IJISET - International Journal of Innovative Science, Engineering & Technology, Vol. 3 Issue 6, June 2016
ISSN (Online) 2348 – 7968 | Impact Factor (2015) - 4.332
www.ijiset.com

Statistical Optimization of Biosorption Of Reactive Dyes By *Rhizopus Arrhizus* Ncim 997 Immobilized In Calcium Alginate

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Abstract
The current study deals with the utilization of dead biomass of *Rhizopus arrhizus* NCIM 997 immobilized in calcium alginate as a low-cost fungal biosorbent and optimization of conditions for the removal of Reactive Orange 13 and Reactive Blue 222 from aqueous solutions using statistically designed sequential experiments. Plackett - Burman design with five independent variables (pH, concentration of dye, temperature, speed of agitation and contact time), was used in the first step to identify the most significant factors influencing dye biosorption. Response surface methodology using Central Composite Design was used successfully in the second step to investigate the mutual interactions between significant factors and to identify their optimum values that would produce maximum biosorption. Based on the analysis of variance, pH was found to be the most important parameter under the experimental ranges examined.Validation experiments showed very good correlation between predicted and experimental values (68.02% for RO13 and 62.09% for RB 222). The fitted quadratic models were used to reach at the best operating conditions to achieve a maximum response. The sequential optimization was successfully used to increase biosorption of both the dyes.

Keywords:
R, arrhizus , Reactive Orange 13 ,Reactive Blue 222 ,Immobilization ,Calcium alginate ,Biosorption Plackett– Burman design ,Response Surface Methodology.

1. Introduction

India's dye industry produces all types of dyes and pigments with production of the dyestuff and pigments being close to 80,000 tons (Mathur et al. 2005). About 7×10^5 tons of dyes per annum, are discharged from textile and associated industries into the water bodies (Swami 1998, Allen et al. 2002).Other than dyes, opportunities exist for the release of potentially perilous compounds such as recalcitrant organics, toxicants and inhibitory compounds, surfactants, chlorinated compounds (AOX), salts at various stages of the operation during textile production (Shin et al. 2002; Sen and Demirer 2003;

Asamudoet al. 2005).Dyes, even at very low concentrations, reduce wastewater transparency and oxygen solubility and are often harmful for a wide range of organisms (Mathur and Bhatnagar 2007).Removal of color from the effluents is thus a major problem and the implementation of tighter constraints on the discharges is forcing waste creators and managers to think about newer options for the effluent treatment and disposal. Unfavorable conditions found in the textile dyeing effluents such as large fluctuations in composition, pH and temperature, high salt loads are known to inhibit the regular biological wastewater treatment processes. Moreover, various azo dyes have been shown to be anaerobically decolorized by the cleavage of the azo bond, resulting in the formation of potentially carcinogenic aromatic amines (Murugesan 2003). Thus, dyes are hardly removed from the effluents by the conventional wastewater treatments.

Among all the wastewater treatments, the adsorption process has been recognized to be an effective and economical procedure for removal of dyes from the industrial effluents. Adsorption can handle large flow rates, producing a high-quality effluent that does not result in the formation of damaging substances. A large variety of adsorbent materials have been tested to remove dyes from the wastewater including activated carbon, which is one of the most widely used adsorbents because of its tremendous adsorption capacity for organic contaminants. However, its high cost, difficulty in regeneration; problems with regard to final disposal, inefficient adsorption of cationic dyes limit its commercial application (Skult 2009; Russo et al. 2009; Erden et al. 2011). Hence, low-cost biosorbent materials viz. waste materials from industry and agriculture, as well as biosorbents produced from microbial biomass have gained increasing attention in reducing the adsorbent dose and minimizing the disposal problem (Chen and Chen 2009). The latter are often found to be even more selective than traditional ion-exchange resins and activated carbons, and potentially provide an inexpensive method for dye elimination (Yang etal. 2012).Application of fungal biosorbent to remove textile dyes from industrial waste water is attractive for industry due to its cheap and constant supply from food and industrial fermentation processes, ease of culturing, and its ability to decrease the overall effluent treatment cost (Ambrosio et al. 2012).

Unfortunately, the use of freely suspended biosorbent is limited due to their inherent disadvantages such as small particle size, possible clogging and low mechanical strength. Immobilized biosorbent overcomes some of these problems and offers greater potential applications. The benefits being better control of particle size, easy separation of biomass and effluent, capability to regenerate, high biomass loadings and minimal clogging under continuous flow (Kapoor and Viraraghavan 1998).Keeping the above reasons in mind, microorganisms have been immobilized using calcium alginate (Pradhan and Rai 2000; Arica et al. 2001), carboxymethylcellulose (CMC) (Wang et al.2008), polyurethane foam, nylon and stainless steel sponge (Couto et al. 2004), polysulphone (Vijayaraghavan and Yun 2008) etc. Alginate, a high molecular weight unbranched binary copolymer of 1-4 linked β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues, is one of the most widely investigated gel matrices for cell entrapment in bioremediation studies (Scott 1987; Tam et al. 1998; Fourest and Volesky 1996; Pradhan and Rai 2000; Arica et al. 2001; Vijaya et al. 2008; Vreeker et al. 2008).Alginate's several properties include its

biodegradability, good substrate diffusion within the matrix, non-toxicity, biocompatibility and ability to form gels with an array of cross-linking agents in mild and aqueous conditions. In alginate beads, the microbial cell density can be maintained as high as 10^{10} to 10^{11}cfu/g of beads (Adzmi et al. 2012).

The aim of the present study was to employ multi-variant experimental design based on statistical approach suchas response surface methodology (RSM) for techno-economical optimization of the biosorption of Reactive Orange 13 (RO 13), a mono-azo reactive dye and Reactive Blue 222 (RB 222) from aqueous solutions using the immobilized dead biomass of *Rhizopus arrhizus* NCIM 997 as a low-cost biosorbent.RO 13 and RB 222 were chosen in this study due to their extensive use in Indian textile industry for dyeing and printing cotton, silk, viscose, wool and nylon fabric (Saraf and Vaidya 2015).

