaters containing various types of dyes.

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**Figure 1**

Chemical structure of Reactive Black B, Reactive Red RB, Methylene Blue

## MATERIALS AND METHODS

### Culture medium

The filamentous fungus *Rhizopus arrhizus* (NRRL 1526) was obtained from the US Department of Agriculture Culture Collection. The pure cultures (*Rhizopus arrhizus*) were maintained on Potato Dextrose Agar (PDA) media, containing 39.0 g/l PDA and dye, at 4°C. PDA was purchased from Merck (1.10130) and the cultivation medium was autoclaved at 121°C for 15 min.

The fungal cells were used in two ways to decolourise dyeing water: growing cells in liquid medium bioremoved dyes, and living cells biosorbed dyes. In order to compare the dye removal rate for growing and living cells, biosorption experiments using living cells were performed with RB dye only, which was maximally bioremoved by growing cells.

### Preparation of dye solutions

Remazol Blue (RB), Reactive Black B (RBB) and Reactive Red RB (RR) were obtained from Aytemizler Textile Co., Turkey, in pure form. Methylene Blue (MB) (C.I: 52030) dye was supplied by Merck. Dyes were selected based on frequency of use in the local textile industries. The dye structures of Reactive Black B (RBB), Reactive Red RB (RR) and Methylene Blue (MB) are shown in Fig. 1 (from Pearce et al., 2003; CII, 2006; Gusmão et al., 2012). The structure of the Remazol Blue dye is not in the public domain. The dye stock solutions (RB, RBB and RR) were prepared by dissolving the powdered dyestuff in distilled water to obtain a final concentration of 2% (w/v). A stock solution of Methylene Blue (MB) was prepared at 1.0 g/l concentration by dissolving the weighed amount in double-distilled water. Appropriate volumes of the stock solutions were added to the media.

**TABLE 1**

| Composition of the liquid growth medium |                     |
|---|---------------------|
| Constituents                            | Concentration (g/l) |
| Yeast extract                           | 2.0                 |
| KH <sub>2</sub> PO <sub>4</sub>         | 2.6                 |
| NH <sub>4</sub> Cl                      | 0.12                |
| MgSO <sub>4</sub> ·7 H <sub>2</sub> O   | 0.5                 |
| CaCl <sub>2</sub> ·2 H <sub>2</sub> O   | 0.1                 |
| Glucose                                 | 10                  |
| Thiamine                                | 0.001               |

### Dye bioremoval experiments with growing *R. arrhizus* in medium

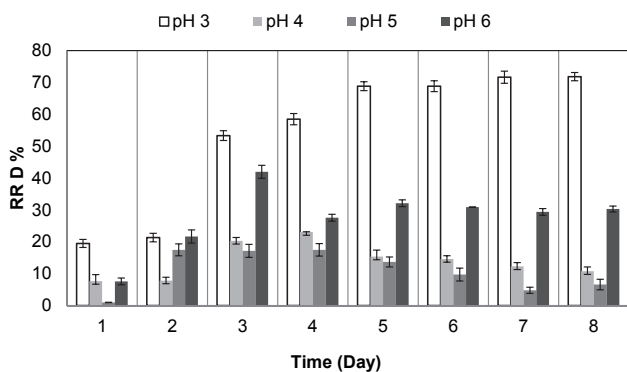
Dye bioremoval experiments with growing fungus were performed in liquid growth medium. The composition of the growth medium is given in Table 1. The pH was adjusted to desired value with 0.1 M NaOH and 1 M H<sub>2</sub>SO<sub>4</sub>. The medium was autoclaved (121°C for 15 min) and then a defined quantity of sterilised dye solution with a known concentration was added to the liquid growth medium. The fungal cells were inoculated twice into 250 ml Erlenmeyer flasks containing 100 ml of liquid medium at 30°C.

Decolourisation experiments were performed with 100 ml culture medium placed in 250 ml Erlenmeyer flasks. In order to examine the effect of initial pH and incubation time, initial pH was varied between 2, 3, 4, 5 and 6 for each dye (initial dye concentration: 100 mg/l) over 8 days at 30°C. All of the experiments were carried out at least twice. The values used in calculations were mostly the arithmetic average of the experimental data.

