

Decolourisation Process of Drimaren Blue CL-BR by Using *Aspergillus oryzae* Biomass and the Toxicity Assessment of the Final Product

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Abstract

Dyes have wide range of applications in the textile printing, leather and paper dyeing, food colouring, pharmaceutical and cosmetic industries. Dyes containing wastewaters originated from these industries results in the pollution of aquatic ecosystems such as lakes and rivers. Decolourisation of these dyes by using physical and chemical methods such as adsorption, oxidation, coagulation-flocculation, chemical degradation and photo-degradation also produce toxic and carcinogenic dye-derivatives. But biological decolourisation of dyes is more economic and ecologically friendly than other methods. In this study, the effects of various conditions such as initial pH, dye concentrations, glucose and yeast extract concentrations; temperature and agitation on decolourising activity of *Aspergillus oryzae* were investigated. These were all found to be important for Drimaren Blue CL-BR decolourising activity of *A. oryzae*. The decolourisation of the dye involved adsorption and absorption of the dye compound by *A. oryzae* pellet at the initial stage, followed by the decolourisation through fungal metabolism. The maximum decolourisation rate of the dye was determined as 94.4% under optimum conditions. The toxicity of Drimaren Blue CL-BR at 300 mg/L and 15.3 mg/L concentrations was also tested by analysis of DNA damage as measured by comet assay in peripheral erythrocytes of *Carassius auratus* exposed to the dye for 48 hours.

Keywords: Drimaren Blue CL-BR, biosorption, decolourisation, *Aspergillus oryzae*

1. Introduction

The first commercial synthetic dyes were discovered by W.H. Perkin in 1856 (1). The use of synthetic dyes has been increasing rapidly since 1900 and currently, 100,000 different dyes and pigments are used. Annual dye production is about 100,000 tons (2). Dyeing is an important step particularly in textile industry. Also, dyeing is used in photography, pharmaceutical, food, cosmetics, paper and other industries (3). During textile dyeing, 2-50% dye is lost as effluent. The average loss rate is approximately 10-15% (4). The amount of the azo dyes in these effluents was 5-1,500 mg/L (5). The amount of dye released into aquatic ecosystems should be quite high given that 280,000 tons of annual textile waste waters (6).

Dyes are grouped in three classes. These are cationic (basic), anionic (direct, acid, reactive), and non ionic (dispersed) dyes (7). Chromophores in anionic and non ionic dyes are generally azo groups or anthraquinone (8). Azo dyes comprise approximately 70% of all dyes used in industry (9). Azo compounds are a broad class of organic compounds with formula $R-N=N-R'$ in which R and R' can be either aryl or alkyl groups (10). The $N=N$ group is called an azo group. Aromatic rings bind to these groups. Due to the fact that reactive dyes so readily soluble in water, easily flow to open ecosystems. It gives rise to visible colour difference on the water surface. Therefore, light permeability, photosynthetic activity and

dissolved oxygen concentration decrease (11). The discharge of these dye effluents into aquatic ecosystems can result in environmental damages. Furthermore, many dyes and their metabolites may be toxic, carcinogenic and even mutagenic for aquatic organisms (12, 13).

The elimination of coloured substances in wastewater is based mainly on physical or chemical methods. Chemical methods comprise oxidation, ozonolysis and electrolysis process. Physical methods involve reverse osmosis, filtration, and coagulation/flocculation. Extensively used coagulation/flocculation techniques produce large amounts of sludge, which require safe disposal (14). All of these methods have shortcomings because of they often involve high capital and operational costs and may also be associated with the generation of secondary wastes, which present treatment problems (15). Therefore an effective, inexpensive and environmentally friendly alternative would be of great value. Textile dyes are also relatively resistant to microbial degradation. However, bacteria such as *Micrococcus* sp., *Pseudomonas* sp., *Bacillus* sp., some *Streptomyces* strains and fungi like *Phanerochaete chrysosporium*, *Coriolus (Trametes) versicolour*, *Funalia trogii*, *Bjerkandera adusta*, *Pleurotus* and *Phlebia* species are used for decolourisation of dyes (16-20). Oxidative and reductive enzymes like azoreductase were involved in bacterial degradation of dyes while, ligninolytic enzymes such as laccase, manganese peroxidase and lignin peroxidase were involved in fungal degradation. (21). Azoreductase catalyzes reductive cleavage of the azo bond to produce aromatic amines (22). Azo dyes are degraded by fungi without direct cleavage of the azo bond through a highly non-specific free radical mechanism, forming phenolic type compounds (23). Dead cells or growing cells can be used for microbial biosorption of dyes (24). A wide variety of microorganisms including *Aeromonas* sp., *Aspergillus niger*, *Candida lipolitica*, *Candida utilis*, *Cryptococcus* sp., *Endothiella aggregata*, *Fonitopsis carnea*, *Geotrichum fici*, *Kluveromyces marxianus*, *Pseudomonas luteola*, *Rhizopus arrhizus*, *Rhizopus oryzae*, *Saccharomyces cerevisiae*, *Streptomyces* BW130 have been reported for their ability to decolourise dyes via biosorption (25-31). If waste waters contain very toxic compounds and high dye concentrations that inhibit cell growth, dead cells using for biosorption of dyes are preferable (32). The advantage of using growing cells is that separate processes of biomass production, harvesting, drying and storage can be avoided. Also, if microorganism is tolerant to different environmental and physiological conditions, decolourisation process time may be shorter. The aim of the present study was to investigate the potential of *Aspergillus oryzae* for the removal of Drimaren Blue CL-BR from aqueous solutions. Various conditions required such as initial pH, initial dye, glucose and yeast extract concentrations; temperature, agitation, carbon sources for decolourisation have been optimized and decolourisation percentages have been analyzed. Also, enzymatic activities of some extracellular enzymes during decolourisation and microscopic examination of fungal biomass has been performed to prove biosorption.

2. Materials and methods

2.1. Organisms and chemicals: Pure cultures of *Aspergillus oryzae* were maintained at 30 °C on Malt Extract Agar. This medium contains 30 g malt extract, 3 g peptone from soymeal and 15 g agar per 1,000 mL of distilled water. Final pH of the medium was 5.5 ± 0.2.