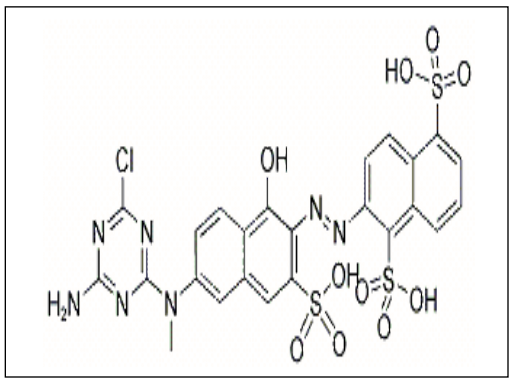
2. Materials and Method

2.1 Preparation of adsorbate

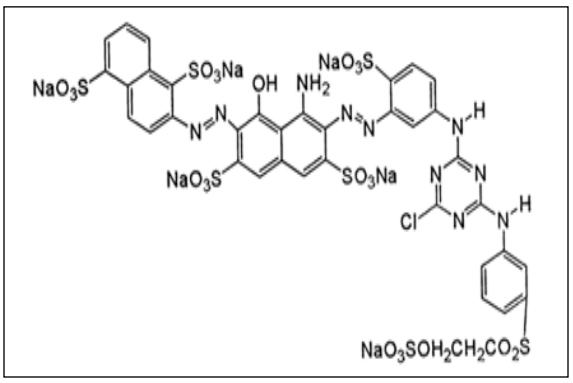
RO 13 (C.I. 18270, CAS 12225-85-3, chemical formula:$C_{24}H_{15}CIN_7Na_3O_{10}S_3$, molecular weight: 762.04 g/mol), a monoazo reactive dye(Fig. 1a) and Reactive Navy Blue BF (C.I. Reactive Blue 222, CAS 93051-44-6, chemical formula: $C_{37}H_{23}ClN_{10}Na_6O_{22}S_7$,MW: 1357.49 g/mol) a bifunctional diazo reactive dye (Fig.1 b) were obtained as a solid powders from Colortex Dyes Pvt. Ltd. (Mumbai, India) and DyStar, (Mumbai, India) respectively and used without further purification.Stock solutions of RO 13 and RB 222 (1000 mg/L) were prepared by separately dissolving the required amount of dye powders in double deionized water. The stock solutionswere diluted with double deionized water as required to obtain the desired concentration ranging from 40 to 100 mg/L. The required pH was adjusted by 0.1 N HCl or 0.1 N NaOH using a pH meter (Labotronics, LT-10, India) (Saraf and Vaidya 2015).

Fig. 1 Chemical structure of a) Monoazo reactive dye Reactive Orange 13 b) Bifunctional diazo dye Reactive Blue 222

1a)



1b)



2.2 Preparation of the biosorbent

R. arrhizus NCIM 997 was obtained from NationalCollection of Industrial Microorganisms (NCIM), NationalChemical Laboratory, Pune, India, and used as a biosorbent. It was routinely maintained at 4^0C onpotato dextrose agar (PDA) (g/L: potato infusion from200 g potatoes, dextrose 20, yeast extract 0.1, agar 20, pH 5.0). For experimental purposes, fungal myceliawere obtained by aseptically transferring mycelia fromthe PDA spread-plate cultures to 100 mL of potatodextrose broth (PDB) containing 0.25 %

Tween 80 (toprevent sporulation) in 250 mL Erlenmeyer flasks. Theflasks were incubated at 30 ± 1^0C under static conditionswith intermittent shaking. The biomass harvestedafter 7 days was washed thoroughly with generousamounts of deionized distilled water and dried at 80^0Cin an oven for 24 h. Care was taken to keep the particlesize of the biomass uniform, by grinding into powderand sieving through a 150-mesh sieve. The biomass wasstored in a desiccator until use for the biosorption experiments (Saraf and Vaidya2015).

2.3 Immobilization of fungal biosorbent

The immobilized fungal biosorbent (IFB) was prepared by using ionotropic gelation technique**.**A homogenous suspensionof 5g of dried biomass of *R. arrhizus* in 100 ml of 6% sodium alginate was extruded dropwise through a 10-ml syringe without a needle from a height of approximately 6 cm and at a rate of approximately one drop per second, into ice-cold solution of 4% $CaCl_2$ solutionunder mild agitation (80 rpm) to avoid aggregation of the beads. The resultant alginate beads were left in $CaCl_2$ solution overnight to enhance their mechanical stability, followed by washing twice with distilled water. The beads with an average diameter of 3.5 mm were kept in refrigerator at 4°C for further use. Alginate beads devoid of *R. arrhizus* served as a control (Binupriya et al. 2010; Charumathi and Das 2010; Adzmi et al. 2012).

2.4 Dye biosorption experiments

The biosorption studies were carried out in duplicate for the evaluation of the immobilized biosorbent of *R. arrhizus* for the removal of RO 13 and RB222 from aqueous solutions. The batch contact biosorption experiments were conducted employing the specified combinations of independent variables using sequential statistically designed experiments consisting of Plackett - Burman design (PBD) and Central Composite Design (CCD).For these experiments, 50 beads of IFBwere added to 50 mL of dye solutions at desired pH andconcentrationsin 250 mL Erlenmeyer flasks. The flasks werekept under agitation in a rotating orbital shaker (Labtop Quality Lab Equipment, India)for desired period of time. After centrifugation at 4000 rpm,the remaining concentration of each dye in the solution was measured using UV–Vis Spectrophotometer (Shimadzu, Japan UV1800 UV/VIS) at 489 nm for RO13 and 609 nm for RB222, corresponding to the maximum absorbance. Beads without biosorbent were used as control.The amount of the dye adsorbed on the fungal biomass at the corresponding equilibrium conditions was determined by using % sorption:

$$\% \text{ Sorption} = \frac{C_0 - C_e}{C_0} * 100 \quad (1)$$

Where,$C_0$is theinitial concentration of the dye(mg/L) and $C_e$is the concentration of dye in solution at equilibrium time(mg/L).

3. Experimental data and design analysis

3.1 Plackett and Burman Design (PBD)

To investigate the effect of various parameters like pH, concentration of dye (mg/L), , temperature (^{0}C), speed of agitation (rpm) and contact time (min) on the biosorption process, a 12-run Plackett - Burman design was employed along with three runs at zero level. Each variable was examined at two levels: –1 for the low level and +1 for the high level (Table 1) (Plackett and Burman 1946).

