Adsorption behavior of Reactive Blue 4, a tri-azine dye on dry cells of 
Rhizopus oryzae in a batch system

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ABSTRACT
The adsorption behavior of Reactive Blue 4, a tri-azine dye, on dry Rhizopus oryzae biomass (ROB) has been investigated in aqueous solution with special reference to physicochemical parameters associated with the adsorption process. Adsorption of dye on the biomass is found to be a function of solution pH (optimum 3.0), while temperature has no significant effect. Adsorption rate of biomass is very fast initially and attains equilibrium within 5 h for 50 and 100 mg L⁻¹ and 6 h for 200 mg L⁻¹ initial dye concentrations, respectively, following pseudo-second-order rate model (R² = 0.99). The equilibrium adsorption isotherm can be best described by Redlich–Peterson model (R² = 0.95). Scanning electron micrograph demonstrates a conspicuous change in surface morphology of ROB after dye adsorption. Fourier transform infrared spectroscopic data reflect the binding of Reactive Blue 4 on the biomass through complexation reaction involving amino and carboxyl groups. Reactive Blue 4 can be desorbed from the dye loaded biomass using 1(N) NaOH solution. Results establish that ROB has the potential to pollution control management involving Reactive Blue 4.

Introduction
Aquatic ecosystem is vigorously affected by the presence of textile dyes as dye is a visible toxic pollutant and the presence of very minute amount of dye makes it undesirable due to its appearance. Industries such as textile, tannery, food, paper and pulp, printing, carpet, and mineral processing use dyes and pigments to color their products. There are greater than 10,000 different commercial dyes and pigments and over 7 x 10⁵ tons are produced annually worldwide.[1] Around 5–10% of these dyes are discharged into water as wastes.[2] The present scenario is very much alarming as dye also reduces photosynthesis by restricting sunlight penetration into the stream.

Reactive Blue 4 is a reactive tri-azine dye, used heavily in textile industries for coloring different cloth materials, is highly toxic and potentially carcinogenic, mutagenic and allergenic, and sometimes causes damage not only to aquatic life but also exposed organisms.[3–6] Most of the tri-azine dye components are resistant to chemical and photochemical degradations.[7–9] So, before releasing it into the field or aqueous phase, the effluent containing textile dyes must be treated properly.

A number of conventional methods (viz., electrofloatation, precipitation, electro-kinetic coagulation, ion exchange, membrane filtration, ozonation, adsorption through activated carbon, chemical oxidation, electrochemical treatment, reverse osmosis, hydrogen peroxide catalysis, etc.) are used for removal of textile dyes from the effluent.[10–13] But these methods are costly, generate huge amount of toxic sludge and are inefficient for very dilute solution. The biosorption is an efficient eco-friendly alternative process for dye removal minimizing toxic sludge production. The dry biomass may be stored for long time [14] and chances of contamination are low. Different textile dyes can be removed using microbial biomass including bacteria, fungi, and algae by biosorption and biodegradation [15] which consume less chemicals and energy. Some low-cost fungal biosorbent has been developed for the removal of dye and metal ions from wastewater, which included Trametes versicolor,[16] Corynebacterium glutamicum,[17] Aspergillus versicolor,[18] Lentinus sajor caju,[19,20] Rhizopus nigricans,[21] Aspergillus niger,[9] Aspergillus fumigates,[22] and Phanerocheate chrysosporium.[23,24]

However, a few studies have been focused on utilization of the fungal biomass for Reactive Blue 4 biosorption.

The earlier reports describe the sorption process as economic and having good performance for toxicity reduction and removal of dyes from industrial effluents. The process may be more economic if the sorbent is...
inexpensive and eliminates the requirement of any expensive pretreatment of wastewater.

The present investigation was undertaken to evaluate the biosorption (Reactive Blue 4) capacity of dry cells of *Rhizopus oryzae* (MTCC 262). The uptake capacity of the selected mold was studied as a function of pH, temperature, dose of biosorbent, initial dye concentration, and time of contact. Equilibrium rate kinetic study and analysis of biosorption data were also carried out. The conspicuous changes in the surface morphology of the biomass as a result of dye adsorption were demonstrated by scanning electron micrograph (SEM) study. The possible mechanisms involved in the dye biosorption were discussed on the basis of FTIR spectroscopic studies.