Drimaren Blue CL-BR, Cibacron Gelp NP, Foran Scharlock and Dispersol Flavine dyes were obtained local textile factory in Turkey. Poly R-478, Methyl Green, Acid Orange 63, Methyl Red, Azure A, Bismarck Brown Y and Acid Blue 74 dyes were purchased from Sigma and Fluka. Media components were obtained from Merck. All other chemicals were obtained analytical grade from Sigma and Merck.

Goldfish, *Carassius auratus* (Linnaeus, 1758) belonging to the family of Cyprinidae was chosen for micronucleus test and comet assay because of sensitivity of the goldfish to genotoxic chemicals.

2.2. Decolourisation experiments: For observation on the time course of Drimaren Blue CL-BR decolourisation, cultures were incubated in basal medium, which described below and supplemented with 300 mg/L dye, 10 g/L glucose and 5 g/L yeast extract at pH 4.0, 30 °C and 150 rpm for up to 7 days. However, for examination of the effects of initial dye concentration (25-1.600 mg/L), incubation pH (3.0-10.0), temperature (30-60 °C), agitation rate (50-200 rpm) and different concentration of glucose (0-10 g/L) and yeast extract (0-5 g/L) as main carbon and energy source, cultures were incubated at 30 °C at 150 rpm for 2 days. Also, effects of different carbon sources on the dye decolourisation were investigated. For this purpose, suspensions of sporulating growth were used to inoculate of basal medium (pH 4.0) which described below, supplemented with one of the eleven different carbon sources (i.e, wheat straw, poplar and oak sawdust and banana leaf) (0,8% wt/v) at pH 4, 30 °C, 150 rpm in the presence of 300 mg/L dye. After 2 days incubation, the culture supernatant fluids were centrifuged at 10.000 x g at 4 °C for 10 min and then used for *decolourisation* assays.

2.3. Decolourisation assays: Decolourisation studies were carried out by using basal medium which consisting of (in g/L): Drimaren Blue CL-BR 0.1; glucose 10; (NH₄)₂HPO₄ 0.5; KNO₃ 0.5; NaCl 1; yeast extract 5 and MgCl 0.5. The experiments were performed in batch mode in 250 mL Erlenmayer flasks. In order to ensure aerobic conditions, the volume of the medium was restricted to 1/5 of the total volume of each flask. All the experiments were carried out in triplicate. Each flask was inoculated with 1 mL of suspension of spores (10⁶ cfu). Decolourisation was determined through measurements of culture supernatant absorption at Drimaren Blue CL-BR dye λ_{max} (595 nm) using a spectrophotometer. Percentage decolourisation was calculated using equation:

$$\% \text{ Decolourisation: } [(A_0 - A_1) / A_0] \times 100$$

A₀: initial absorbance, A₁: final absorbance

2.4. Harvesting of biomass and culture supernatant: After 48 hours incubation period, culture samples were filtered through Whatman filter paper No 1 and 1.5 mL of filtrate in eppendorf tubes was centrifuged at 10.000 rpm for 10 minute to remove any particulate material. The weight of harvested biomass was determined gravimetrically. After filtration, residual biomass was washed and completely dried at 100 °C for 12 hours. *A. oryzae* biomass (dyeless as control) and biomass after bioabsorption of dye was illustrated with light microscopy.

2.5. Determination of enzyme activities: Laccase activity was analyzed by the oxidation of ABTS (2,2'-azino-bis (3-ethylthiazoline-6-sulfanote). The reaction mixture contained 600 μL supernatant, 300 μL sodium acetate buffer (pH 5, 0.1 M) and 100 μL ABTS solution (1 mM). Oxidation of ABTS was monitored at 30 °C for 1 min at a wavelength of 420 nm (33). Tyrosinase activity was determined in 1 mL reaction mixture containing 200 μL supernatant, 700 μL phosphate buffer (pH 7, 0.1 M) and 100 μL catechol solution (0.1%) and the reaction was monitored at 30 °C for 5 min at a wavelength of 420 nm (34). Peroxidase activity was assayed using 2,4-dichlorophenol (2,4-DCP) as the substrate. The reaction mixture (total volume 1 mL) contained equal volumes (0.2 mL) of potassium phosphate buffer (100 mmol/L, pH 7.0) 2,4-DCP (25 mmol/L), 4-aminoantipyrine (16 mmol/L), culture supernatant as enzyme solution and H₂O₂ (50 mmol/L). The reaction was initiated with the addition of H₂O₂ and the reaction was monitored at 30 °C for 1 min at a wavelength of 510 nm (35, 36).

One unit (U) of laccase, tyrosinase and peroxidase activities were defined as the amount of enzyme required for an increase in absorbance of 1 unit per minute. The absorbance values obtained at the end of the incubations were corrected by subtracting corresponding controls absorbance values obtained by omitting of substrates in the reaction mixture.

2.6. Micronucleus test and comet assay: The micronucleus test and comet assay were performed using goldfish (*Carassius auratus*) because of its common availability in most fish markets and due to proven sensitivity to genotoxic chemicals (37). Five goldfishes were placed in each of four different aquaria. First aquarium containing dechlorinated tap water was used as negative control. Cyclophosphamide (5 mg/L) was used as positive control in second aquarium. For the evaluate toxicity of initial 300 mg/L Drimaren Blue CL-BR concentration, which was used in experimental studies and the final 15.3 mg/L dye concentration, which retained in culture media after 2 days incubation, were added to third and fourth aquaria, respectively.

3. Results and discussion

3.1. Effect of incubation time on decolourising activity of *A. oryzae*: A typical growth curve and decolourising activity of *A. oryzae* in basal medium containing 300 mg/L of Drimaren Blue CL-BR dye was obtained at pH 4.0, 30 °C, 150 rpm. The decolourisation of the dye increased significantly during the growth phase of culture and followed by an insignificant change in decolorization for the next 2 days, which corresponded to the stationary phase of fungal growth cycle. After this point of incubation, decolourising activity of the fungus sharply decreased, while dye content of culture medium increased gradually until end of the incubation (10 days) (data not shown). Decolourisation of Drimaren Blue CL-BR dye by *A. oryzae* could be due to adsorption onto the fungal cell surfaces or biodegradation. To confirm the mode of decolourisation by *A. oryzae* the absorption spectrum of the dye, which remained in culture supernatants during incubation period, was examined and results reveal that all peaks decreased approximately in proportion to each other. Therefore, dye removal by *A. oryzae* was attributed to biosorption. Dye adsorption onto the fungal cell surfaces was also judged clearly by inspecting the fungal hyphae by microscopic examinations (figure 1). Moreover, some enzymes which could involve in dye decolourising activity of organisms such as laccase, tyrosinase and peroxidase were also assayed during decolourisation by 24 hours intervals. But enzymatic activities weren't detected related to dye decolourisation. Consequently, according to the above results, the colour removal by *A. oryzae* might be largely attributed to biosorption onto the fungal cell surfaces, and the biodegradation was not significant.