Table 1: Experimental range and levels of independent process variables tested in PBD for biosorption of RO 13 and RB 222 by immobilized *R. arrhizus*

Designation	Variable	Variable values		
		-1	0	1
X_1	pH	2.0	4.0	6.0
X_2	Concentration of the dye (mg/L)	40	60	80
X_3	Temperature(^{0}C)	35	40	45
X_4	Speed of agitation (rpm)	80	100	120
X_5	Contact time (min)	30	75	120

The values of the variables were selected based on the previous preliminary experimental results. The Plackett–Burman design and the response values of biosorption are shown in Table 2. All the experiments were carried out in duplicate and the average of percent sorption was taken as response. The effect of individual variable on biosorption was calculated by the following Eq. (2):

$$E(X_i) = \frac{2\,(\sum M^+{}_i - \sum M^-{}_i)}{N} \qquad (2)$$

Where, *E(Xi)*is the effect of the tested variable *(Xi)* and $M^+{}_i$ and $M^-{}_i$ are responses (biosorption) of trials at which the variable is at its high or low levels respectively, *N* is the total number of trials (Plackett and Burman 1946).

Table 2: Plackett–Burman design of variables (in coded levels) with experimental and predicted values of biosorption of RO 13 and RB 222 by immobilized *R. arrhizus*

Run No.	**Coded values**					**Biosorption (%)**			
	X_1	X_2	X_3	X_4	X_5	**RO 13**		**RB 222**	
						Experimental	**Predicted**	**Experimental**	**Predicted**
1	1	-1	-1	-1	1	30.45	33.19	29.67	33.51
2	-1	1	-1	-1	-1	25.99	25.45	23.67	24.86
3	-1	-1	-1	1	1	65.79	63.19	61.23	60.09
4	1	-1	1	1	-1	29.88	30.65	27.02	27.31
5	-1	1	1	1	-1	39.55	38.92	34.19	36.12
6	1	1	1	-1	1	35.61	32.00	34.42	33.56
7	1	1	-1	1	-1	13.89	13.63	11.09	10.98
8	-1	-1	-1	-1	-1	33.92	34.55	33.78	32.91
9	1	-1	1	-1	-1	27.58	26.79	27.46	26.76
10	-1	1	1	-1	1	48.17	49.38	35.97	34.05
11	-1	-1	1	1	1	60.79	60.19	59.03	58.95
12	1	1	-1	1	1	28.03	29.65	11.14	10.55
13	0	0	0	0	0	35.00	36.06	31.98	33.51

14	0	0	0	0	0	35.01	36.06	32.20	33.24
15	0	0	0	0	0	35.00	36.05	31.98	33.51

Significant factors affecting biosorption of both the dyes were determined from the regression analyses and the contribution of the factors towards the biosorption of RO 13 and RB 222 was determined based on the *t*-values (main effect). The sign of the effect indicates the level at which it is considered for further improvement.

3.2 Central Composite Design (CCD)

The optimal levels of the significant variables and their interactions on the biosorptionprocess were investigated using Central Composite Design (CCD) (Plackett and Burman 1946). In order to make the designs rotatable, values of α were chosen to be 1.633 for RO 13 and 2 for RB 222.A three factor, five level CCD consisting of 20 runs, (with 6 centre points, 4 cube points and 2 axial points)was conducted to establish the accuratebest possible values of pH (X_1), speed of agitation(X_4) and contact time(X_5)influencing biosorption of RO 13(Table 3). A four factor, five level CCD consisting of 30 runs (with 6 centre points, 4 cube points and 2 axial points) was conducted to locate the true optimum values of pH (X_1), concentration of dye (X_2), temperature (X_3) and contact time (X_5) affecting biosorption of RB222. The runs were made in a random order to minimize the effects of variability in the observed responses due to extraneous factors. Replicates were used to estimate the experimental error and to check for the adequacy of the model.

Table 3: Experimental range and levels of independent processvariables tested in Central Composite Design for biosorption of Reactive Orange 13 by IFB

Designation	**Variable**	**Variable values**				
		-1.633	**-1**	**0**	**1**	**1.633**
X_1	pH	1.36	2.0	3.0	4.0	4.63
X_4	Speed of agitation (rpm)	109	113	120	127	131
X_5	Contact time (min)	63	75	94	113	125

Table 4: Experimental range and levels of independent processvariables tested in CentralComposite Design for biosorption of Reactive Blue 222 by IFB

Designation	**Variable**	**Variable values**				
		-2	**-1**	**0**	**1**	**2**
X_1	pH	1.0	2.0	3.0	4.0	5.0
X_2	Concentration of dye (mg/L)	35	40	45	50	55
X_3	Temperature (^{0}C)	27	30	33	36	39

X_5	Contact time (min)	56	75	94	113	132

The factors were coded according to the following Eq. (3):

$$x_i = \frac{X_i - X_o}{\Delta X_i}, i = 1, 2, \ldots, k \quad (3)$$

Where, $x_i$is the coded independent factor, $X_i$is the real independent factor; X_0 is the value of X_i at the centre point; ΔX is the step change value.

The second-order model used to fit the response to the independent variables is shown in Eq. (4):

$$Y = \beta_0 + \Sigma\beta_i x_i + \Sigma\beta_{ii} x_i^2 + \Sigma\beta_{ij} x_i x_j, \text{ i= 1, 2, 3, . . . ,}k \quad (4)$$

Where, Y is the predicted response, β_0 is the constant coefficient, x_i and x_j are the coded values of the independent factors, β_i is the linear coefficient, $\beta_{ii}$is the quadratic coefficient and β_{ij} is the interaction coefficient (Guo et al. 2012; Yazdani et al. 2014). The quality of the polynomial equation was judged by determination coefficient (R^2), and its statistical significance was checked by Fischer's F-test. Analysis of variance (ANOVA) was conducted to determine the significance of the model. The response surface plots of the model-predicted responses were utilized to assess the interactive relationships between the significant variables. The results obtained from the statistical analyses of CCD were verified by validation tests, using the predicted optimized conditions against the basal conditions. Minitab 16 (State College, PA, USA) and Design Expert Version 6.0.8 (Stat-Ease Inc., Minneapolis, USA) software were used for experimental design, construction of quadratic models and graphical analysis of the experimental data.

4. Results and discussion

4.1 Screening of parameters using PBD

PBD is an efficient two-level screening design, requiring fewer runs than a comparable fractional design and can be used to spot the more important independent variables from a list of candidate factors. It allows unbiased estimation of main effects with least possible variance of components. The PBD is based on the first-order model, with no interaction among the factors (Myers and Montgomery2002).The data represented in Table 2 shows a wide range of sorption values i.e. from 13.89 to 65.79 %for RO 13 and from 11.09 to 61.23% for RB 222 in 12 run trials. Regression analysis was performed on the results and the first order polynomial equations were derived (Eq.5):

$$Y_{RO\ 13} = 29.17 + (-15.46*X_1) + (-2.29X_2) + (-3.21X_3) + (6.08*X_4) + (7.05X_5) \quad (5)$$

$$Y_{RB\ 222} = 33.97 + (-8.43*X_1) + (-4.03X_2) + (-5.36X_3) + (0.03*X_4) + (7.42X_5) \quad (6)$$

Regression analyses of Plackett-Burman design for both RO 13 and RB 222 areshown in Table 5 and 6.

