Enhanced Decolorization of Reactive black 5 by Laccase-Natural Redox Mediator System using Response Surface Methodology

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Summary: Response surface methodology (RSM) was applied for the decolourization of the azo dye Reactive black 5 (RB-5) using commercial laccase from a white rot fungus *Trametes versicolor*. It was observed that the presence of syringaldehyde is essential for decolourization of RB-5 because laccase alone did not decolorize the dye. Syringaldehyde (SYD) was found to be an effective natural redox mediator. The effect and interaction of dye, mediator, and enzyme concentration on RB-5 decolorization was evaluated by Response Surface Methodology using Box-Behnken design. Seventeen experiments were conducted as designed by the above design and a quadratic model was obtained for dye decolour-ization through this design. The experimental values were in good agreement with predicted values and the model was highly significant, the correlation coefficient being 0.994. SYD showed main effect on RB-5 decolorization whereas enzyme had low effect. The optimum concentration of dye, enzyme and SYD were found to be 84 μM, 53 mg/L and 150.3 μM, respectively for maximum decolourization (92 %) of the dye. The validation experiment also showed good correlation between experimental and predicted responses.

Keywords: Kinetics; Reactive black 5; Decolori-zation; Laccase; Modeling.

Introduction

The presence of dyes in effluents is a major concern due to their adverse effect to many forms of life. Colored waters are also objectionable on aesthetic grounds for drinking and other municipal and agricultural purposes [1]. Industries such as textile, leather, paper, plastics, etc., are some of the sources for dye effluents. The treatment of aqueous water containing soluble dyes thus requires complete removal followed by secure disposal [2]. The most commonly used techniques for colour removal include chemical precipitation, ion exchange, reverse osmosis, ozonation and solvent extraction, etc. However, these techniques have certain disadvantages such as high capital cost and operational costs or secondary sludge disposal problem, time-consuming and methodologically demanding [3]. Reactive dyes are typically azo-based chromophores combined with different types of reactive groups, e.g., vinyl sulfone, chlorotriazine, trichloropyrimidine and difluorochloropyrimidine [4]. They have poor fixation rates and hence may be hard to remove from wastewaters because of their low biodegradability and their weak absorption into activated sludge [5]. In recent years, biological decolourization method has been considered as an alternative and eco-friendly method to dye degradation and colour removal [6-10].White rot fungi are a heterogeneous group of organisms but have in common the capacity to degrade lignin as well as other wood components and wide variety of recalcitrant compounds including synthetic dyes [8,11-14]. The dye degrading ability of the white rot fungi is due to their ligninolytic enzyme system consisting of lignin peroxidase, manganese dependent peroxidases, and laccases [15-17] as well as H_2O_2 producing oxidases [18]. A number of white rot fungi have been explored for decolourization of various industrial dyes and treatment of dye effluent [7, 8, 10, 12, 19]. Majority of these studies were conducted with fungal mycelia. One of the major disadvantages of using fungal cultures to decolourization is the accumulation of biomass, which would cost the wastewater treatment in industrial scale. Therefore, innovative treatment technologies need to be investigated. To overcome this disadvantage the application of isolated enzymes for dye decolourization has increased in recent years [17, 20-22]. Decolourization of dye wastewater by the action of the enzyme laccase is the subject of many studies [23–25]. Laccase-based decolourization treatments are potentially advantageous to bioremediation technologies since the enzyme is produced in larger amounts. Laccase (p-diphenol oxidase, EC1.10.3.2) catalyzes the oxidation of phenolic compounds and aromatic amines and accepts a broad range of substrates [26-28]. The number of substrates can further be extended by using laccase in combination with mediators [24]. Laccase requires only molecular oxygen as a co-substrate which is concomitantly