Figure 1. Biosorption of Drimaren Blue CL-BR by *A. oryzae* hyphae after 2 days incubation

3.2. Effect of incubation pH and temperature on decolourising activity of *A. oryzae*:

The effect of pH on the decolourisation of Drimaren Blue CL-BR by *A. oryzae* was investigated by adjusting the pH of the growth medium in a pH range of 3.0 to 10.0. *A. oryzae* was also capable of growth in basal medium containing the dye at these pH ranges (figure 2 a). However, decolourisation of the dye by *A. oryzae* was found to vary between the different pH values. The optimum pH for the decolourisation of the dye occurred in the pH range of 4.0 and 7.0. The highest decolourisation of the dye (83.5% and 77%) was detected at these pH points, respectively. These also corresponded to the maximum growth yield for *A. oryzae* biomass (7.39 and 7.06 g/L). At pH 3.0 and pH 10.0, the decolourisations of the dye (71% and 9.7%, respectively) were significantly decreased. These findings indicating that the *A. oryzae* could be used in treatment of effluents which are either acidic or neutral. The optimum pH for decolorization of Drimaren Blue CL-BR by *A. oryzae* however remains 4.0. The optimum pH for decolourisation Drimaren Blue CL-BR by *A. oryzae* was found to occur at pH 4.0. This result is similar to those reported for *Aspergillus foetidus*, *Rhizopus stolonifer* and *Lentinus sajor-caju* (38) and generally in accordance with fungal physiology.

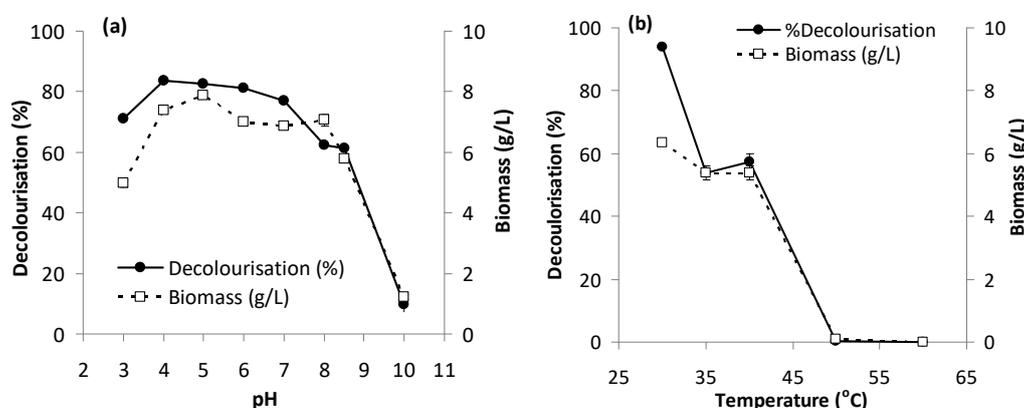


Figure 2. Effect of initial pH (a) and incubation temperature (b) on decolourisation of Drimaren Blue CL-BR by *A. oryzae* and fungal growth as measure of biomass after 2 days incubation at different pH values.

All data are presented as means of three replicates with SEs

The effect of temperature on the decolourisation activity of Drimaren Blue CL-BR by *A. oryzae* was investigated by growing the organism at a temperature range of 30 °C to 60 °C. From the analysis of the results, it is evident that the highest decolourisation (94%) of the dye by *A. oryzae* occurred at 30 °C (figure 2 b). Fungal growth was also greater at 30 °C (6.36 g/L) and it reduced with higher temperatures. There wasn't significant alteration on fungal growth between 30 °C to 40 °C (5.39 g/L). However, at 35 °C and 40 °C the decolourisations of the dye (53.8% and 57.3%, respectively) were significantly decreased, while at 50 °C and 60 °C decolourisation and fungal growth weren't detected at all. According to the reports (39-41) the optimum growth temperature of *A. oryzae* is 32-36 °C.

3.3. Effect of Drimaren Blue CL-BR concentrations on decolourising activity of *A. Oryzae* Different concentrations of Drimaren Blue CL-BR on decolourising activity of *A. oryzae* was investigated by adjusting the initial dye concentrations of the growth medium ranges from 25 to 1.600 mg/L. In 48 hours it has been found that with increase in dye concentration up to 1.000 mg/L the dye decolourising efficiency of *A. oryzae* remained fairly constant (between 82.2% and 85.3%), but slightly decreased (80.5%) on 1.200 mg/L and (71.2%) on 1.400 mg/L concentrations. The maximum decolourisation (87.8%) was found on 300 mg/L concentration and minimum decolourisation (1%) was found on 1.600 mg/L concentration (figure 3). These findings indicating that the *A. oryzae* could be used in

treatment of effluents which has dye concentration up to 1.000 mg/L. The optimum dye concentrations for decolorization of Drimaren Blue CL-BR by *A. oryzae* however remain between 50 to 1.000 mg/L. There were no significant differences at fungal growth between 50-800 mg/L initial dye concentration and after this point, biomass yield decreased gradually. In parallel to this, decolourisation was also decreased by *A. oryzae*.