Table 5: Linear multiple regression analysis of Plackett-Burman design for biosorption of RO13

Term	Effect	Coefficient	Standard error	*t* Statistics	*P*-value
Constant		29.17	0.1909	152.78	0.000
Block		-0.02	0.1708	-0.14	0.893
pH	-30.92	-15.46	0.1909	-80.97	0.000
Concentration of dye	-4.58	-2.29	0.1909	-12.00	0.000
Temperature (^oC)	-6.43	-3.21	0.1909	16.83	0.000
Speed of agitation	12.16	6.08	0.1909	31.84	0.000
Contact time	14.10	7.05	0.1909	36.93	0.000

RO 13: R^2 = 0.9876, Pred-R^2= 0.9849, Adj-R^2 = 0.976

Table 6:Linear multiple regression analysis of Plackett-Burman design for biosorption of RB 222

Term	Effect	Coefficient	Standard error	*t* Statistics	*P*-value
Constant		33.965	0.2343	144.98	0.000
Block		-0.201	0.2095	-0.96	0.348
pH	-16.861	-8.43	0.2343	-35.99	0.000
Concentration of dye	-8.049	-4.025	0.2343	-17.18	0.000
Temperature (^oC)	-10.714	-5.357	0.2343	22.87	0.000
Speed of agitation	0.552	0.0276	0.2343	1.18	0.251
Contact time	14.846	7.423	0.2343	31.69	0.000

RB 222: R^2 = 0.9866, Pred-R^2= 0.9843, Adj-R^2= 0.9838

It was seen that pH (X_1), concentration of dye (X_2) and temperature (X_3) had a negative effect on the process whereas speed of agitation (X_4) and contact time (X_5) had a positive effect on the biosorption of both RO 13 and RB 222 by IFB, (Table 5 and 6).The corresponding probability values (*P*) indicate the importance of each of the coefficients. The smaller the value of *P*, the more significant is the corresponding coefficient. Table 5 showed that all the parameters were significant (P<0.001) in increasing the sorption of RO 13 whereas in case of RB 222 speed of agitation played a very insignificant role.R^2values of 0.9876 and 0.9866 for RO 13 and RB 222 respectively, indicated that 98.76 % and 98.66% of the total variability in the response could be explained using these models. An analysis of the effects of variables on biosorption was carried out using the main effects plots of all the variables used in this work to plot data means and also to compare magnitudes of marginal means as shown in Fig 2.

Fig.2. Main effects plot of parameters for biosorption of RO 13 by *R. arrhizus* using Plackett - Burman Design

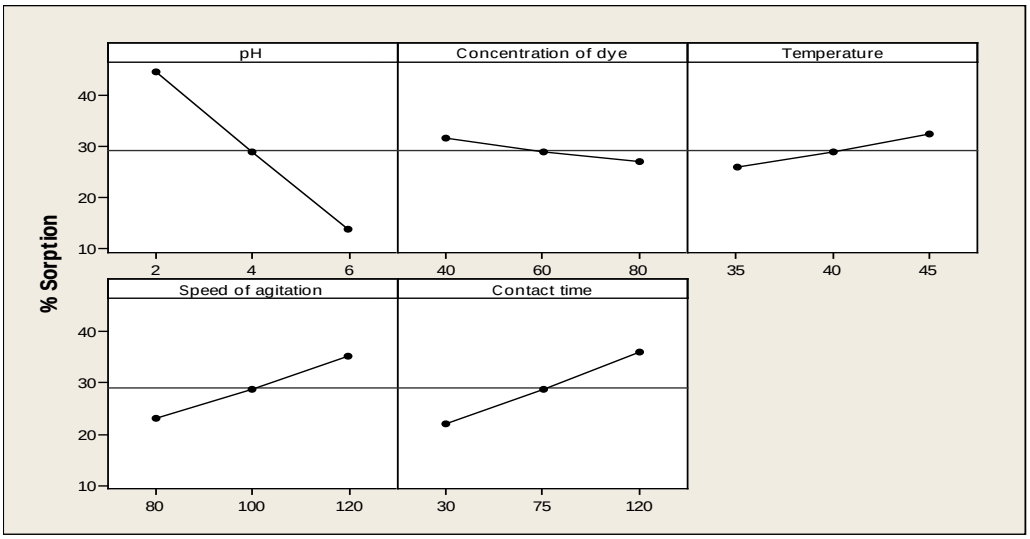
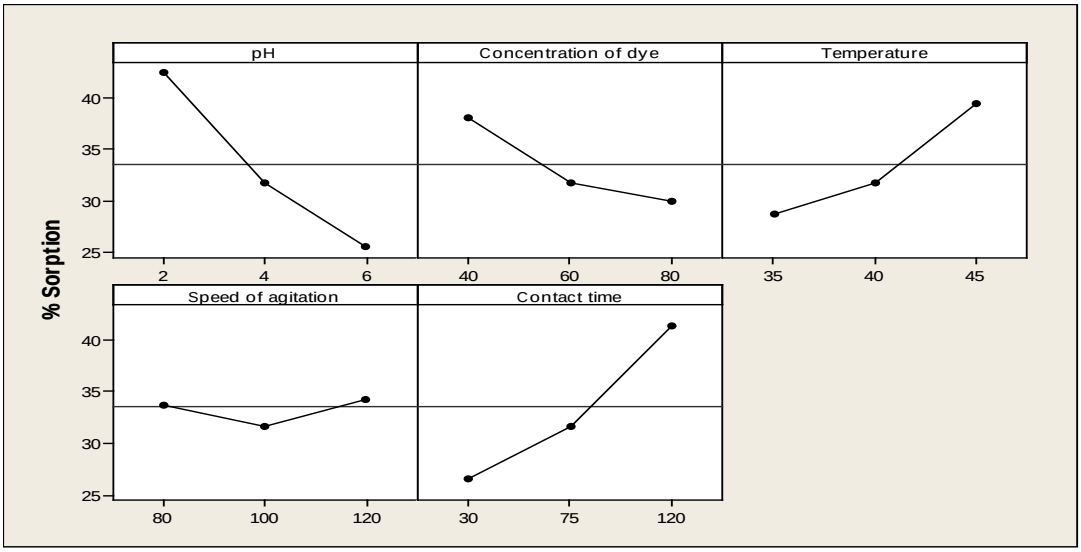


