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Bio removal of malachite green by mangrove-derived *Aplanochytrium* sp., KGA2512

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Effect of mangrove-derived *Aplanochytrium* sp., was studied for the removal of malachite green in aqueous solution under controlled experimental conditions. The dye removal was measured at regular interval by measuring its color intensity. This was confirmed further by Fourier Transform Infra Red (FT-IR) and also to find out the change in the chemical groups. To enhance the dye removal, a statistical optimization was done by two phases of response surface methodology (RSM): (i) Plackett-Burman design for selection of the important process enhancing factors of dye removal, such as pH, temperature, incubation period, dye concentration, glucose, peptone and yeast extract and (ii) Centre composite design to study optimized condition, interaction and combined effect of the selected factors on dye removal. *Aplanochytrium* sp., was found to remove the azo-dye significantly up to 86.32% within five and half days of incubation under optimized conditions of pH 7.8 and at temperature of 27.8°C. This study proved that mangrove-derived *Aplanochytrium* sp., was found promising for its potential of synthetic dye removal

Key words: Mangroves, bioremoval, malachite green, *Aplanochytrium* sp and response surface methodology.

INTRODUCTION

Chemical dyes are widely used in many industries related to paper, printing, textile and leather industries (Korbahti and Rauf, 2008a). After usage of the dyes, the remaining dye effluents are directly discharged into the river, sea or lake. The discharged dyes, in particular azo-dyes, are of great environmental concern. During natural removal of the azo-dyes, some toxic chemicals are produced and they are highly carcinogenic and mutagenic to the flora and fauna (Bali et al., 2004).

In order to remove the toxic dye chemicals, many treatment methods are applied. The methods include flocculation, coagulation, activated carbon adsorption, membrane filtration and sedimentation. However, these methods are not successful due to several reasons: (i) the chemicals are only partially degraded; (ii) the azo-dyes are converted into the toxic metabolites and (iii) the toxic chemi-cals are just converted to the secondary solid wastes due to their complex binding structure; and this secondary waste has to be either treated again or dumped as such (Behnajady et al., 2006).

The biological treatments in the dyes are more important to the dye removal process for removing the toxic compounds (Peralta-Hernandez et al., 2008). Microorganisms are capable of removing azo-dyes in *vallismortis* and *B. megaterium* (Tony et al., 2009a and b), *Ganoderma* sp. (Mohammadian Fazli et al., 2010), *Proteus vulgaris* and *Micrococcus glutamicus* (Saratale et al., 2010a), *Oscillatoria curviceps* (Balakrishnan Priya et al., 2011), *Bacillus lentus* (Chetan et al., 2011), *Sphingomonas paucimobilis* (Lamia Ayed et al., 2011), *Halomonas* sp. (Balamurugan et al., 2011), and *Schizophyllum* sp. (Wenzhong Tang et al., 2011).

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The classical and conventional methods of studying the dye removal as influenced factors individually and in combinations are difficult. These methods also consume more time and require numerous amounts of experiments to represent the combinational effects of the parameters. With the large number of experiments, the results will be unreliable. These limitations of conventional methods can be solved by optimizing the parameters using the response surface methodology (RSM). RSM is a statistical tool for determining the optimum conditions for a target product. The application of statistical experimental design techniques can improve product's yield, reduce process variability and experimental time (Montgomery, 1997). The present investigation therefore, was made to study the dye-degrading effect of *Aplanochytrium* sp., and to optimize the factors that were responsible for dye removal with the following objectives: to find out the potential *Aplanochytrium* sp., for degrading the dye using plating methods, and identify the important parameters for the dye removal by using statistical model (Plackett-Burman design of RSM) and optimize the identified parameters by using statistical model (center composite design of RSM).

MATERIALS AND METHODS

Preparation of dye stock solution

Malachite green dye was prepared by dissolving 250 mg of the dye powder in 100 ml of sterilized distilled water with concentration of 2.5 mg per ml. From the stock, different concentrations of the dye solution were incorporated in the culture medium.

Bioremoval of the azo-dye by Aplanochytrium sp.

The pure strain of *Aplanochytrium* sp., KGA2512 (JQ284385) was maintained at -4 to 1°C on glucose peptone agar slants (Gomathi, 2009). For experimental purpose, *Aplanochytrium* sp. was aseptically transferring to the 100 ml glucose peptone broth medium in 250 ml Erlenmeyer flasks. The flasks were incubated at 27±1°C in shaking incubator. The biological treatment experiments were performed in the Erlenmeyer flasks containing 250 ml of the synthetic dye solution and live *Aplanochytrium* sp., biomass under controlled environment. At appropriate reaction times, cell-free culture filtrate samples were drawn and the colour intensity was determined by using a spectrophotometer.