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reduced to water. This makes it very interesting for use in enzyme-based bioreactors [29, 30].The present study aims to determine the ability of commercial laccase in the decolourization of reactive dye. However, although a large number of structurally diverse dyes have been successfully oxidized by laccases, decolorization take place at different rates and to different extents and many dyes are not degraded at all [29]. Generally, initial enzyme, dye and mediator concentration are the most important parameters that significantly influence the enzymatic degradation process. Since the conventional method of optimization, "one factor at a time" approach is laborious, time-consuming and incomplete, response surface methodology (RSM) was applied to model the decolourization process, to identify possible interactions and to determine the optimum operational conditions. RSM is an advanced tool, commonly applied nowadays as it involves three factorial designs giving number of input (independent) factors and their corresponding relationship between one or more measured dependent responses [31]. RSM has been extensively studied in biotechnology namely for optimization of medium composition [32, 33], fermentations [34, 35], catalyzed reaction conditions [36], oxidation [37], production [35] and food processes [38]. However, few reports have been presented for dye degradation optimization by enzymatic catalysis with RSM. The most common and efficient design used in response surface modeling is Box–Behnken design. It has three levels per factor, but avoids the corners of the space, and fills in the combinations of centre and extreme levels in which the optimal conditions for an experiment are found [39, 40].

To our knowledge there has been no study for the optimization of parameters in enzymatic dye decolourization using RSM. The aim of this study was to optimize the concentrations of dye, enzyme, and SYD in order to obtain best possible results in reactive azo dye decolourization by commercial laccase from *Trametes versicolor*. In this study, we selected reactive black 5 (RB-5) (Fig. 1), a widely used reactive di-azo textile dye, as a model dye.

Results and Discussion

Reactive dye RB-5 is a widely used azo dye which is more recalcitrant to microbial biodegradation. In the present study, decolorization of RB-5 using commercial laccase from *Trametes versicolor* was evaluated in the presence of natural redox mediator syringaldehyde (SYD), and response surface methodology was employed for optimization of decolorization process. There was no decolourization occurred with laccase alone without redox mediator. It requires addition redox mediator compound for laccase mediated decolourization. Preliminary studies show that SYD was found to be very effective redox mediator than other synthetic mediators.

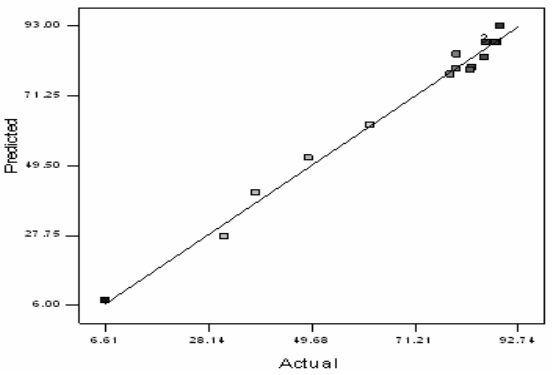

Fig. 1: Plot of actual versus predicted values.

Similar to this study, previous studies also show that without redox mediators no RB-5 decolorization was observed for other laccases [41]. The effectiveness of SYD as potential redox mediator has also been shown for different organic compounds including dyes, antimicrobials [42, 43]. For effective decolourization of redox mediator optimization is important. Thus, we optimized the experimental parameters, such as concentration enzymes, mediator and dyes using RSM.

RSM has been applied widely for optimization bioprocess for production of byproducts. RSM has also been used for enzymatic dye decolorization process [41, 44]. Box-Behnken design was chosen for optimization of three variables (dye, enzyme, and mediator). Table-1 shows the levels of process factors as designed by Box-Behnken model. The experimental response was analyzed by RSM to obtain an empirical model for the maximum response. The quadratic model was used to explain the mathematical relationship between the independent process variables and decolourization and obtained in terms of coded factors as;

$$\text{Decolourization} = 87.58 - 13.13\,A + 7.24\,B + 22.84\,C + 12.70\,AB + 14.13\,AC - 4.04\,BC - 10.47\,A^2 - 3.92\,B^2 - 2.032\,C^2 \quad (1)$$

Table-1: Process factors and their levels for response surface.

Factor	Variable	Unit	Level of actual and coded values		
			-1	0	+1
A	Dye	µM	25	112.5	200
B	Enzyme	mg/L	10	55	100
C	Mediator	µM	25	112.5	200

The experimental and predicted responses for RB-5 decolorization based on the quadratic model are shown in Table-2. The results of analysis of variance (ANOVA) for fitting second-order response surface model by a least squares method are shown in Table-3. The high F-ratio for model (F_{model}=135.94) with very small probability value ($p < 0.0001$) indicates that the model is statistically highly significant for optimizing RB-5 decolorization from three process variables. The high value of adjusted coefficient of determination (R^2_{Adj} = 98.70%) indicates that about 98 % of the variability in response variable has been explained by linear and quadratic terms of three process variables and only 2% explained by other uncontrollable factors. The significance of linear and quadratic terms was established by p-values for each term shown in Table-3. The smaller the p-value for a term the more significant is the term in the model. As p-values for all linear as well as quadratic terms are small ($p < 0.05$) so all terms made significant contribution to the fitted model. Furthermore, as p-values for all first order interaction effects of the three predictors are very small so interaction effects of three independent variables were also statically significant. Moreover, the validation experiments, their experimental and predicted response is shown in Table-4.