Fig.3. Main effects plot of parameters for biosorption of RB 222 by *R. arrhizus* using Plackett - Burman Design



Main effect is the effect of an independent variable on the dependent variable averaging across the levels of any other independent variables. In the main effects plot, a horizontal line (parallel to the X-axis), represents no main effect, while a non-horizontal line indicates a main effect. The magnitude of the main effect is indicated by the steepness of the slope of the line.Analysis of the main effects plot showed that the pH of the solution was the most influential factor in the biosorption of both RO 13 and RB 222 exerting a negative effect on biosorption. A decrease of 68.29 % and 39.76 % in percent biosorption was seen when pH was increased from -1 level to +1 level for RO 13 and RB 222 respectively. The effect of pH can be

attributed to the change in the surface charge as well as the dissociation of functional groups on the active sites of the biosorbent and the degree of ionization of the dyes present in the solution (Bayramoglu and Arica 2006; Aksu and Balibek 2010; Mezenner et al. 2013). Reactive dyes release colored dye anions in the solution. More protons are available to protonate the amino groups when the pH of the solution decreases. These positively charged amine groups provide cationic anchor points with which the anionic sulphonic acid groups ($-SO_3^-$) of the dyes bind by electrostatic interactions (Suteu and Bilba 2005). As the pH of the system is decreased, the number of positively charged adsorption sites increase and the number of negatively charged surface sites decrease. At very low pH value, the surface of the biosorbent would also be surrounded by the hydronium ions which enhance the dye interaction with the binding sites of the biosorbent by greater attractive forces. This chemical affinity between the positive charge on the biosorbent and negative charges in the structures of RO 13 and RB 222 weaken the resistance of the boundary layer surrounding the biosorbent leading to more sorption under acidic conditions (Donmez and Aksu 2002; Crini and Badot2008; Sun et al. 2013; Saraf and Vaidya 2015). The deprotonation of surface groups at high pH results in the electrostatic repulsion between the anionic dyes and negatively charged sites contributing to the decreased uptake of the dyes (Wong et al. 2009).

The concentration of the dye seemed to have a negative effect on the biosorption of RO 13 and RB 222 showing a decrease in the mean response from 31.46% to 26.88 % for RO 13 and from 37.99 to 29.94 % for RB 222 with increase in the concentration of the dyes.This may be attributed to the increase of dye molecules onto the external surface of the alginate beads, significantly increasing the local dye concentration, thereby giving rise to the formation of aggregates of the dye on the alginate beads (El-Latif et al. 2010). Accumulation of dye ions at higher dye concentration decreased the availability of the total surface area of the biosorbent particles for biosorption (Gong et al.2005). This probably led to the saturation of the adsorption sites and increase in the number of ions competing for the available binding sites on the biosorbent for binding of RO 13 and RB 222 at higher dye concentration (Bai and Abraham 2003).Chu et al. (2009) showed similar results for biosorption of Lanaset Red 2G by alginate immobilized *Chlorella vulgaris*.

Temperature also exerted a negative effect on the percent biosorption showing a decrease of 19.83% and 27.24% in biosorption of RO13 and RB 222 with its increase. An increase in temperature causes an increase in Brownian movement of the molecules in solution. Thisin turn causes their departure from the solid surface to liquid surface, thereby causing a decrease in percent sorption. High temperature may also lead to the breaking of existing intermolecular hydrogen bonding between the dyes and the biosorbent surface(Bhattacharyya and Sharma 200). Similar trend was shown by Ergene et al. (2009) in sorption of Remazol Brilliant Blue R dye from aqueous solution onto the immobilized *Scenedesmus quadricauda*.

Speed of agitation showed a positive effect on biosorption of RO 13 showing an increase in mean percent biosorption values from 23.095 from -1 level to 35.25 % at +1 level. This can be attributed to the fact that agitation facilitates proper contact between the dye ions in solution and the biomass binding sites.

It is reported that, external film diffusion can impact the rate of a biosorption process. With proper agitation, this mass transfer resistance thickness and the liquid boundary layer around the adsorbent particles are minimized (Bai and Abraham 2001; Ahalya et al. 2005; Al-Qodah, 2006). The diffusion rate of a solute from the bulk liquid to the liquid boundary layer surrounding particles becomes higher due to the enhanced turbulence thereby promoting effective transfer of sorbate ions to the sorbent sites (Evans et al. 2002; Shen and Duvnjak 2005).The effect of contact time on the removal of RO 13 and RB 222 indicated that a longer contact time favoured the reaction towards the equilibrium between the dyes and the immobilized biosorbent. This may be due to the availability of the uncovered surface area on the biosorbent and the progressive occupation of active binding sites over a longer period of time (Saraf and Vaidya 2015).

The highest responses of percent sorption of 65.79% for RO 13and 61.23% for RB 222 were obtained, after running Plackett-Burman experiments, under following conditions: pH 2.0; concentration of dye 40 mg/L; temperature 35^{o}C; speed of agitation 120 rpm and contact time 120min.

4.2 Central Composite Design

Once the ranges of the relevant variables were selected through the Plackett-Burman design, RSM consisting of trials plus a star configuration was used to appraise quadratic model and central points to estimate the pure process variability and reassess gross curvature. To determine the optimum levels of the factors and to study their interactions, specified combinations of threeindependent significant factors i.e. pH (X_1), speed of agitation (X_4) and contact time (X_5) for RO 13 at five levels (- 1.633, -1, 0, +1, +1.633) and four factors viz. pH (X_1), concentration of dye (X_2), temperature (X_3) and contact time (X_5) for RB 222 at five levels (-2,-1,0,+1,+2)were used in CCD experiments. The design matrix of tested variables in 20 experimental runs for RO13 and 30 runs for RB 222 along with the experimental results and the results of theoretically predicted responses (using the model equation) are shown in Tables7 and 8.