**Methodology**

**Micro-organism**

*R. oryzae* (MTCC 262), *A. versicolor* (MTCC 280), and *A. niger* (MTCC 281) were procured from Institute of Microbial Technology, Chandigarh, India. *Termitomyces clypeatus* was kindly provided by Dr S. Sengupta, Indian Institute of Chemical Biology, Kolkata, India. The organisms were maintained on potato dextrose agar slant by monthly subculturing at 30 °C for 120 h and stored at 4 °C.

**Chemicals and dye**

All the chemicals and ingredients of microbiological media used in the present study were purchased from E. Merck, Germany and Hi Media, India, respectively. Reactive Blue 4 [Chemical formula: C_{22}H_{22}N_{6}S_{2}Cl_{2}, molecular weight: 637.4, and $\lambda_{\text{max}}$ (nm): 595] was procured from Sigma-Aldrich Chemical Co., St. Louis, MO, USA.

**Biomass production**

Composition of maintenance medium (potato dextrose agar) was as follows (g L^{-1}): potato extracts: 200.0, dextrose: 20.0, agar: 20.0, and pH 5.0. Composition of inoculum and growth medium was (g L^{-1}): potato extracts: 200.0, dextrose: 20.0, and pH 5.0. Inoculum was prepared by transferring one loopful of biomass from slant culture to 50 mL of inoculum medium in 250 mL Erlenmeyer flask and incubating at 30 °C, 120 rpm for 48 h. 1 mL of inoculum was added to 250 mL Erlenmeyer flask containing 50 mL of growth medium and the flasks were incubated for 72 h under the above mentioned conditions for biomass production.

**Preparation of dry cells**

The fungal mycelia were harvested by centrifugation at 5000 rpm for 15 min, washed thrice with double distilled water, and dried by lyophilization. After that the dried biomass was ground and used for adsorption study.

**Cell size measurement**

Size and shape of dry biomass were determined under microscope using stage and ocular micrometers.[25]

**Preparation of dye solution**

A stock solution of dye (1000 mg L^{-1}) was prepared by dissolving the required amount of Reactive Blue 4 in distilled water and diluted to get desired concentrations.

**Biosorption experiment using dry cells**

0.1 g of each adsorbent [viz., *R. oryzae* (MTCC 262), *A. versicolor* (MTCC 280), *A. niger* (MTCC 281), and *Termitomyces clypeatus*] was added separately to each of 250 mL erlenmeyer flasks containing 50 mL of dye solution (100 mg L^{-1}) having different pH viz., 3.0, 5.0, 7.0, and 9.0 and incubated at 30 °C and 120 rpm for 24 h unless stated otherwise. The flask containing only dye solution (without biomass) served as control. After incubation, the biomass was separated by centrifugation at 5000 rpm for 15 min and the concentration of residual dye in the supernatant was measured.

**Estimation of dye concentration**

The concentration of dye in the solution was determined using a UV spectrophotometer (HITACHI U-2000) at maximum absorption wavelength, ($\lambda_{\text{max}}$) 595 nm, which was determined by spectrophotometrical scanning of the dye over a range of wavelength (200–800 nm). An absorbance calibration curve of standard dye solutions was prepared and used as the reference standard for determination of dye concentration.

The uptake of dye by the biomass was calculated using the following mass balance equation:

$$ q_e = \left( \frac{C_0 - C_f}{V} \right) \frac{1000W}{1000W} \quad (1) $$

where $q_e$ is the amount of dye uptake by the biomass in mg g^{-1}. $C_0$ and $C_f$ are the initial and final dye concentrations in mg L^{-1} in the solution, respectively. V and W represent the volume of solution (L) and weight of the biomass (g), respectively.

**Effect of pH**

To determine the effect of pH on biosorption, the dried biomass (0.1 g) of the selected strain was suspended in 50 mL of dye solution (100 mg L^{-1}) having different pH (range 3.0–9.0) in 250 mL Erlenmeyer flasks separately and incubated at 30 °C and 120 rpm for 24 h. After
harvesting the cell mass by centrifugation at 5000 rpm for 15 min, residual dye concentration in the supernatant was estimated.