Table-2: The actual design of experiment, experimental and predicted responses for RB-5 decolorization.

Experiments	Variables			Response Decolorization (%)	
	Dye (µM)	Enzyme (mg/L)	Mediator (µM)	Experimental	Predicted
1	25	55	25	61.76	61.2
2	25	55	200	78.7	78.6
3	112.5	55	112.5	88.0	87.5
4	200	55	25	6.61	6.7
5	112.5	55	112.5	88	87.6
6	25	10	112.5	89.0	91.7
7	112.5	100	200	80.0	80.6
8	112.5	10	200	85.8	83.0
9	112.5	55	112.5	86.0	87.6
10	112.5	55	112.5	88.3	87.6
11	200	100	112.5	82.87	80
12	200	55	200	80.0	80.6
13	25	100	112.5	83.09	81.0
14	200	10	112.5	37.89	40.1
15	112.5	10	25	31.53	29.2
16	112.5	100	25	49.0	51.7
17	112.5	55	112.5	88	87.5

Fig. 1 shows that the predicted values of the response variable from the empirical model are in agreement with the observed values with a high value of correlation coefficient 0.985 between them.

Using RSM, the effect of three independent variables and their combined interaction on dye decolorization can be predicted. By keeping one variable at constant level, the interaction of other two variables at different levels can be explored through response surface plots. Fig. 2 shows the interaction between different concentrations of dyes and enzymes on RB-5 decolorization at constant SYD concentration (112.5 µM). RB-5 decolourization decreased with increasing concentration of dye. Particularly at low enzyme concentration (10 mg/L) and dye at 200 µM (mediator: dye ratio at 0.562), the decolorization was drastically decreased. However, this effect was diminished when increasing the enzyme concentration. The enzyme concentration had little effect on decolorization except at higher dye concentration.

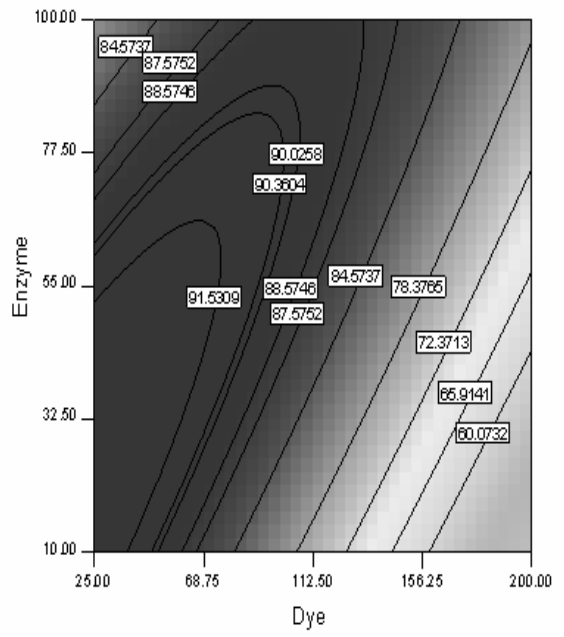

Fig. 2: Response surface plot showing the effect of different concentrations of Dye and Enzyme on RB-5 decolourization.

Fig. 3 shows the interaction between different concentration of dye and SYD on RB-5 decolourization at constant enzyme concentration (55 mg/L). This response plot clearly shows that both dye and mediator had influence on decolorization. At low dye and mediator (25 µM each; ratio1) decolorization was 61% which increased up to 91% while increasing the SYD concentration up to 146.5 µM (mediator: dye ratio up to 5.86) but about this level RB-5 decolorization decreased. At SYD: dye ratio 8, the decolorization was 78%. This might be due to interaction of SYD radicals with its own radicals instead of dye molecules. When mediator dye ratio decrease from 1 to 0.125 the dye decolorization decreased from 61 % to 7%.