A 3-ml sample was taken daily from each flask. Samples were centrifuged by Med. Instruments MPW-351 R model centrifuge to remove suspended biomass and the concentration of dye in the supernatant was determined by reading absorbance at the maximum absorption peak for each dye: at 600 nm for Remazol Blue (RB), at 590 nm for Reactive Black B (RBB), at 495 nm for Reactive Red (RR) and at 663 nm for Methylene Blue (MB). The calibration curve of absorbance versus concentration for each dye solution was plotted. Absorbance measurements were done using a Shimadzu UV 2001 model spectrophotometer. Dye-containing liquid medium was used as the blank. For determination of fungal growth, dry weight of fungal biomass was measured at the end of the incubation period. Dry weight of the fungal biomass was obtained by filtering the contents of each flask through pre-weighed filter paper, drying to a constant weight at 80°C overnight and measuring the dry weight of biomass. Dry weight was expressed in terms of grams of biomass per litre of culture.

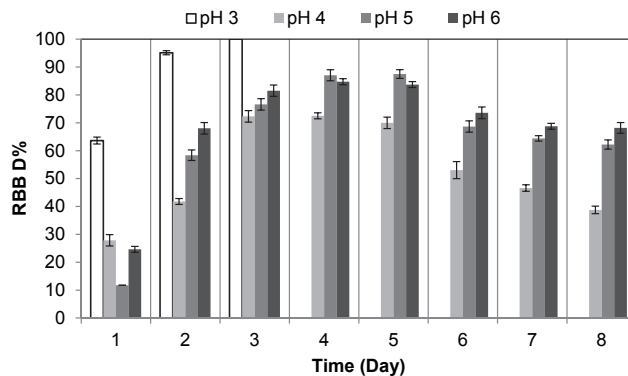
### Dye biosorption experiments with living fungal cells

To examine the biosorption of RB dye by *R. arrhizus*, living fungal cells were prepared. The fungal biomass was filtered from liquid medium (pH 3) after 2 days incubation period without dye. The cell samples were transferred into 250-ml



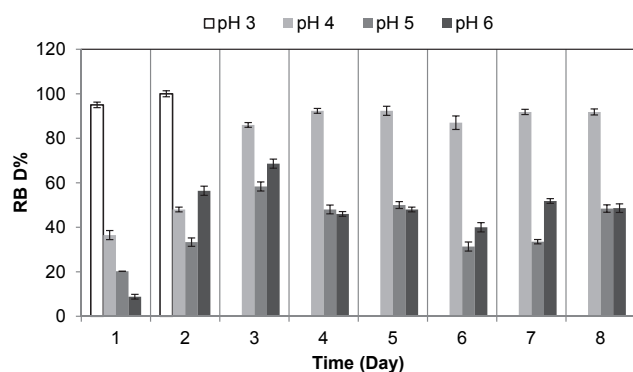
**Figure 2**

The effect of pH and incubation time on Reactive Red RB (RR) dye bioremoval (D%) by growing *R. arrhizus* in liquid medium with 100 mg/l dye at 30 ± 1°C



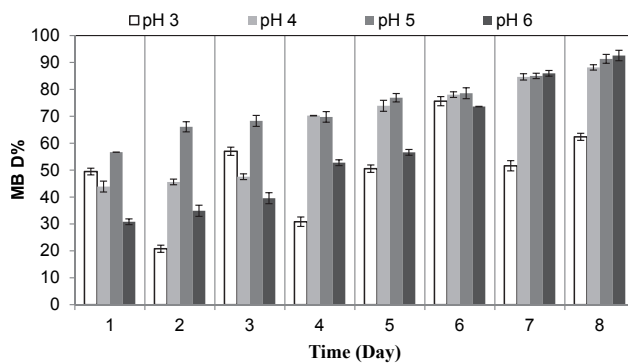
**Figure 4**

The effect of pH and incubation time on Reactive Black (RBB) dye bioremoval (D%) by growing *R. arrhizus* in liquid medium with 100 mg/l dye at 30 ± 1°C