**Effect of temperature**

To study the effect of temperature on biosorption, experiment was carried out at optimized pH and at different temperatures (viz., 20, 25, 30, 35 °C), other conditions remaining the same. Residual dye concentration was measured as usual.

**Effect of dry biomass concentration**

Dried powdered cells at different concentrations (2–8 g L\(^{-1}\)) were added to 50 mL of dye solution (100 mg L\(^{-1}\)) in 250 mL Erlenmeyer flask separately and incubated for 24 h at 30 °C and 120 rpm. Residual dye concentration was measured as usual.

**Adsorption kinetic study**

Kinetic studies were carried out with three initial dye concentrations viz. 50, 100, and 200 mg L\(^{-1}\) at pH 3.0, temperature 30 °C and biomass concentration 2.0 g L\(^{-1}\). The solute uptake rate by the biosorbent from solid–solution interface is described by the kinetic study of adsorption. The adsorption kinetics of Reactive Blue 4 were investigated using Lagergren’s pseudo-first (Equation (2)) and pseudo-second-order (Equation (3)) rate models. The pseudo-first-order kinetic model of Lagergren is based on solid capacity.[26–28] It is expressed as follows:

\[
\log (q_e - q_t) = \log q_e - k_1 t / 2.303 \tag{2}
\]

where \(q_e\) and \(q_t\) represent the amount of dye adsorbed at equilibrium (mg g\(^{-1}\)) and dye adsorbed (mg g\(^{-1}\)) at time \(t\), respectively, and \(k_1\) is the first-order rate constant (min\(^{-1}\)). It has been reported that as pseudo-first-order equation is not applicable to several observations, in that case, the use of pseudo-second-order equation has been suggested,[29] and it has been considered the rate-limiting step through the formation of chemisorptive bond between the adsorbate and the adsorbent. The generalized equation of pseudo-second-order kinetic model is given as:

\[
t / q_t = 1 / k_2 q^2_e t / q_e \tag{3}
\]

where \(q_e\) and \(q_t\) are the amount of dye adsorbed at equilibrium (mg g\(^{-1}\)) and dye adsorbed (mg g\(^{-1}\)) at time \(t\), respectively, and \(k_2\) is the pseudo-second-order rate constant.

**Adsorption isotherm**

The efficiency of an adsorbent depends on its capacity to adsorb a particular adsorbate. So, it is necessary to design an efficient operating system to analyze the experimental data from the angle of different isotherm models to understand the adsorbate-adsorbent interaction. The biosorption equilibrium defines the distribution of a solute phase between the liquid phase and solid phase after the sorption reaction reached equilibrium condition. In the present study, equilibrium data were analyzed using three of the most commonly used isotherm equations, Freundlich, Langmuir, and Redlich-Peterson isotherm models. The isotherm expressions of the respective models are given by Equations (4)–(6), as follows:

Langmuir: \(q_e = q_0 K_L C_e / (1 + K_L C_e) \tag{4}\)

Freundlich: \(q_e = K_F C_e^{1/n} \tag{5}\)

Redlich–Peterson: \(q_e = K_{RP} C_e / (1 + a C_e^{1/g}) \tag{6}\)

where \(C_e\) is the equilibrium concentration (mg L\(^{-1}\)) and \(q_e\) is the adsorbed amount of metal ion per gram of the biomass at equilibrium (mg g\(^{-1}\)). \(K_L\), \(q_0\), and \(K_F\) are the Langmuir and Freundlich constants, respectively, whereas \(1/n\) is the heterogeneity factor. The Redlich-Peterson model deals with three parameters: where \(K_{RP}\) (L g\(^{-1}\)) and \(a\) (L mg\(^{-1}\)) g are the Redlich-Peterson constants and ‘\(g\)’ is the Redlich-Peterson isotherm exponent. The value of ‘\(g\)’ lies between 0 and 1, and when \(g = 1\), this model converts to the Langmuir model.