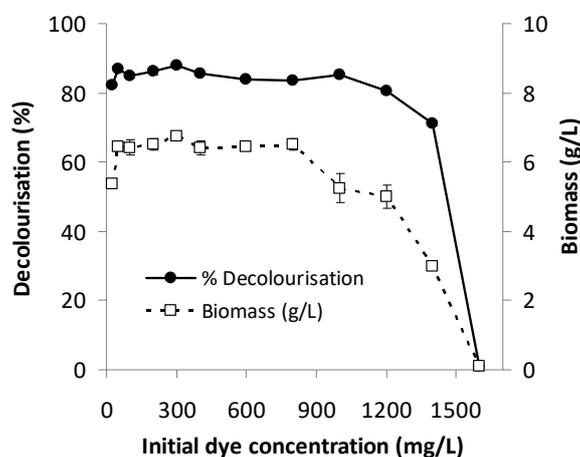


Figure 3. Effect of initial dye concentration on decolourisation of Drimaren Blue CL-BR by *A. oryzae* and fungal growth after 2 days incubation. Data are presented as means of three replicates with SEs.

Zeroyal *et al.* reported similar results for biosorption of Bromophenol Blue with *R. solonifer* (15). When initial dye concentration was set as 1.600 mg/L, there wasn't any fungal growth in medium. Because of chemical structure of Drimaren Blue CL-BR, fungal growth was inhibited by high concentration of the dye. This is also supported by earliest study. Parshetti *et al.* determined that higher dye concentration (0.6-1 g/L) showed a toxic effect that adversely affected adsorption and decolourisation performance of *Aspergillus ochraceus* (34). This is most important disadvantage for bioadsorption with growing cells.

3.4. Effect of glucose and yeast extract concentrations on decolourising activity of *A. oryzae*: Different concentrations of glucose (0-10 g/L) and yeast extract (0-5 g/L) have been utilized the determination of effect of glucose as main carbon source and yeast extract as nitrogen source for decolourisation efficiency of *A. oryzae* (figure 4). With increase concentration of glucose from 0 to 8 g/L achieved decolourisation was also increased from 27.6% to 94.9% (figure 4 a). However, at 10 g/L glucose concentration the decolourisation was slightly decreased and found to be 91.1% with the maximum growth yield for *A. oryzae* biomass (8.13 g/L). Glucose has been added to enhance the decolourisation performance of biological systems in some studies (42-44). However, others reported that glucose inhibited the decolourising activity (45, 46). The reason for this variability may be due to the different microbial characteristics. In this study, various concentrations of glucose (0-8 g/L) were first evaluated for decolorization of Drimaren Blue CL-BR by *A. oryzae* (figure 4 a). Dong *et al.* tested effect of different glucose concentrations on decolourisation of Direct Black 22 by *Aspergillus ficuum* and they obtained 61.63% and 98.17% decolourisation when glucose in medium were 0 g/L and 20 g/L, respectively (47). However, figure 4a clearly indicates that glucose concentration of higher than 8 g/L inhibited slightly the decolourisation of Drimaren Blue CL-BR by *A. oryzae*. Fig. 4b shows the influence of yeast extract as a nitrogen source on the efficiency of decolourisation of Drimaren Blue CL-BR by *A. oryzae*. The metabolism of yeast extract is considered essential to the regeneration of NADH that acts as the electron

donor for the reduction of azo bonds [43]. Therefore, yeast extract was tested as a part of culture medium for further experiments because yeast extract is cheap nitrogen source. At 300 mg/L dye concentration and in presence of yeast extract from 0 to 3 g/L concentrations achieved decolourisation was also increased from 6.4% to 94.0% (figure 4 b). However at 5 g/L of yeast extract concentration in medium showed no any more beneficial effects on decolourisation (89.3%).

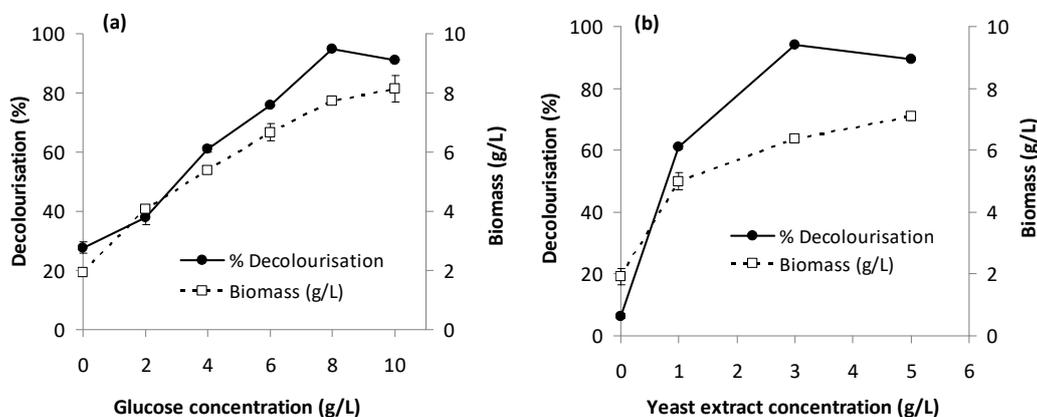


Figure 4. Effect of glucose (a) and yeast extract (b) concentrations on decolourisation of Drimaren Blue CL-BR by *A. oryzae* and fungal growth after 2 days incubation. All data are presented as means of three replicates with SEs.

The optimum concentration of yeast extract on decolourisation activity of *A. oryzae* was 3 g/L with a biomass of 6.36 g/L, while the highest fungal growth was observed in the medium containing 5 g/L yeast extract (7.1 g/L of biomass). The results clearly indicate that optimal glucose and yeast extract concentrations for the maximum decolourisation of the dye by the fungus were 8 g/L and 3 g/L, respectively (figure 4). Therefore, yeast extract at a concentration of 3 g/L as nitrogen source for decolorization of Drimaren Blue CL-BR by *A. oryzae* was added in further experiments.

3.5. Effect of agitation on decolourising activity of *A. oryzae*: The effect of incubation conditions namely shaking (50, 100, 150 and 200 rpm) and stationary condition on decolourisation of Drimaren Blue CL-BR by *A. oryzae*, revealed that shaking condition was more suitable for decolorization, where the activity was found to be 90.9% and in stationary condition it was 16.7%. The data suggest that at shaking condition (150 rpm) is more appropriate for the decolourisation of the dye by the fungus (figure 5). This probably because of different growth form of *A. oryzae* in stationary and shaking conditions, where fungal biomass growth was occurred on the medium surface as a layer in stationary condition, while fungal biomass formed as pellet like beads in shaking condition. Moreover, decolourisation rate was also higher in agitated cultures. This could be due to the physiological state of *A. oryzae* as pellets and increased mass transfer between the cells. Kaushik and Malik reported that the higher decolourisation was occurred in agitated cultures because of better oxygen transfer and nutrient distribution compared to the stationary cultures (38).