Determination of dye removal based on colour intensity of dye solution

5 ml of culture filtrate from each group of flasks was drawn and centrifuged at 3,000 rpm for 60 min. After centrifugation, the supernatant was collected and the absorbance was determined at 640 nm by using a spectrophotometer (Elico, Model 301). This was done for 20 days at an interval of three days. The change of absorbance value was converted into concentration of dye value for determining the removal of malachite green dye.

FT-IR analysis

Fourier transform infrared (FT-IR) spectroscopy was performed in the presence of toxic chemicals in the degraded dye samples. For FT-IR analysis, biological treatment process was performed with 250 ml solution containing 25 $mg.l^{-1}$ of malachite green and 5 g of *Aplanochytrium* sp., biomass. At the reaction times of 0 h (control) and 20 days, samples were taken and the biological removal products were extracted with 30 ml of diethyl ether in three times, then crystallized and used for FT-IR analysis.

Laccase enzyme assay

To find out the reason of the potential bioremoval, the laccase activity was tested. It was assessed by growing the fungi on glucose yeast peptone agar medium amended with 1-naphthol, 0.005% (pH, 6) and incubated. On oxidation of 1-naphthol by laccase, the medium changed from clear to blue, indicating positive reaction for the presence of laccase.

Selection of the important parameters for bioremoval of dye using Plackett-Burman design

To select the important medium constituents and environmental factors, the Plakett-Burman design (Plackett and Burman, 1946) with the 16 runs was used. The culture medium constituents and environmental parameters tested were pH, temperauture, incubition period, dye concentration, glucose ($mg.l^{-1}$), peptone and yeast extract. From these, important process influencing factors were selected. The experimental design along with the experimental and statistically predicted response of the dye removal is presented in the Table 1. Finally, the selection was made based on positive coefficient values. The effect of individual parameters on dye removal was calculated by the following equation:

$$E = (\Sigma M_{+} - \Sigma M_{-})/N \quad (1)$$

Where, E is the effect of parameter under study and M (+) and M (-) are responses of dye removal in trials, and N is the total number of trials. The selected factors were used for further optimization study.

Optimization of the biological dye removal

The significant changes were observed with pH, temperature, incubation period, yeast extract. This indicated that importance of these parameters on dye removal (Table 2). These parameters were selected for the further optimization. For the optimization, 30 runs of center composite design were used. Of these, each factor was assessed at five coded levels (-2, -1, 0, +1 and +2). In the whole experiment, minimum and maximum actual values of the production medium were used and are presented in Table 2. The response value (Y) in each trial was the average of the duplicates (Table 3).

Statistical analysis and modeling

The obtained data from RSM on dye removal were subjected to analysis of variance (ANOVA). The experimental results of RSM were fit via the response surface regression procedure, using the following second order polynomial equation.

$$Yi = \beta_0 + \Sigma_i \beta_i X_i + \Sigma_i \beta_{ii} X_i^2 + \Sigma_{ij} \beta_{ij} X_i X_j \quad (2)$$

Where, Yi is the predicted response, $X_i X_j$ are independent variables, β_0 is the offset term, β_i is the i^{th} linear coefficient, βii is the i^{th} quadratic coefficient and β_{ij} is the ij^{th} interaction coefficient. However, in this experiment, the independent variables were coded as

Table 1. Selection of dye removal responsible parameters and predicted and actual percentage of dye removal by using Plackett-Burman experimental design.

Number	A	B	C	D	E	F	G	Percentage (%) of dye removal	
								Experimental	Predicted
1	7.8	20	0	10	2	2	1	0.9401	-1.88776
2	8.2	20	0	10	4	2	4	0.6996	4.55945
3	7.8	26	0	10	4	4	1	0.7702	3.598063
4	8.2	26	0	10	2	4	4	0.2321	-3.62775
5	7.8	20	10	10	4	4	4	48.3	45.47214
6	8.2	20	10	10	2	4	1	21.35	25.20985
7	7.8	26	10	10	2	2	4	45.138	47.96586
8	8.2	26	10	10	4	2	1	46.6706	42.81075
9	7.8	20	0	50	2	4	4	0.4305	3.258363
10	8.2	20	0	50	4	4	1	2.6654	-1.19445
11	7.8	26	0	50	4	2	4	0.9429	-1.88496
12	8.2	26	0	50	2	2	1	0.2312	4.09105
13	7.8	20	10	50	4	2	1	27.3117	30.13956
14	8.2	20	10	50	2	2	4	57.2532	53.39335
15	7.8	26	10	50	2	4	1	46.0903	43.26244
16	8.2	26	10	50	4	4	4	53.6617	57.52155