**Desorption and regeneration of biosorbent**

Regeneration of biosorbent for repeated use is important to make the process cost effective, eco-friendly and thereby decrease the dependency of the process on the continuous supply of the biosorbent. For successful regeneration, the selection of an appropriate eluant is important, which strongly depends on the type of biosorbent and the mechanism of biosorption. Desorption studies were carried out with four desorption media viz., 1(N) NaOH solution, 70% ethanol, 70% acetone, and distilled water separately.[30]

In the present study, 5 mL of desorption media was added separately to 0.5 g of dye loaded biomass in the test tubes, shaken vigorously and left for 12 h. Desorption medium with dye extract was then filtered into test tubes. Eluant and dye mixture was left for 1 h at 65 °C to evaporate the solvent eluant. The remaining volume in all test tubes was made up to 5 mL with distilled water and the concentration of desorbed dye was estimated using a spectrophotometer (HITACHI U-2000) at 595 nm.[31].
FTIR spectra analysis
The Fourier transform infrared spectra of pristine and dye laden biomass of *R. oryzae* (MTCC 262) were obtained using IR spectrophotometer (SHIMADZU CORPORATION, IR-Prestige 21, resolution 4 cm⁻¹). For FTIR spectra analysis, approximately, fungal biomass (0.01 g) was mixed with KBr (0.1 g) and the mixture was pressed into a tablet form by pressing the ground mixed material with the aid of a bench press. The FTIR spectrum was analyzed in the region of 4000–400 cm⁻¹ with a scanning frequency of 200 kHz (i.e. 0.0805 s scan⁻¹, 40 s total for an experiment).

Scanning electron microscopy study
SEMs of the pristine and dye loaded biomass of *R. oryzae* were obtained using a JEOL, JMS 5600 scanning electron microscope after coating with a thin layer of platinum under reduced pressure.

Results and discussions
Screening of biosorbent
The four fungal strains used in the present experiment, absorb dye from the aqueous solution to the extent of 48–99% depending on the type of the species and pH of the solution (Figure 1). *R. oryzae* biomass (ROB) was found to be the most efficient and adsorbed 98.95% of dye (initial concentration 100 mg L⁻¹) at pH 3.0. The noted difference in adsorption capacity may be attributed to difference in surface structure and functional groups present on the cell wall of the fungi.[32] The shape and size of dry ROB were found to be spherical and 0.00645 mm³, respectively.

Effect of pH on biosorption
To study the effect of pH on dye biosorption, experiment was conducted at different pH (3.0–9.0). From Figure 2 it was observed that biosorption of dye on ROB biomass increased with decrease in pH and maximum biosorption occurred at pH 3.0. Reactive Blue 4 dye molecule has two sulfonate and a primary amino groups. The pKₐ values of the sulfonate and amino groups of the dye molecule are around 0.8 and 7.0, respectively. These functional groups can be easily dissociated and thus, the dye molecule has negative and positive charges under the working experimental conditions. Therefore, the positive and negative sites of the fungal biomass such as protonated form of amino groups (i.e. −NH₃⁺; pKₐ values between 7.0 and 10.0), and deprotonated form of carboxylic and phosphate groups (the pKₐ values around 4.0 and 6.5, respectively) [33] can play a role in Reactive Blue 4 biosorption. The zero point charge (ZPC) is found to be at pH 3.5 in this study with the strain *R. oryzae*, MTCC 262. [34] This means that at pH 3.5, the net surface charge is zero. When the pH is higher than ZPC, there is a net negative charge, which results from deprotonation of carboxylate and phosphate groups. When the pH is less than the ZPC, protonation of all three major functional groups (carboxylic, phosphate, and amino) results in a positive charge. Therefore, with decreasing pH, the binding sites increased, and thereby the biosorption of Reactive Blue 4 increased.

Effect of temperature
As shown in Figure 3, temperature has no significant effect on biosorption. The present study showed slight increase in uptake capacity (q value) from 41.32 mg g⁻¹ (at 20 °C) to 43.80 mg g⁻¹ (at 35 °C) due to increased surface activity of cells and kinetic energy of solute molecules at higher temperature. Thus, an economically feasible temperature within the above range may be suitably used for the biosorption process.
Adsorption isotherm analysis