**Figure 3**

The effect of pH and incubation time on Remazol Blue (RB) dye bioremoval (D%) by growing *R. arrhizus* in liquid medium with 100 mg/l dye at 30 ± 1°C



**Figure 5**

The effect of pH and incubation time on Methylene Blue (MB) dye bioremoval (D%) by growing *R. arrhizus* in liquid medium with 100 mg/l dye at 30 ± 1°C

Erlenmeyer flasks containing 100 ml distilled water with 100 mg/l RB. Then, 3-ml samples were taken every 2 h and centrifuged at 10 000 r/min for 15 min in order to remove suspended biomass, and dye concentration in the supernatant was analysed spectrophotometrically (600 nm for RB) over a 48-h period. The 100-ml distilled water flask containing only dye and surfactant without fungus was used as control.

### Analytical methods

The percentage bioremoval of dye was calculated using the following equation:

$$\text{Dye decolourisation (\%)} = (C_o - C_f) / C_o \times 100$$

where:

$C_o$  and  $C_f$  represent the initial and final dye concentrations (mg/l), respectively.

## RESULTS

### Effect of pH and incubation time on dye bioremoval by growing fungal cells

To investigate the effect of pH and incubation time on dye bioremoval by growing *R. arrhizus*, the medium pH was varied to pH 2, 3, 4, 5 and 6 for all of the dyes under investigation.

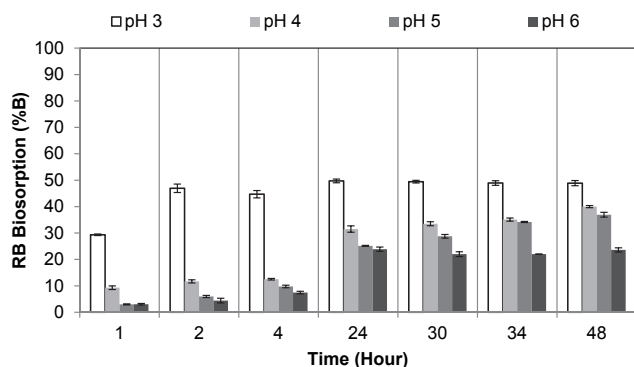
Fungal strain was inoculated into Erlenmeyer flasks containing 100 ml liquid medium with 100 mg/l dye and incubated for 8 days. No fungal growth occurred at pH 2. Maximum RR decolourisation (71.83% after 8 days) occurred at pH 3, as (Fig. 2). Decolourisation rates were very similar between 7 and 8 days of incubation. The fungal strain decolourised 100% of 100 mg/l RB at pH 3 after 2 days of incubation (Fig. 3) and removed 100% of RBB at pH 3 after 3 days (Fig. 4). MB (100 mg/l growing) removal by *R. arrhizus* strain was 92.5% at pH 6 after 8 days (Fig. 5).

### Effect of pH on biosorption of dye by living fungal cells

The effect of pH on biosorption of Remazol Blue (RB) by *R. arrhizus* was examined in Erlenmeyer flasks contained 100 ml double-distilled water with 100 mg/l RB at pH 3, 4, 5 and 6 over 48 h. Maximum dye biosorption (49.7% in 24 h) occurred at pH 3 (Fig. 6). There was no significant change in biosorption (B%) of RB by *R. arrhizus* after 24 h.

## DISCUSSION

RR, RB, RBB bioremoval by *Candida tropicalis* has been demonstrated to achieve a removal rates of 87.9% at 103.1 mg/l RR, 98.8% at 91.3 mg/l RB, and 98.3% at 101.7 mg/l RBB concentration, in 6 days at pH 3 (Dönmez, 2002). *Saccaromyces cerevisiae* decolourised 74.3% of 102.5 mg/l RR in 16 days, 91.2% of



**Figure 6**

The effect of pH and incubation time on Remazol Blue (RB) Biosorption (B%) by living *R. arrhizus* with 100 mg/l dye at  $30 \pm 1^\circ\text{C}$

78.9 mg/l RB in 11 days, 88.4% of 94.3 mg/l RBB in 14 days at pH 3 (Aksu, 2003). Çetin et al. (2008) have reported that mixed culture removed about 20% of 98.3 mg/l RR, 24% of 95.8 mg/l RB and 26 % of 97.4 mg/l RBB after 3 days. Recently, Namdhari et al. (2012) showed that Reactive Blue decolourisation was 95.13%, 93.01% and 82.62% by *Aspergillus allhabadii*, *A. suphureus* and *A. niger*, respectively, in 10 days. In this study it was found that *R. arrhizus* decolourised 100% of 100 mg/l RB in 2 days and 100% of 100 mg/l RBB in 3 days at pH 3.