Table7: Central composite design matrix with experimental and predicted values for the biosorption of RO 13 by IFB

Run No.	Coded values			Biosorption(%)	
	X_1	X_4	X_5	Experimental	Predicted
1	1.633	0	0	33.77	32.54
2	0	1.633	0	34.77	34.11
3	0	0	0	42.09	42.66
4	0	-1.633	0	50.97	51.74
5	0	0	1.633	58.38	59.71
6	0	0	0	43.56	42.66
7	0	0	-1.633	29.09	27.87
8	-1.633	0	0	48.89	50.22
9	1	-1	1	46.98	48.73
10	1	1	1	36.98	35.16

11	-1	-1	1	68.02	67.58
12	1	-1	-1	37.98	36.62
13	-1	-1	-1	35.54	37.28
14	0	0	0	40.02	41.37
15	-1	1	1	54.87	56.16
16	0	0	0	41.06	41.37
17	-1	1	-1	31.09	29.27
18	1	1	-1	20.09	23.46
19	0	0	0	42.13	41.37
20	0	0	0	41.97	41.37

Table 8: Central composite design matrix with experimental and predicted values for the biosorption of RB 222 by IFB

Run No.	**Coded values**				**Biosorption (%)**	
	X_1	X_2	X_3	X_5	**Experimental**	**Predicted**
1	-1	-1	1	1	62.09	59.65
2	-1	-1	-1	1	30.18	41.06
3	1	1	1	1	25.90	23.99
4	-1	1	1	-1	32.63	32.22
5	-1	-1	-1	-1	36.50	36.32
6	1	1	-1	1	25.15	24.78
7	1	-1	1	1	28.43	29.62
8	1	-1	1	-1	25.01	23.75
9	1	1	1	-1	20.67	19.66
10	1	-1	-1	1	22.12	20.44
11	-1	1	-1	-1	32.70	32.38
12	-1	-1	1	-1	44.89	46.13
13	-1	1	-1	1	37.42	36.58
14	1	-1	-1	-1	20.09	20.34
15	-1	1	1	1	41.56	42.19
16	1	1	-1	-1	23.89	24.23
17	0	-2	0	0	24.70	25.59
18	2	0	0	0	21.20	21.81
19	0	0	2	0	20.89	21.26
20	0	0	0	2	26.80	27.95
21	0	2	0	0	15.70	16.03
22	0	0	0	-2	19.82	18.88

23	0	0	-2	0	15.39	14.24
24	-2	0	0	0	55.39	55.99
25	0	0	0	0	23.09	22.44
26	0	0	0	0	24.34	25.00
27	0	0	0	0	25.09	25.50
28	0	0	0	0	24.89	25.05
29	0	0	0	0	24.79	25.00
30	0	0	0	0	24.67	25.04

It can be seen that percent sorption of RO 13increased from 65.79% to 68.02% under the following conditions: pH 2.0; concentration of dye 40 mg/L; temperature 35^0C; speed of agitation 113rpm and contact time 113min.For RB 222,percent sorption increased from 61.23% to 62.09% under following conditions: pH 2.0; concentration of dye 40 mg/L; temperature 36^0C; speed of agitation 120rpm and contact time 113min.The data were analyzed using multiple regression analysis in order to obtain empirical models for the best responses and to derive second-order polynomial equations (Eq. 7 and 8) as follows:

$$Y_{RO13}=42.01+(-5.41*X_1)+(-5.40*X_4)+(9.75*X_5)+X_1*(X_1*-0.48)+X_4*(X_4*0.09)+X_5*(X_5*0.42)+X_1*(X_4*-1.28)+X_1*(X_5*-3.79)+X_5*(X_4*-0.10) \quad (7)$$

$$Y_{RB222}=22.72+(-8.54*X_1)+(-2.39*X_2)+(2.25*X_3)+(2.51*X_5)+X_1*(X_1*4.86)+X_2(X_2*0.34)+X_3*(X_3*-0.17)+X_5*(X_5*1.12)+X_1*(X_2*2.45)+X_1*(X_3*-1.60)+X_1*(X_5*-1.41)+X_3*(X_2*-2.49)+X_5*(X_2*-0.38)+X_5*(X_3*1.44) \quad (8)$$

RSM gives an insight into the quadratic and interaction effects of the parameters apart from the linear effect of the factors on the dye biosorption. The analyses were performed by using Fisher's *F* test and Student's *t* test. The regression coefficient, the *F* and *P* values for all the linear, quadratic, and interaction effects of the factors used in the study are shown in Tables9 and 10.

Table 9: Estimated regression coefficients and corresponding *t* and *P* values of the CCD for the biosorption of RO 13 by IFB

Term	Coefficient	SE Coefficient	*t* -Statistics	*P*-value
Constant	42.015	0.974	43.137	0.000
Block	-0.6479	0.5432	-1.193	0.263
pH (X_1)	-5.4136	0.6518	-8.305	0.000
Speed of agitation (X_4)	-5.3958	0.6518	-8.278	0.000
Contact time (X_5)	9.7485	0.6518	14.956	0.000
pH*pH	-0.4796	0.6549	-0.732	0.483

Speed of agitation*Speed of agitation	0.0979	0.6549	0.15	0.884
Contact time* Contact time	0.4223	0.6549	0.645	0.535
pH*Speed of agitation	-1.2863	0.8415	-1.529	0.161
pH*Contact time	-3.7962	0.8415	-4.511	0.001
Contact time*Speed of agitation	-0.1012	0.8415	-0.12	0.907

Table 10: Estimated regression coefficients and corresponding *t* and *P* values of the CCD for the biosorption of RB 222 dye by IFB

Term	Coefficient	SE Coefficient	*t* -Statistics	*P*-value
Constant	22.7197	0.9726	23.361	0.000
Block	3.276	0.4557	7.189	0.000
pH (X_1)	-8.5454	0.4803	-17.791	0.000
Concentration of dye (X_2)	-2.3912	0.4803	-4.979	0.000
Temperature (X_3)	2.2554	0.4803	4.696	0.000
Contact time (X_5)	2.5179	0.4803	5.242	0.000
pH*pH	4.8651	0.4493	10.828	0.000
Concentration of dye* Concentration of dye	0.3414	0.4493	0.76	0.460
Temperature*Temperature	-0.1736	0.4493	-0.386	0.705
Contact time* Contact time	1.1189	0.4493	2.49	0.026
pH*Concentration of dye	2.4569	0.5883	4.176	0.001
pH*Temperature	-1.6006	0.5883	-2.721	0.017
pH*Contact time	-1.4119	0.5883	-2.3	0.031
Temperature*Concentration of dye	-2.4956	0.5883	-4.242	0.001
Contact time*Concentration of dye	-0.3869	0.5883	-0.658	0.521
Contact time*Temperature	1.4431	0.5883	2.453	0.028

The coefficients for all the linear and interaction effects for pH and contact time were highly significant for the biosorption of RO 13. On the other hand, the coefficients for all the linear, quadratic [except temperature (P =0.705) and concentration of dye (P= 0.460)]and the interaction effects [except contact time and concentration of dye (P = 0.521)]were significant for biosorption of RB 222. Accuracy and general ability of the polynomial models were good as suggested by $P< 0.05$. The analyses of the response trends using the models were reasonable. In order to ensure the statistical significance of the quadratic model employed for explaining the experimental data at a 95% confidence level the model was tested by analysis of variance (ANOVA) results (Sener et al., 2014) (Table 11 and 12).