Table-3: ANOVA for decolourization of RB-5 with quadratic model.

Source	Degree of freedom	Sum of squares	Mean square	F-value	p-value	
Model	**9**	**9916.84**	**1101.87**	**135.94**	**0.000**	**Significant**
A-Dye	**1**	**1379.44**	**1379.44**	**170.18**	**0.000**	
B-Enzyme	**1**	**419.49**	**419.49**	**51.75**	**0.000**	
C-Mediator	**1**	**4174.24**	**4174.24**	**514.97**	**0.000**	
AB	**1**	**645.41**	**645.41**	**79.62**	**0.000**	
AC	**1**	**798.06**	**798.06**	**98.46**	**0.000**	
BC	**1**	**65.12**	**65.12**	**8.03**	**0.025**	
A^2	**1**	**461.45**	**461.45**	**56.93**	**0.000**	
B^2	**1**	**64.66**	**64.66**	**7.98**	**0.026**	
C^2	**1**	**1738.75**	**1738.75**	**214.51**	**0.000**	
Residual	**7**	**56.74**	**8.11**			
Lack of fit	**3**	**53.13**	**17.71**	**19.63**	**0.007**	**Significant**
Pure error	**4**	**3.61**	**0.90**			
Total	**16**	**9973.58**				

SE= 2.85 ; $R^2 = 0.994$; $R^2_{Adj} = 0.987$; $R^2_{Pre} = 0.914$.

Table-4: Validation experiments: experimental and predicted responses for RB-5 decolorization.

Experiments	Variables			ResponseDecolorization (%)	
	Dye(µM)	Enzyme(mg/L)	Mediator(µM)	Experimental	Predicted
1	**25**	**55**	**112.5**	**86**	**90**
2	**112.5**	**55**	**112.5**	**89**	**88**
3	**200**	**100**	**200**	**93**	**87**
4	**25**	**30**	**25**	**61**	**61**
5	**75**	**50**	**100**	**87.5**	**88**
6	**100**	**60**	**100**	**87**	**86**
7	**120**	**100**	**200**	**90**	**89**
8	**25**	**70**	**40**	**88**	**68**
9	**60**	**40**	**60**	**87**	**75**
10	**25**	**10**	**25**	**77.3**	**59**
11	**200**	**10**	**25**	**16**	**0**
12	**25**	**55**	**25**	**84.43**	**61**
13	**150**	**25**	**70**	**86.1**	**50**
14	**50**	**25**	**30**	**84.6**	**58**

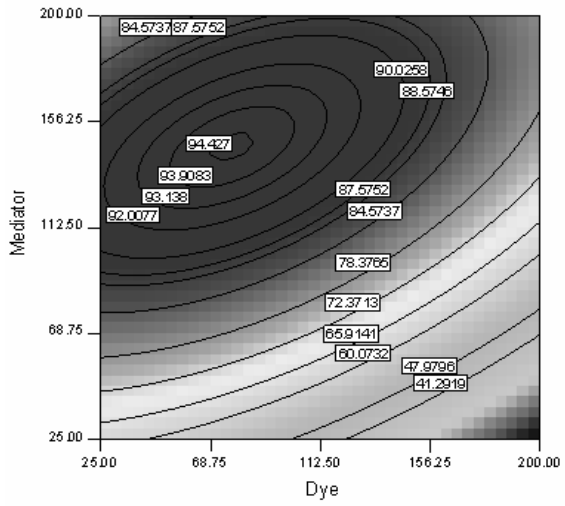

Fig. 3: Response surface plot showing the effect of different concentrations of Dye and Mediator on RB-5 decolourization.

The interaction between mediator and enzyme at dye concentration 112.5 µM is shown in response surface plot (Fig. 4). This result also clearly shows the effect of SYD on decolorization RB-5. At 112.5 µM RB-5 the mediator to dye ratio increases from 0.22 to 1.77 for 25 µM to 200 µM SYD, respectively. With 10 mg/L laccase, the decolorization was 29 % at mediator to dye ratio 0.22. The extent of decolorization continuously increased with increasing concentration of SYD. At mediator to dye ratio 1.77, maximum decolorization was 83 %. Similar to the redox mediator, the laccase also had effect on decolorization. When increasing the enzyme concentration from 10 to 100 mg/L the decolorization also increase. However, the enhancement was up to 15-20 % indicating that enzyme had little effect on decolorization.