Dyes belonging to different classes were used in this study in order to compare dye-type effects on the bioremoval properties of *R. arrhizus*. Remazol Blue and Reactive Black B were able to be decolourised to a greater extent (100% removal), while Reactive Red was only able to be bioremoved to a lesser extent in a short incubation period (Table 2). Remazol Blue appeared to be the dye most effectively bioremoved by the fungus. This difference in bioremoval was due to the smaller molecular structure of Remazol Blue relative to the other reactive dyes (Dönmez, 2002).

*R. arrhizus* decolourised anionic reactive dyes more effectively than the cationic thiazine dye. Fungal decolourisation of anionic dyes occurred at low pH values (pH 3), but cationic dye bioremoval was maximum at pH 6 (Table 2). Medium pH had a significant effect on decolourisation, associated with the chemical properties of the dyes and fungal surface. The pH value of the medium influenced the bioremoval properties of the fungal biomass (Aksu and Tezer, 2000). The bioremoval behaviour of the fungal biomass was mainly linked to the composition of the cell wall, which was considered as the primary site of bioavailability. The pH dependence of dye removal was related to protonation or deprotonation of the functional groups of the biopolymers on the biomass surface and the ionisation potential of complex dye molecules in solution (Maurya et al., 2006). The fungal cell walls had both cation- and anion-exchange

properties. The cell walls contained amino groups with low  $pK_a$  values and cation exchange groups with high  $pK_a$  values, which enabled a cell wall to be a natural anion and cation exchanger depending on the environmental conditions (Feofilova, 2010). The electrical charge of the fungal surface can be measured in the form of a zeta potential isoelectric point, depending on the solution pH (Aksu and Karabayir, 2008). The surface charge on each fungal biomass is predominantly positive at low pH values, due to the presence of ionised groups such as carboxyl, phosphate, and amino groups (Aksu and Tezer, 2000). The surface of fungal cells charged positively at pH values below the isoelectronic point due to protonation of nitrogen-containing functional groups such as amines or imidazoles that are major adsorption sites for dye removal (Wu and Yu, 2006).

The isoelectric point of *R. arrhizus* has been shown to be below pH 5.5 (Tobin and Cooper, 1984). The surface charge of *R. arrhizus* was positive at  $\text{pH} < 5.5$ , and anionic reactive dye removal maximum at pH 3, because of electrostatic interactions (Table 2). The surface charge became negative at  $\text{pH} > 5.5$  and cationic MB dye removal was maximal at pH 6 (Table2). Recently, it has been reported that the filamentous fungus *R. arrhizus* has chitin and chitosan polymers in the structure of its cell wall (Cardoso et al., 2012). Chitin/chitosan is a component of fungal cell walls and the major site of sorption (Fu and Viraraghavan, 2001). Chatterjee et al. (2007) studied the sorption capacity of chitosan for the anionic dye Congo Red, as well as the ionic interaction between Congo Red and chitosan, and concluded that the dye removal capacity of chitosan decreased with an increase in the initial pH value. The dye removal mechanism was related to the presence of chitosan as a component of the fungal cell, and electrostatic interactions between chitosan and dye molecules, depending on environmental conditions.

The difference in dye removal related to removal mechanism was investigated by comparing the removal rates for growing cell biomass in liquid medium and living cell biomass in water. MB sorption by dead *R. arrhizus* biomass was 12.4%; growing *R. arrhizus* in liquid medium with thiamine decolourised 56.7% MB in 24 h (Table 3). RB biosorption by living culture in water was 62.1% but decolourisation by growing culture in liquid media with thiamine was 100% in 48 h (Table 3). Dye bioremoval rate by growing biomass was higher than dye biosorption by living and dead biomass in the same incubation time.