Table 11:ANOVA for response surface quadratic model for biosorption of RO 13 by IFB

Source	DF	Seq SS	Adj SS	Adj MS	*F*	*P*
Blocks	1	8.06	8.06	8.06	1.42	0.263
Regression	9	2180.62	2180.62	242.292	42.77	0
Linear	3	2046.07	2046.07	682.024	120.4	0
Square	3	5.94	5.94	1.981	0.35	0.791
Interaction	3	128.61	128.61	42.87	7.57	0.008
Residual error	9	50.98	50.98	5.665		
Lack-of-fit	5	47.07	47.07	9.414	9.62	0.024
Pure error	4	3.91	3.91	0.979		
Total	19	2239.67				

DF: Degree of freedom; SS: Sum of squares; MS: Mean sum of square.

R^2=0.9727; Pred-R^2=0.9634; Adj-R^2=0.9519; CV= 5.20; Adequate precision = 29.822

Table 12:ANOVA for response surface quadratic model for biosorptionof RB 222 by IFB

Source	DF	Seq SS	Adj SS	Adj MS	*F*	*P*
Blocks	1	286.19	286.19	286.191	51.69	0.000
Regression	14	3149.96	3149.96	224.997	30.64	0.000
Linear	4	2164.06	2164.06	541.014	97.71	0.000
Square	4	681.07	681.07	170.268	30.75	0.000
Interaction	6	304.83	304.83	50.805	9.18	0.000
Residual error	14	77.52	77.52	5.537		
Lack-of-fit	10	74.29	74.29	7.429	9.2	0.023
Pure error	4	3.23	3.23	0.808		
Total	29	3513.67				

DF: Degree of freedom; SS: Sum of squares; MS: Mean sum of square.

R_2=0.9779 Pred-R^2 = 0.9620; Adj-R^2 = 0.9543; CV=8.19; Adequate precision=25.264

The low *P*-value of the Fisher's *F*-test (F_{model}, 42.77 for RO13 and 30.64 for RB 222) [(P_{model}>*F*) = 0.000]demonstrated that the models were highly significant. The lower calculated $F_{9,9}$- value (17.99)for RO 13 and $F_{14,14}$-value (8.99) for RB 222 than their respective tabulated *F*-values even at the 0.0001 confidence level showed a statistically insignificant lack of fit. The models were found to be adequate for prediction within the range of variables employed. The values of R^2 and *Adj*-$R^2$are very high and advocate a high correlation between the observed values and the predicted values. The Pred-R^2 of 0.9634for RO 13 and 0.9620 for RB 222 was in reasonable agreement with Adj-R^2 in both the cases (0.9519 for RO 13 and 0.9543 for RB 222), indicating that the models are significant. The adjusted coefficient of determination represents the proportion of the variation in the response explained by the regression model. It is thus envisaged that Eq. 7 and 8 can capture <96.34% and 96.20%of the variation in the measured values of

percentsorption as function of the three independent variables for RO 13 and four for RB 222 within the ranges considered in the present study. The values of CV 5.20 and 8.19 for RO 13 and RB 222 respectively, demonstrated that the performed experiments were reliable. "Adeq Precision" of 29.822 for RO 13 and 25.264 for RB 222 obtained in this study indicated an adequate signal. Therefore, these models can be used to navigate the design space. The ANOVA thus indicated that the second-order polynomial models were highly significant and adequate to represent the actual relationship between the response (% sorption) and variables, with $P< 0.0001$ and high values of the coefficient of determination.

In order to gain a better understanding of the influence ofthe independent variables and their interactions on the dependent variables, 3D response surface plots for the measured responseswere formed based on the model equations for the removal of RO13 and RB 222 (Fig. 4 and 5)(Sener et al. 2014). A surface plot can be used to explore the relationship between three variables. Here, each response surface plot represented the effect of two independent variables varying in the factorial part of the experimental space (between -1 and +1),with the other independent variable set at its central level. These surface plots provide a method to predict the biosorption efficiency for different values of the tested variables.

Fig.4: Three-dimensional response surface plot of biosorption of RO 13 by IFBshowing interaction of pH and contact time

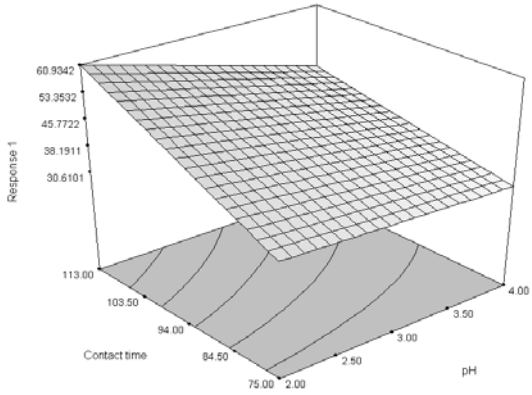
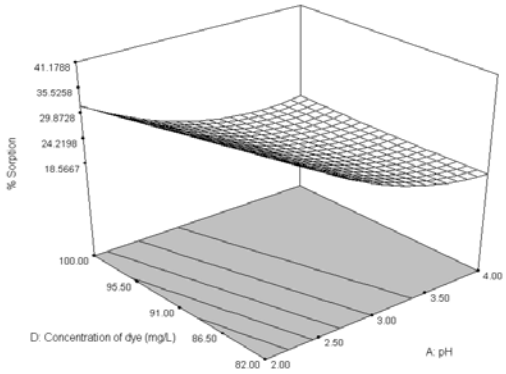
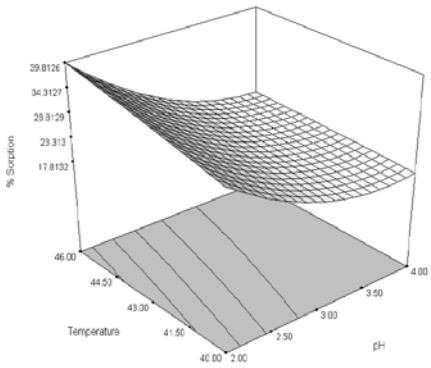