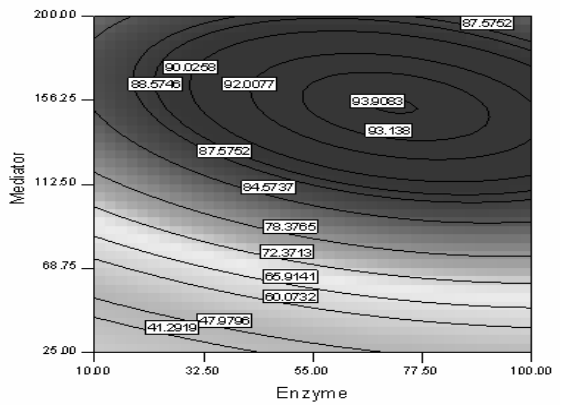

Fig. 4: Response surface plot showing the effect of different concentrations of Enzyme and Mediator on RB-5 decolourization.

Adequacy of the Model

Generally, it is important to confirm the fitted model to make sure that it gives sufficient approximation to the actual test. Unless the model shows a satisfactory fit, proceeding with investigation and optimization of the fitted response surface likely gives poor or misleading results. The residuals from the least squares fit play an important role in judging model adequacy. By constructing a normal probability plot of the residuals, a check was made for the normality assumption as shown in Fig. 5. The normality assumption was satisfied as the residual plot approximated along a straight line. Also, as only 1 out of the 17 values is outside the range ±2.0 on the x-axis, this satisfies the requirement that 95 % of the values are inside the range ±2.0 if the data is normally distributed. In Fig 6, the plot of studentized residuals versus the predicted values from the model suggests that residuals are of constant variance, as the points appear evenly scattered around zero without any systematic changes in spread. Finally independence of residuals is evident from non systematic pattern of residuals against the run order of observations in Fig 7.

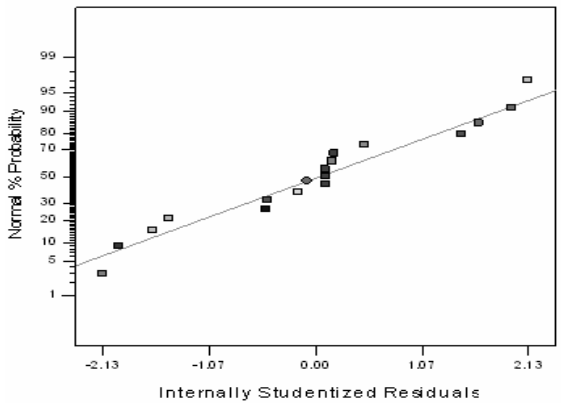

Fig. 5: Normal probability plot of Residuals.

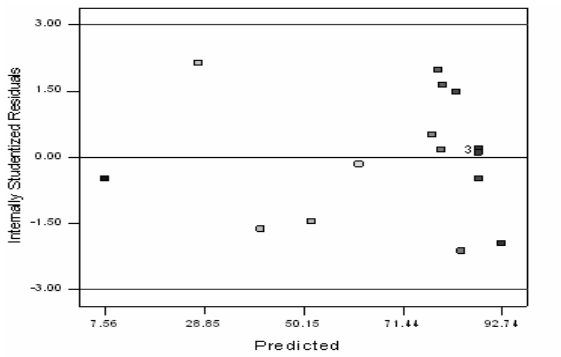

Fig. 6: Plot of Residuals versus Predicted values.

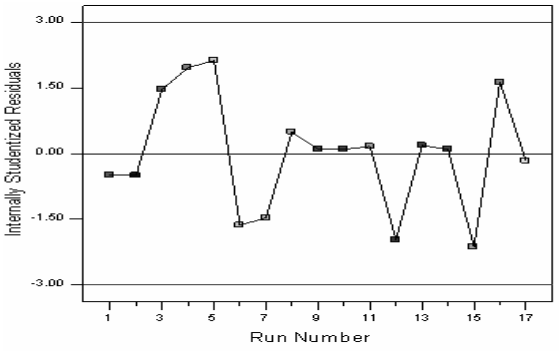

Fig. 7: Plot of Residuals versus Run order.