Decolourisation rate changed according to the composition of the growth medium ingredients for a particular culture. As seen in Table 3, the growing fungal biomass in liquid media with thiamine removed 100% of RB in 48 h but growing fungal biomass in molasses media decolourised 39.2% of RB in 120 h (Gül and Dönmez, 2012). It is assumed that the difference in decolourisation rate was related to the composition of the medium, and ingredients such as thiamine, carbon and

| Dye                    | Type     | Chemical character | $C_{\text{odye}}$ (mg/l) | Maximum D% | pH | Time (day) |
|------------------------|----------|--------------------|--------------------------|------------|----|------------|
| Reactive Red (RR)      | Reactive | Anionic            | 100                      | 71.83      | 3  | 8          |
| Reactive Blue (RB)     | Reactive | Anionic            | 100                      | 100        | 3  | 2          |
| Reactive Black B (RBB) | Reactive | Anionic            | 100                      | 100        | 3  | 3          |
| Methylene Blue (MB)    | Thiazine | Cationic           | 100                      | 92.5       | 6  | 8          |

| Fungus | Dye | C <sub>odye</sub> (mg/l) | Mechanism                             | D%   | Time (h) | Reference            |
|--------|-----|--------------------------|---------------------------------------|------|----------|----------------------|
| Ra     | MB  | 50                       | Biosorption in water (dead cell)      | 12.4 | 24       | Aksu et al., 2010    |
| Ra     | RB  | 100                      | Growing in molasses media             | 39.2 | 120      | Gül and Dönmez, 2011 |
| Ra     | RB  | 100                      | Biosorption in water (living cell)    | 62.1 | 48       | This study           |
| Ra     | RB  | 100                      | Growing in liquid media with thiamine | 100  | 48       | This study           |
| Ra     | MB  | 100                      | Growing in liquid media with thiamine | 56.7 | 24       | This study           |

nitrogen sources. In molasses medium carbon and nitrogen sources were sucrose and ammonium sulphate (Gül and Dönmez, 2012). The liquid medium, which was used in this study, contained glucose and yeast extract as carbon and nitrogen source. Hadibarata and Kristati (2011) studied the effect of carbon source on removal of Remazol Brilliant Blue R (RBBR) and showed that maximum RBBR decolourisation (100% in 6 days) was observed with glucose (as carbon source). In the current study the vitamin thiamine was used as a component of the liquid medium. It has been reported that adding thiamine to the medium contributes to fungal growth (Chung and Tzeng, 2004). Thiamine is required for lignin metabolism and growth of fungus (Kirk et al., 1978). Decolourisation and ligninolytic activity of fungus is known to be correlated (Glenn and Gold, 1983). According to the results of this study, using thiamine in the medium increased fungal growth, with the dry weight of *R. arrhizus* biomass recorded as 2.604 mg/l in 100 ml liquid medium with thiamine, 100 mg/l RB, at pH 3 after 2 days. Gül and Dönmez (2012) reported that growing *R. arrhizus* biomass in molasses medium with 100 mg/l RB resulted in 2.01 mg/l dry weight at pH 3 after 3 days. Addition of components to the growth medium, such as glucose, yeast extract and thiamine, enhanced both microbial growth and decolourisation.

## CONCLUSION

The results obtained show that growing *R. arrhizus* in liquid media with thiamine is able to remove all of the selected dyes, but to differing extents. Addition of thiamine to the growth medium had a positive effect on fungal decolourisation. The bioremoval efficiency for 4 dyes differed significantly depending on the structure of the dye. Decolourisation of RB and RBB *R. arrhizus* was 100% in short incubation periods. The shortened incubation period enables the energy savings and a reduction in energy costs for the treatment of dyeing wastewater. The fungus also removed 92.5% of cationic Methylene Blue dye.

From the above results, it was concluded that *R. arrhizus* can efficiently remove various types of dyes, including reactive and basic dyes. Future work will concentrate on using the fungal biomass in the development of a textile effluent treatment system.

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