Fig.5: Three-dimensional response surface plots of biosorption of RB 222 by IFBshowing variable interactions of (a) pH and concentration of dye (b) pH and temperature (c) pH and contact time (d) Contact time and temperature (e) Temperature and concentration of dye

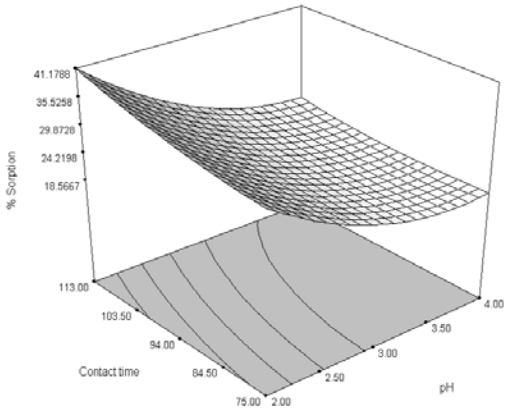
a)pH and concentration of dye	b) pH and temperature

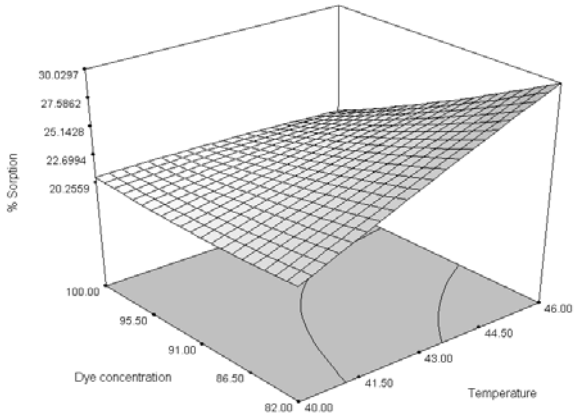




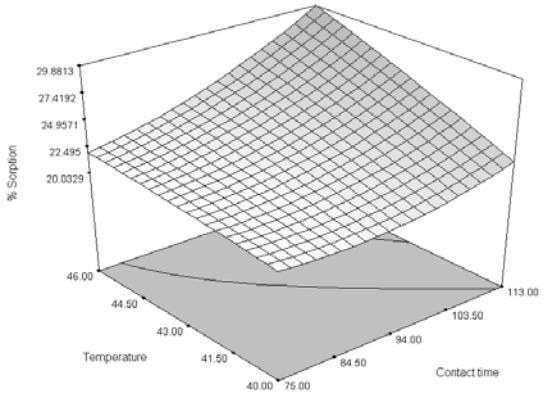
c) Contact time and pH

d) Contact time and temperature





e) Temperature and concentration of dye



As seen in Figs. 4 and 5, each response surface yield showed a clear peak suggesting that the optimum point was inside the design boundary. Examination of the response surface plot for biosorption of RO 13 based on independent variables i.e. pH and contact time, with the other independent variable concentration of dye kept at zero level is shown in Fig. 4. The figure revealed an interaction behavior with a negative main effect of pH and positive main effect of contact time. This indicated that when the pH decreased from 3.0 to 2.0 and contact time increased from 94 min to 113 min, there was an increase in percent biosorption of RO 13. The profile shown in Fig.5 (a-e) for biosorption of RB 222 revealed that pH

proved to be the most important factor and was involved in a three way interaction with concentration of RB 222, temperature and contact time. Highest percent biosorption was observed withthe decrease in pH from 3.0 to 2.0, decrease in concentration of dye from 45 to 40 mg/L and increase in contact time from94 to 113 min and increase in temperature from 33^{o}C to 36^{o}C.The two-dimensional contour plots showed clearly elongated lines running diagonally on plot, suggesting that the interactions shown in Fig 5 (a-e)were significant on percent biosorption of RB 222.

4.3 Validation of models

To assess the prediction capability of the models developed, several validation experiments were carried out in the region of experimentation delimitated by the factorial points (x_i varying between -1 and +1)(Zuorro et al. 2013). The special feature of the RSM tool, 'Point prediction' was employed to find optimum value of the combination of the four factors for the maximum biosorption. The observed values of % sorption (68.02% for RO 13 and 62.09% for RB 222) were in good agreement with the predicted values (65.15% and 60.35%, respectively) and fell into the 95%prediction intervals, further validating the model presented above. The predicted optimal conditions for RO 13 were as follows: pH 2.0, contact time 113 min and speed of agitation 113 rpm; and for RB 222 pH 2.0, contact time 113 min, concentration of dye 42 mg/L and temperature 36^{0}C. Thus, the sequential optimization strategy could be successfully employed to increase biosorption of RO 13 and RB 222.

5. Conclusion

In the present study, alginate immobilized fungal biosorbent *R. arrhizus* was used successfully for the sorption of two reactive dyes i.e. RO13 and RB 222 from aqueous solutions using sequential statistical optimization strategy. Plackett-Burman analysis employed for rapid screening and evaluation of critical variables affecting biosorption identified pH, speed of agitation and contact time as the most significant factors for biosorption of RO 13. On the other hand, pH, concentration of RB 222, temperature and contact time proved to be the significant factors affecting biosorption of RB 222. PBD allowed unbiased estimation of main effects with smallest possible variance of components. The RSM approach allowed estimation of the influence of the main process variables on biosorption of RO 13 and RB 222 dyes and provided useful indications on the optimal set of operating conditions to be used. Optimum conditions for biosorption of RO 13 (68.02%): pH 2.0; concentration of dye 40 mg/L; temperature 35^{0}C; speed of agitation 113rpm and contact time 113min. For RB 222 (62.09%) optimized conditions were:pH 2.0; concentration of dye 42 mg/L; temperature 36^{0}C; speed of agitation 120 rpm and contact time 113 min. The excellent correlation between predicted and experimental values confirmed the validity and practicability of this statistical optimum strategy. Thus, the use of RSM approach in combination with a mechanistic model can be a useful tool for a better analysis of dye biosorption data and a more effective design of experiments. The study indicated that the immobilized fungal biosorbent is an effective and economical alternative for the removal of both the dyes from the aqueous solutions.

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