3.6. Effect of various carbon sources on decolourising activity of *A. oryzae*: To investigate effects of different carbon and energy sources on Drimaren Blue CL-BR decolourisation were investigated by replacing glucose with varying carbon sources at 8 g/L, while maintaining a fixed yeast-extract concentration (3 g/L) (figure 6). Decolourisation of Drimaren Blue CL-BR by *A. oryzae* reached the maximum levels on sucrose (84.9%) and cotton pulp (84.0%), while the highest decolourisation of the dye was detected on glucose (90.7%). However, decolourisation performance of used carbon sources weren't effective and obtained as follows: starch (7.6%), lactose (27.9%), banana leaf (34.5%), lentil straw (38.7%), corn stalk

(69.7%), wheat straw (56.0%), oak sawdust (43.5%) and poplar sawdust (46.6%). Ramya *et al.* reported that sucrose, mannitol and glucose were the best carbon sources for decolourisation of Reactive Blue by *Aspergillus sp.* (48). Hadiborata *et al.* reported to 85% and 63% decolourisation of RBBR by growing *Polyporus sp.* S133, when glucose and sucrose were used as carbon sources, respectively (49). Glucose is the most readily usable carbon source for most fungi. However, it is not commonly used in wastewater treatment since it is an expensive carbon source. Therefore, as can be seen from Fig. 6, cotton pulp and corn straw could occupied as carbon sources for alternative to glucose for decolourisation of Drimaren Blue CL-BR *A. oryzae*.

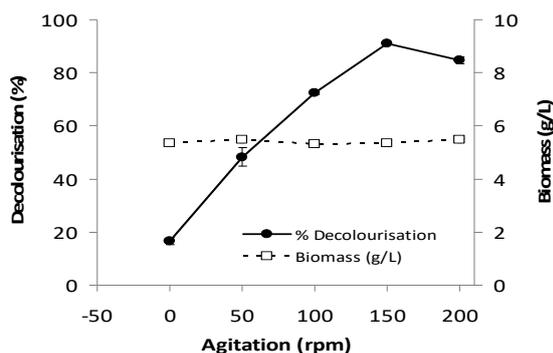


Figure 5. Effect of agitation on decolourisation of Drimaren Blue CL-BR by *A. oryzae* and fungal growth after 2 days incubation. Data are presented as means of three replicates with SEs.

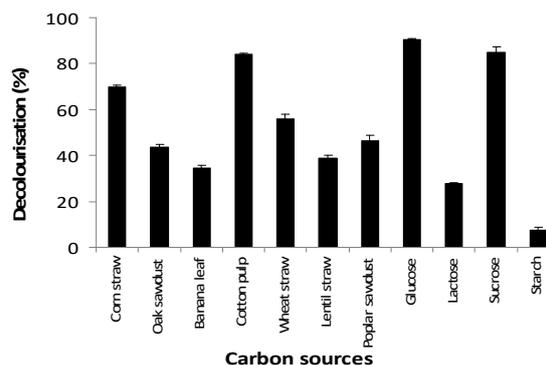


Figure 6. Effect of additional carbon sources on decolourisation of Drimaren Blue CL-BR by *A. oryzae*. Data are presented as means of three replicates with SEs.

A. oryzae was also tested for decolourisation of different dyes. Decolourisation was examined at 30 °C, 150 rpm for 2 days (Initial dye, glucose and yeast extract concentrations were 300 mg/L, 8 g/L and 5 g/L, respectively). Most of these dyes such as Methyl Red (83%), Acid Orange 63 (83,2%), Lewofix Brillant Blue (82,4%), Cibacron Gelp NP (88.7%), Poly R 478 (88.7%) and Acid Blue 74 (85.6%) were decolourised by *A. oryzae* significantly. However, decolourisation of some dyes, like Bismark Brawn (11.9%), Foran Scharlock (23.6%) and Azure A (32.2%) by *A. oryzae* was occurred quite inefficiently. Methyl Green (45.4%) and Dispersol Flavine (73.3%) were decolourised moderately (figure 7).

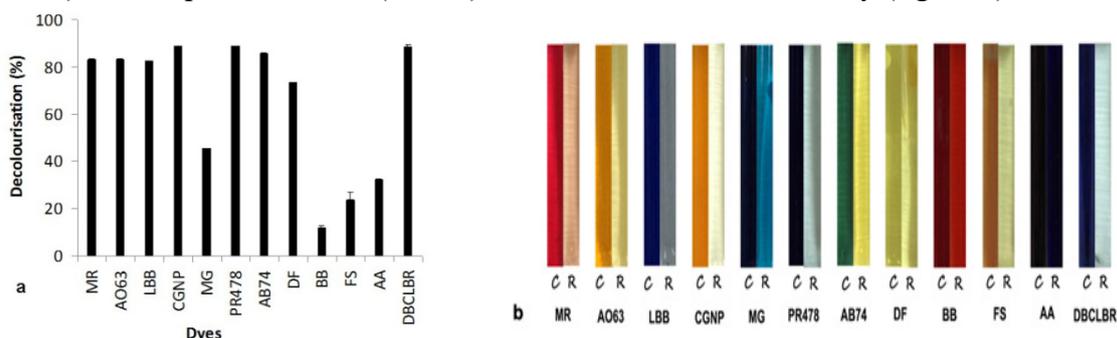


Figure 7. (a) Decolourisation (%) of different dyes by *A. oryzae* at pH 4, 30 °C, 150 rpm for 2 days (Initial dye, glucose and yeast extract concentrations were 300 mg/L, 8 g/L and 5 g/L, respectively). (b) Visual appearance of different dyes before (C) and after (R) decolourisation by *A. oryzae*. (CK: control, R: after decolourisation). Dyes are MR: Methyl Red, AO63: Acid Orange 63, LBB: Lewofix Brillant Blue, CGNP: Cibacron Gelp NP, PR478: Poly R 478, AB74: Acid Blue 74, DF: Dispersol flavine, BB: Bismark Brawn, FS: Foran Scharlock, AA: Azure A, and DBCLBR: Drimaren Blue CL-BR

3.7. The toxicity of Drimaren Blue CL-BR As shown in Table 1, micronucleus frequencies of lowest dosage of Drimaren: Blue CL-BR (15.3 mg/L), which correspond to

dye concentration that left in culture media after 2 days incubation, was similar to negative control. But, frequency of blebbed nuclei are increasing significantly in the 15.3 mg/L dye exposed group. However, other frequencies of nuclear anomalies (lobed nuclei, notched nuclei, binucleated cells) were observed more than negative control (figure 8 b). For the micronucleus test, nuclear anomalies lower than positive control. Goldfishes in aquaria with 300 mg/L Drimaren Blue CL-BR were died at the end of 18 hours.