A , pH; B , temperauture (C); C, incubation period (Days); D, dye concentration ($mg.l^{-1}$); E, glucose ($mg.l^{-1}$); F, peptone ($mg.l^{-1}$); G, yeast extract ($mg.l^{-1}$).

Table 2. Statistical parameters for selected the linear polynomial model using Plackett-Burman design.

Parameter	Coefficient	SE Coefficient	Actual	Probability
pH	22.04297	2.392449	22.04	0.013
Temperature (°C)	0.802506	2.392449	0.80	0.007
Incubation period (days)	2.174156	2.392449	2.17	0.045
Dye concentration ($mg.l^{-1}$)	-0.17897	2.392449	21.18	0.012
Glucose($g.l^{-1}$)	-0.530394	2.392449	1.53	0.587
Peptone ($mg.l^{-1}$)	-0.584794	2.392449	0.58	0.829
Yeast extract ($mg.l^{-1}$)	12.35544	2.392449	-0.36	0.008

X_1, X_2, X_3 and X_4. Thus, the second order polynomial equation can be presented as follows:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{44} X_4^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{14} X_1 X_4 + \beta_{23} X_2 X_3 + \beta_{24} X_2 X_4 + \beta_{34} X_3 X_4 \quad (3)$$

Statistical software namely the Design expert (8.0.6 package) was used for the regression analysis and to plot the response surface graphs of the experimental data. The statistical significance of the model equation and the model terms were evaluated by the Fisher's test. The quality of fit in the second-order polynomial model equation was expressed by the coefficient of determination (R^2) and the adjusted R^2. The fitted polynomial equation was then expressed in the form of three-dimensional surface plots, in order to illustrate the relationship between the responses and the experimental levels of each of the variables used in this experiment. The point optimazation method was employed in order to optimize the level of each variable for maximum dye removal. The combination of different optimized variables, which yielded the maximum response, was determined in an attempt to verify the validity of the model.

RESULTS

The removal of the malachite green dye by *Aplanochytrium* sp., was tested and in this connection, the presence of oxidative enzyme laccase was tested in the culture filtrate of *Aplanochytrium* sp. The presence of laccase enzyme was observed in the culture filtrate (Figure 1a). Before the dye decolouration and after decolouration processes, dye concentration was determined by using a spectrophotometer. It revealed 73.23% of dye decolouration (Figure 1b).

Selection of important factors for dye removal using Plackett–Burman design

The important factors for the maximum dye removal were selected by using 16 runs of the Plackett-Burman design and data are presented in the Table 1. The F value of

Figure 1a. A view of dye decolouration before the addition of *Aplanochytrium* sp. and with *Aplanochytrium* sp. after 20 days of incubation.

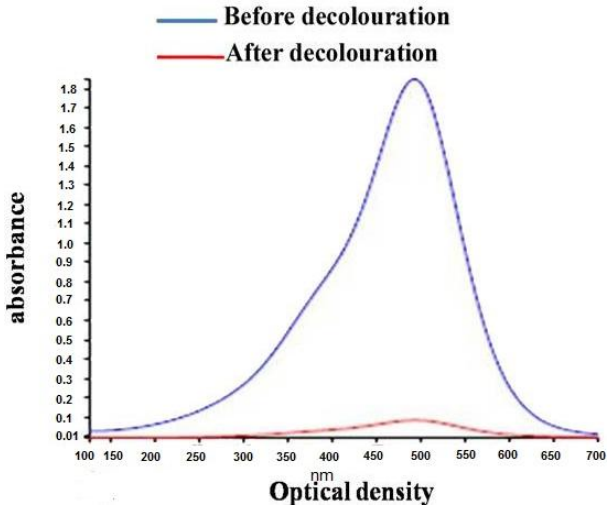

Figure 1b. Spectrophotometer determination of dye removal by *Aplanochytrium* sp.

8.56 implies that the model was significant. The probability value of < 0.0143 indicates model terms were significant. The magnitude of the effects indicated the level of the significance of the variables on dye removal. The selection of the important factors was made based on the significant response on dye removal and positive coeffi-cients values (Table 2).