In the present work, adsorption isotherm studies were carried out at a range of 25–600 mg L⁻¹ concentration of dye solution. Figure 5 shows the equilibrium plot for biosorption of Reactive Blue 4 by dry ROB (0.1 g) using 50 mL of dye solution (concentration range 25–600 mg L⁻¹), at pH 3.0 and temperature 30 °C. All the model parameters are described in Table 2 as the ‘goodness of fit’ of the experimental data with the calculated data from the isotherm model can be assessed by $R^2$ (linear coefficient) and $\chi^2$ (nonlinear coefficient) values. The value of $R^2$ varies from 0 to 1 and will be very low or zero, if the experimental data differs from the data obtained from the model, whereas the perfect matching of these values yields a coefficient of 1.0. And in case of $\chi^2$, the coefficient value will be small if the experimental data is very close to the obtained data from the model and will be higher if they differ. So, it is essential to evaluate the data with both for $R^2$ and $\chi^2$ values.

From the Table 2, it is observed that the adsorption data are very well represented by Redlich-Peterson isotherm with an average higher correlation coefficient $R^2$ of 0.95 followed by Langmuir and Freundlich isotherms with correlation coefficient $R^2$ of 0.93 and 0.82, respectively, and the nonlinear regression coefficient ($\chi^2$) of 44.97, 57.17, 155.89, respectively. Therefore, the present sorption data are found to be best fitted to Redlich-Peterson model [35] (Figure 5) in comparison with the other two models considering high correlation coefficient ($R^2 = 0.95$) and low $\chi^2$ value (44.97). This indicates that the adsorption mechanism is a hybrid one and does not follow the ideal monolayer adsorption behavior.[36] The value of Freundlich exponent $n$ is 2.85 and, in the range of 1–10, indicates the favorable adsorption. Also, the higher adsorption capacity, $q_0$ ($\gg 1$) indicates the strong electrostatic force of attraction.[37]

Effect of dry cell mass concentration

Reactive Blue 4 biosorption by ROB was studied at different biomass concentrations (range 2.0–8.0 g L⁻¹), with other conditions remaining the same. The adsorption rate on the biomass was rapid initially due to availability of abundant binding sites on the bare cell surface thus diffusion of dye molecules onto the fungal cell surface was faster. Gradually, the process became slow due to less availability of bare adsorption sites. It is evident from the Figure 4 that with an increase in biomass load, uptake capacity ($q$ value) decreased while percentage removal of dye increased. As the number of biosorption sites (or total surface area) increased with the increase in biomass load, percentage removal increased and attained saturation at 8.0 g L⁻¹. On the other hand, the Reactive Blue 4 uptake capacity ($q$) increased initially with an increase in biomass concentration up to 4.0 g L⁻¹ followed by a gradual decrease on further increasing biomass concentration. From the above study, biomass load of 2.0 g L⁻¹ was selected as optimum for removal of Reactive Blue 4 by ROB.
**Desorption and regeneration**

Desorption of dye from the dye laden biomass for repeated use is extremely important for any successful biosorption process development.[38] In the present study, it was observed that about 100% desorption was possible using 1(N) NaOH (Figure 8). The removal and recovery efficiencies of the dry fungal biomass of ROB during four regeneration cycles are shown in Figure 9. It shows that biomass is reusable up to four cycles with 68.26% adsorption.

**FTIR spectroscopic studies**

Fourier transform infrared spectroscopy was employed to get an idea about the possible mechanism of adsorption by identifying the functional groups present on the cell surface of ROB because, each group has a unique energy absorption band [18] and thus, FTIR spectrum of pristine ROB (Figure 10) exhibits distinct peaks suggesting the presence of amine, carbonyl, phosphate, and hydroxyl groups. The broad mixed stretching vibrations frequency of N–H and O–H are observed in the region of 3500–3300 cm⁻¹ and those for alkyl chains are found around 2920–2850 cm⁻¹. The sharp peak at 1652.88 cm⁻¹ can be attributed to C=O stretching of carboxyl or amide groups. The band at 1550.66 cm⁻¹ is assigned to N–H bending. The presence of COO⁻ of the carboxylate can be attributed to the peak positions at 1452.30 and 1400.22 cm⁻¹ on the biomass. The complex amide III band is located near 1319.22 cm⁻¹. The wave number at 1026.06 cm⁻¹ arises due to the presence of P–O–C link of the organo phosphorous groups on the biomass. The shift of around 4 cm⁻¹ in the FTIR analysis may be due to the resolution of the device. In the present case, the shift of more than 4 cm⁻¹ was only considered. It is evident from the FTIR spectra (Figure 10) that peak at about 3404.52 cm⁻¹ due to amino group has been shifted to 3409.61 cm⁻¹ after dye adsorption. Similarly, peak due to carboxylate ion in pristine biomass at 1738 cm⁻¹ has...
that the dye had been adsorbed throughout the surface, especially along the edge of the cell boundary.