Table 1. Micronucleus frequencies in peripheral blood erythrocytes of *C. auratus* exposed to Drimaren Blue CL-BR (15.3 mg/L) for 2 days.

Negative control	Positive control	Drimaren Blue CL-BR (15.3 mg/L)
3.15 ± 0.46	20.1 ± 3.98	3.17 ± 0.48

Table 2 summarizes the proportion of damaged nuclei and genetic damage index (GDI) as measured in the comet assay. Compared to the negative control, increase of DNA migration was determined in the Drimaren Blue CL-BR (15.3 mg/L) exposed group. But, detected DNA damage was less than positive control. As these data indicates, Drimaren Blue CL-BR dye may cause significant DNA damage at 15.3 mg/L in aquatic biota. Undamaged and damaged fish erythrocytes cells were classified in to 5 categories (Type 0: undamaged, Type 1: low damage, Type 2: medium damage, Type 3: large damage and Type 4: complete damage) (figure 8a).

Table 2. Analysis of DNA damage as measured by comet assay in peripheral erythrocytes of *C. auratus* exposed to Drimaren Blue CL-BR (15.3 mg/L) for 2 days. Percentage of damaged cells = Type 2 + 3 + 4. GDI = (Type 1 + 2 x Type 2 + 3 x Type 3 + 4 x Type 4)/(Type 0 + 1 + 2 + 3 + 4) (37).

Treatment group	Type 0	Type 1	Type 2	Type 3	Type 4	% of damaged cells	GDI
Negative control	75.67	17.00	4.67	1.67	1.00	7.33 ± 1.20	0.35 ± 0.04
Positive control	56.33	19.67	10.37	6.63	7.00	24.00 ± 2.08	0.88 ± 0.04
Drimaren Blue CL-BR(15.3 mg/L)	56.00	23.00	12.66	9.33	5.33	21.00 ± 2.08	0.85 ± 0.09

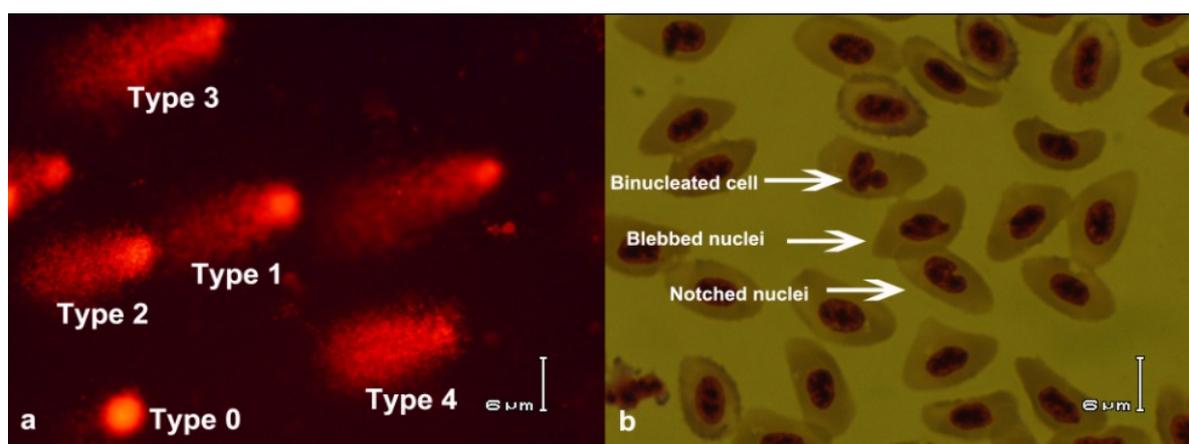


Figure 8. (a) Images of Type 0, 1, 2, 3 and 4 cells of comet assay and (b) binucleated cell, blebbed nuclei and notched nuclei of micronucleus test

4. Conclusions

The decolourisation process was affected by various experimental conditions such as pH, initial dye concentration, glucose and yeast extract concentrations, incubation temperature,

agitation rate and carbon sources. Maximum decolourisation (94.9%) was observed at pH 4, 30 °C, 150 rpm, with 300 mg/L initial dye concentration in the presence of 8 g/L glucose and 3 g/L yeast extract concentrations. Enzymatic studies indicated that there was not involvement of some enzymes, which could involve in dye decolourising activity of organisms such as laccase, tyrosinase and peroxidase. Despite the fact that untreated dyeing effluents might cause serious environmental and health hazards, they are being disposed off in aquatic ecosystems. Use of untreated dyeing effluents in the agriculture has also direct impact on fertility of soil. Thus, it was of concern to assess the toxicity of the effluent before and after decolourisation. Therefore, especially when consider the toxicity of Drimaren Blue CL-BR as indicted by analysis of DNA damage as measured by comet assay in peripheral erythrocytes of *C. auratus* exposed to the dye (15.3 mg/L) for 2 days, it may be urged that *A. oryzae* is good candidate for the decolourisation of the dye and possibly other dyes form effluents for environmental-friendly applications. Such a biosorption process could be adopted as a cost effective, sustainable, and safer for effluents that have dyes and dyestuff like textile wastewater. Furthermore, adsorbed dye by fungal biomass may be recovered with convenient recovery processes.