As the fitted model in Equation 1 provides a good approximation to the experimental conditions, the model was employed to find the values of the process variables for maximum decolourization of RB-5. The optimal values of the predictors corresponding to maximum decolourization are dye: 84 µM , enzyme : 53 mg/L and SYD: 150.3 µM. for which maximum decolourization was 92 %.

The adequacy of the tested model (Eq 1) for dye decolorization by commercial laccase was evaluated using the predicted conditions. Experiments were performed with different set of variables and evaluated the response. The experimental response had good agreement with the decolourization of RB-5 indicating that the model used is highly adequate for dye decolorization.

Experimental

Reactive dyes represent the dyes which are mostly used in the textile industries. The dyes used in this study reactive black 5 (RB-5) and syringaldehyde (SYD) were purchased from Sigma-Aldrich Co. USA. Laccase (from *T. versicolor* EC: 1.10.3.2) was obtained from Fluka chemicals, USA.

Response Surface Methodology

Response surface methodology is an empirical modeling technique used to evaluate the relationship between a set of controllable experimental factors and observed results. This optimization process involves three major steps: (i) performing statistically designed experiments, (ii) estimating the coefficients in a mathematical model, and (iii) predicting the response and checking the adequacy of the model [45]. A class of three level complete factorial design for the estimation of the parameters in a second-order model was developed by Box-Behnken [46]. In this study we selected Box-

Behnken design for the optimization of laccase mediated reactive dye decolourization. This design was applied using Design Expert 8.0.2 to our study with three variables at three levels. Three different concentrations of dye (25, 112.5, 200 μM), enzyme (10, 55, 100 U/ml), and SYD (25, 112.5, 200 μM) were chosen as the critical variables and designated as A, B, and C, respectively, as shown in Table 1. The three significant variables can be approximated by the quadratic model equation as follows:

$$Y = k_0 + k_aA + k_bB + kcC + k_{aa}A^2 + k_{bb}B^2 + k_{cc}C^2 + k_{ab}AB + k_{ac}AC + k_{bc}BC + Є$$

where Y is the predicted response; k_0 is a constant; k_a, k_b, k_c are the linear coefficients; k_{aa}, k_{bb}, k_{cc} are the quadratic coefficients; k_{ab}, k_{ac}, k_{bc} are the cross-product coefficients and Є is the random error . This design is preferred because a relatively few experimental combinations of the variables are adequate to estimate potentially complex response function. A total number of 17 experiments including 5 experiments replicated at the center of three study factors for estimation of lack of fit were necessarily carried out to estimate the 10 coefficients for response surface model. Data were analyzed using Design Expert 8.0.2 program including ANOVA to test the statistical significance of the fitted model and its goodness of fit by the coefficient of determination (R^2).

Dye Decolorization Experiment

Dye decolorization experiments were carried out in 2 ml Eppendorf tube. Reaction mixture (1 ml) containing 100 mM citrate-phosphate buffer (pH 4.0), purified laccase at various concentrations (10, 55, 100 mg/L), dye (25, 112.5, 200 μM), SYD (25, 112.5, 200 μM) was prepared as described in Table 1. The reaction tubes were incubated at 25° C under dark and the decolourization was monitored spectrophotometrically (Cary Win 300, Australia) after 1 h incubation by recording the absorbance at the λmax of the dye (596 nm). Before starting the actual designed experiments preliminary tests were conducted using 10 mg/L laccase, 25 μM dye and various redox mediators. The reaction mixture was incubated at 2 h and monitored for decolorization. The control sample received no enzyme.

Conclusions

Laccase-natural redox mediator mediated RB-5 dye decolorization was studied by response surface methodology using Box-Behnken methodology to evaluate the interactions between the enzyme, dye and mediator concentrations. The results clearly reveal that SYD had main effect on decolorization of RB-5 while enzyme had low effect. A quadratic model was obtained for this design using Design Expert 8.0.2. The model employed provided good quality of predictions for the tested variables in terms of effective decolourization, and a good squared correlation coefficient (R^2 0.9943). The validation of model also showed good correlation between experimental and predicted responses.

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