Table 1. Effect of initial dye concentration on kinetic parameters for sorption on dry cells of R. oryzae (MTCC 262).

| Initial dye concentration (mg L⁻¹) | Pseudo-first-order kinetic model | Pseudo-second-order kinetic model |
|-----------------------------------|----------------------------------|----------------------------------|
|                                   | $k_1$ (min⁻¹)  | $R^2$       | $q_e$ (mg g⁻¹) | $k_2$ (g mg min⁻¹) | $R^2$       |
| 50                                | 0.004             | 0.992       | 24.64              | 0.040             | 0.999       |
| 100                               | 0.005             | 0.996       | 38.97              | 0.025             | 0.999       |
| 200                               | 0.005             | 0.991       | 67.74              | 0.014             | 0.999       |

Table 2. Sorption isotherm coefficients correlation and constants for removal of Reactive Blue 4 by dry biomass of R. oryzae (MTCC 262).

| Langmuir | Freundlich | Redlich-Peterson |
|----------|------------|------------------|
| $q_0$ (mg g⁻¹) | $K_L$ (L mg⁻¹) | $R^2$ | $\chi^2$ | $K_F$ (L g⁻¹) | $R^2$ | $\chi^2$ | $K_RP$ (L g⁻¹) | $\alpha$ (L mg⁻¹) | $\beta$ (g) | $R^2$ | $\chi^2$ |
| 101.1    | 0.009      | 0.935 | 57.17       | 0.35 | 8.491 | 0.82 | 155.89 | 0.664 | 0.01 | 1.27 | 0.95 | 44.97 |

Figure 8. Selection of a potent desorption medium.

Figure 9. Adsorption–desorption cycle of Reactive Blue 4 using R. oryzae dry biomass as biosorbent and 1(N) NaOH as desorption medium.

Scanning electron microscopy

The surface morphology of a biosorbent can be characterized extensively using scanning electron microscopy as a tool.[39] Figure 11, paneled A and C, showed the surface morphology of the pristine R. oryzae biomass, which appeared to be rough and had an irregular structure with large area of dye surface interaction. Significant changes in surface morphology were noted after dye adsorption (Figure 11(B)). SEM image of the dye adsorbed biomass (Figure 11(D)) at higher magnification depicted

Figure 10. FTIR spectroscopy study of pristine biomass and dye loaded biomass of R. oryzae (MTCC 262).

that the dye had been adsorbed throughout the surface, especially along the edge of the cell boundary.
Conclusions

From the present investigations, it may be concluded that ROB was capable of removing the carcinogenic, tri-azine textile dye Reactive Blue 4 from aqueous solution efficiently. The biosorption process was found to be influenced by environmental factors viz., initial pH of the solution, biomass load, initial dye concentration, and contact time between the dry fungal biomass and dye solution. Dye solution having pH 3.0 and biomass load of 4 g L\(^{-1}\) was found to be optimum for removal of the dye (initial concentration 100 mg L\(^{-1}\)) by ROB. Temperature showed no significant effect on biosorption. The equilibrium time were found to be 5 h for 50 and 100 mg L\(^{-1}\) and 6 h for 200 mg L\(^{-1}\) initial dye concentrations, respectively. The kinetics of the overall adsorption process was best described by pseudo-second-order kinetic model. At equilibrium, experimental results were found to be fit best to Redlich-Peterson isotherm model followed by the Langmuir isotherm model. Desorption efficiency was found to be 68.26% up to the fourth cycle. FTIR spectroscopy study showed that amino, and carboxyl groups were the functional groups responsible for the adsorption process. Scanning electron microscopy showed significant changes in surface morphology of ROB after adsorption. Thus, removal of Reactive Blue 4 using ROB may be viewed as a considerable alternative process for the removal of dye from industrial effluents in near future.

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Disclosure statement

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