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References

1. I. HOLME, Sir William Henry Perkin: A Review of His Life, *Work And Legacy. Color. Technol.*, 122: 235-251 (2006).
2. A. ÜNYAYAR, M.A. MAZMANCI, H. ATAÇAĞ, E.A. ERKURT, G. CORAL, A Drimaren Blue X3LR Dye Decolorizing Enzyme From *Funalia trogii*: One Step Isolation and Identification. *Enzyme Microb. Technol.*, 36: 10-16 (2005).
3. A. ASAD, M.A. AMOOZEGAR, A.A. POURBABAEE, M.N. SARBOLOUKI, S.M.M. DASTGHEIB, Decolorization of Textile Azo Dyes by Newly Isolated Halophilic and Halotolerant Bacteria. *Bioresource. Technol.*, 98: 2082-2088 (2007).
4. L. TAN, S. NING, H. XIA, J. SU, Aerobic Decolorization and Mineralization of Azo Dyes by A Microbial Community In The Absence Of An External Carbon Source. *Int. Biodeterior. Biodegrad.*, 85: 210-216 (2013).
5. R. KHAN, P. BHAWANA, M.H. FULEKAR, Microbial Decolorization And Degradation of Synthetic Dyes: A Review. *Rev Environ. Sci. Biotechnol.*, 12: 75-97 (2013).
6. A. PANDEY, P. SINGH, L. LYENGAR, Bacterial Decolorization And Degradation Of Azo Dyes. *Int. Biodeterior. Biodegradation*, 59: 73-84 (2007).
7. Y. FU, T. VIRARAGHAVAN, Removal of Congo Red From An Aqueous Solution by Fungus *Aspergillus niger*. *Adv. Environ. Res.*, 7: 239-247 (2002).
8. S. SUMATHI, B.S. MANJU, Uptake of Reactive Textile Dyes by *Aspergillus foetidus*. *Enzyme Microb. Technol.*, 27: 347-355 (2000).
9. Y.D. ARACAGÖK, N. CIHANGIR, Decolorization of Reactive Black 5 by *Yarrowia lipolytica* NBRC 1658. *A.J.M.R.*, 1(2): 16-20 (2013).
10. S. ILYAS, A. REHMAN A, Decolorization and Detoxification of Synozol Red HF-6BN Azo Dye, by *Aspergillus niger* And *Nigrospora sp.* *I.J.E.H.S.E.*, 10(12): 1-9 (2013).
11. G. DÖNMEZ, Bioaccumulation of The Reactive Textile Dyes by *Candida tropicalis* Growing In Molasses Medium. *Enzyme Microb. Technol.*, 30: 363-366 (2002).
12. S. PADMAVATHY, S. SANDHYA, K. SWAMINATHAN, Y.M. SUBRAHMANYAM, T. CHAKRABARTI, S.N. KAUL, Aerobic Decolorization of Reactive Azo Dyes In Presence Of Various Cosubstrates. *Chem. Biochem. Eng. Q.*, 17(2): 147-151 (2003).
13. M.A. KHALAF, Biosorption of Reactive Dye From Textile Wastewater by Non-viable Biomass of *Aspergillus niger* and *Spirogyra sp.* *Bioresour. Technol.*, 99: 6631-6634 (2008).

14. J.Y. FARAH, N.S. EL-GENDY, L.A. FARAHAT, Biosorption of Astrazone Blue Basic Dye From An Aqueous Solution Using Dried Biomass of Baker's Yeast. *J. Hazard. Mater.*, 148, 402-408 (2007).
15. Y. ZEROUAL, B.S. KIM, C.S. KIM, M. BLAGHEN, K.M. LEE, Biosorption of Bromophenol Blue From Aqueous Solutions by *Rhizopus Stolonifer* Biomass. *Water Air Soil Poll.*, 177: 135-146 (2006).
16. B.D. Bhole, B. GANGULY, A. MADHURAM, D. DESHPANDE, J. JOSHI, Biosorption of Methyl Violet, Basic Fuchsin And Their Mixture Using Dead Fungal Biomass. *Curr. Sci. India.*, 86(12): 1641-1645 (2004).
17. Ö. YESILADA, B. ÖZCAN, Decolorization of Orange II Dye With The Crude Culture Filtrate of White Rot Fungus, *Coriolus versicolor*. *Tr. J. of Biology.*, 22: 463-476 (1998).
18. N. SRI KUMARAN, G. DHARANI, Decolorization of Textile Dyes By White Rot Fungi *Phanerocheate chrysosporium* and *Pleurotus sajor-caju*. *J. Appl. Technol. Environ. Sanit.*, 1(4): 361-370 (2011).
19. H.D. ÖZSOY, A. ÜNYAYAR, M.A. MAZMANCI, Decolourisation of Reactive Textile Dyes Drimarene Blue X3LR And Remazol Brilliant Blue R by *Funalia trogii* ATCC 200800. *Biodegradation*, 16: 195-204 (2005).
20. P.S. MAULIN, K.A. PATEL, S.S. NAIR, A.M. DARJI, Microbial Degradation of Textile Dye (Remazol Black B) by *Bacillus spp.* ETL-2012. *J. Bioremed. Biodeg.*, 4(2): 1-5 (2013).
21. H. H. BIYIK, G. BASBULBUL, F. KALYONCU, E. KALMIS, E. ORYASIN, Biological Decolorization of Textile Dyes From Isolated Microfungi. *J. Environ. Biol.*, 33: 667-671 (2012).
21. YAMAMOTO A, UEDA J, YAMAMOTO N, HASHIKAWA N, SAKURAI H, Role of Heat Shock Transcription Factor in *Saccharomyces cerevisiae* Oxidative Stress Response, *Eukaryotic Cell*, 6(8): 1373–1379 (2007).
22. A. MOUTAOUAKKIL, Y. ZEROUAL, F.Z. DZAYRI, M. TALBI, K. LEE, M. BLAGHEN, Bacterial Decolorization of The Azo Dye Methyl Red by *Enterobacter agglomerans*. *Ann. Microbiol.*, 53: 161-169 (2003).
23. S. ERUM, S. AHMED, Comparison of Dye Decolorization Efficiencies of Indigenous Fungal Isolates. *Afr. J. Biotechnol.*, 10(17): 3399-3411 (2011).
24. M. RAFATULLAH, O. SULAIMAN, R. HASHIM, A. AHMAD, Adsorption of Methylene Blue On Low-Cost Adsorbents: A Review. *J. Hazard. Mater.*, 177: 70-80 (2010).
25. Z. AKSU, S. TEZER, Equilibrium And Kinetic Modelling of Biosorption of Remazol Black B by *Rhizopus arrhizus* In A Batch System: Effect of Temperature. *Process Biochem.*, 36: 431-439 (2000).
26. A.K. MITTAL, S.K. GUPTA, Biosorption of Cationic Dyes by Dead Macro Fungus *Fomitopsis carnea*: Batch Studies. *Water Sci. Technol.*, 34(10): 81-87 (1996).
27. T.L. HU, Removal of Reactive Dyes From Aqueous Solution by Different Bacterial Genera. *Water Sci. Technol.*, 34(10): 89-95 (1996).
28. J.K. POLMAN, C.R. BRECKENRIDGE, Biomass-mediated binding and recovery of Textile Dyes From Waste Effluents. *Text. Chem. Color.*, 31-35 (1996).
29. W. ZHOU., W. ZIMMERMANN, Decolorization of Industrial Effluents Containing Reactive Dyes by *Actinomyces*. *FEMS Microbiol. Lett.*, 107(2-3): 157-161 (1993).
30. Z. AKSU, G. DÖNMEZ, A Comparative Study On The Biosorption Characteristics of Some Yeasts For Remazol Blue Reactive Dye. *Chemosphere*, 50: 1075-1083 (2003).
31. M. BUSTARD, G. MCMULLAN, A.P. MCHALE, Biosorption Of Textile Dyes by Biomass Derived From *Kluyveromyces marxianus* IMB3, *Bioprocess Eng.*, 19: 427-430 (1998).
32. Z. AKSU, Application of Biosorption for The Removal of Organic Pollutants: A Review, *Process Biochem.*, 40: 997-1026 (2005).
33. A. SINGH, S. BAJAR, R.N. BISHNOI, N. SINGH, Laccase Production by *Aspergillus heteromorphus* Using Distillery Spent Wash And Lignocellulosic Biomass. *J. Hazard. Mater.*, 176: 1079-1082 (2010).
34. G.K. PARSHETTI, S.D. KALME, S.S. GOMARE, S.P. GOVINDWAR, Biodegradation of Reactive Blue-25 by *Aspergillus ochraceus* NCIM-1146. *Bioresour. Technol.*, 98: 3638-3642 (2007).
35. M. RAMACHANDRA, D.L. CRAWFORD, G. HERTEL, Characterization of An Extracellular Lignin Peroxidase of The Lignocellulolytic Actinomyces *Streptomyces viridosporus*. *Appl. Environ. Microbiol.* 54: 3057-3063 (1988).
36. M. Iqbal, D. K. Mercer, P.G.G. Miller, A.J. McCarthy, Thermostable Extra-cellular Peroxidases from *Streptomyces thermoviolaceus*. *Microbiology*. 140: 1457-1465 (1994).
37. T. ÇAVAŞ, S. KÖNEN, Detection of Cytogenetic And DNA Damage In Peripheral Erythrocytes of Goldfish (*Carassius auratus*) Exposed To A Glyphosate Formulation Using The Micronucleus Test And The Comet Assay. *Mutagenesis*, 22(4): 263-268 (2007).
38. P. KAUSHİK, A. MALIK, Fungal Dye Decolourization: Recent Advances And Future Potential. *Environ Int.*, 35: 127-141 (2009).

39. P. BARBESGAARD, H.P.H. HANSEN, B. DIDERICHSEN, On the Safety of *Aspergillus oryzae*: A Review. *App. Microbiol. Biotechnol.*, 36: 569-572 (1992).
40. R. BIESEBEKE, G. RUIJTER, Y.S.P. RAHARDJO, M.J. HOOGSCHAGEN, M. HEERIKHUISEN, A. LEVIN, K.G.A. DRIEL, M.A.I. SCHUTYSER, J. DIJKSTERHUIS, Y. ZHU, F.J. WEBER, W.M. VOS, K.A.M.J.J. HONDEL, A. RINZEMA, P.J. PUNT, *Aspergillus oryzae* in Solid-State and Submerged Fermentations Progress Report on A Multi-Disciplinary Project, *Fems Yeast Res.*, 2: 245-248 (2002).
41. M.M. ASSADI, M.R. JAHANGIRI, Textile Wastewater Treatment by *Aspergillus niger*. *Desalination*, 141: 1-6 (2001).
42. W. HAUG, A. SCHMIDT, B. NO"RTEMANN, D.C. HEMPEL, A. STOLZ, H.J. KNACKMUSS, Mineralization of The Sulfonated Azo Dye Mordant Yellow 3 by A 6-Aminophthalene-2-Sulfonated-Degrading Bacterial Consortium, *Appl. Environ. Microbiol.*, 57: 3144-3149 (1991).
43. C.M. CARLIELL, S.J. BARCLAY, N. NAIDOO, C.A. BUCKLEY, D.A. MULHOLLAND, E. SENIOR, Microbial Decolorization of A Reactive Azo Dye Under Anaerobic Conditions. *Water SA*, 21: 61-69 (1995).
44. I.K. KAPDAN, F. KARGI, G. MCMULLAN, R. MARCHANT, Effect of Environmental Conditions on Biological Decolorization of Textile Dyestuff by *C. versicolor*, *Enzyme. Microb. Technol.*, 26: 381-387 (2000).
45. K.T. CHUNG, G.E. FULK, M. EGAN, Reduction of Azo Dyes by Intestinal Anaerobes. *Appl. Environ. Microbiol.*, 35: 558-562 (1978).
46. J.S. KNAPP, P.S. NEWBY, The Microbiological Decolorization of An Industrial Effluent Containing A Diazo-Linked Chromophore, *Water Res.*, 29: 1807-1809 (1995).
47. X. DONG, Z. DU, Z. CHEN, Decolorization of Direct Black 22 by *Aspergillus ficuum*, *J. Environ. Sci.*, 13(4): 472-475 (2001).
48. M. RAMYA, B. ANUSHA, S. KALAVATHY, S. DEVILAKSMI, Biodecolorization And Biodegradation of Reactive Blue by *Aspergillus sp.*. *Afr. J. Biotechnol.*, 6(12): 1441-1445 (2007).
49. T. HADIBARATA, R.A. KRISTANTI, Effect of Environmental Factors In The Decolorization Of Remazol Brilliant Blue R by *Polyporus sp.* S133. *J. Chil. Chem. Soc.*, 57(2): 1095-1098 (2011).