RSM approach for optimization of dye removal

Center composite design (CCD) was used to design the experiments to evaluate the interactive effects of process parameters for optimizing dye removal by *Aplanochytrium* sp. The empirical relationship between the response and various input variables in the RSM approach obtained from the center composite model along with the predicted and experimental response of the percentage of the dye removal is presented in Table 3. The statistical significance was tested with the individual and interactions effects of variables at various levels of concentration on the dye removal probability values are shown in Table 4 based on Fisher's test and analysis of variance fitted to second order polynomial equation. The probability value of <0.05 revealed that the variables were statistically significant (Montgomery, 1997). The variables such as pH, temperature and incubation period were observed as significant parameters for the dye removal. Regarding interactions of the parameters, the interactions of temperature and incubation period, temperature and yeasts extract were found significant indicating that the variables were vital for dye removal process. The parameters were then fitted into second order polynomial equation as follows:

$$\text{Percentage of dye removal} = 83.20 + 0.97B + 1.29C + 21.52D + 0.66AB + 0.62AC - 0.57AD + 0.56BC + 3.09BD - 0.81CD \quad (5)$$

The determinations of the model accuracy was tested by second order polynomial model and significant F-Value ($F = 6.34$, degree of freedom = 14, $P < 0.0005$) and a low value of standard deviation (0.17) between the measured and modeled results were observed. This showed that the equation adequately represented actual relationship between the response (percentage of the dye removal) and significant variables. High value of R^2 (0.72) was very close to the predicted value of R^2 which indicated a high dependence and correlation between the observed and the predicted values of response. The lack-of-fit term was non-significant as it was desired. The non-significant value of lack of fit observed was 8.112 more than 0.05 showing that the quadratic model was valid for the present study.

Validation of the model was also carried out by plotting standard error in response as a function of a pair of factors. A plot of the standard errors in bioremoval of the dye as a function of temperature and pH is shown in Figure 2a, b, c and d. The shape of the standard error plot was not only found to fit on the design points but also the polynomial showed low and flat errors exhibiting circular contours and symmetrical shape around the centroid, representing best condition. The standard error value around the centroid was 0.401, which was the best value. The inferences obtained from the response surface methodology based on the CCD experimental design model in relation to dye removal by *Aplanochytrium* sp., with respect to each variable are as follows.

Effect of temperature and incubation period on dye removal

The interactions and individual effects of the temperatures

Table 3. Central composite design matrix for the experimental design and predicted and actual responses for Azo-dye removal.

Standard	pH	Temperature (°C)	Incubation period (Day)	Yeast extract ($mg.l^{-1}$)	% of dye removal	
					Experimental	Predicted
1	7.8	23	5.5	1	5.3	13.84
2	8.2	23	5.5	1	5.52	12.08583
3	8	26	1	5.5	5.2	7.824167
4	8	26	5.5	1	5.1	8.705
5	8	23	5.5	5.5	68.52	56.16583
6	8.2	23	5.5	10	68.5	56.87167
7	8	23	5.5	5.5	55.6	52.37
8	8	20	5.5	10	62.54	55.71083
9	8	26	10	5.5	1.59	15.2275
10	8	23	1	1	5.12	15.75333
11	7.8	23	10	5.5	2.53	21.56167
12	8.2	26	5.5	5.5	5.56	24.7225
13	8.2	23	10	5.5	50.52	54.31833
14	7.8	20	5.5	5.5	53.12	57.30417
15	7.8	23	5.5	10	62.63	62.8725
16	8	23	10	1	69.63	68.49333
17	8	20	1	5.5	18.56	9.520833
18	7.8	26	5.5	5.5	18.56	13.3875
19	8	23	5.5	5.5	52.1	47.5175
20	8	23	10	10	62.32	52.69083
21	8	26	5.5	10	55.6	20.80583
22	8	23	5.5	5.5	86.32	106.9025
23	8	20	10	5.5	0	13.45917
24	8.2	23	1	5.5	55.3	27.62917
25	8.2	20	5.5	5.5	82.5	83.195
26	8	20	5.5	1	85.12	83.195
27	8	23	5.5	5.5	82.12	83.195
28	7.8	23	1	5.5	82.53	83.195
29	8	23	1	10	83.32	83.195
30	7.8	23	5.5	1	83.58	83.195

Table 4a. Analysis of variance table (ANOVA) for response surface methodology of main effects and interacting effects of parameters in quadratic model.

Source	Sum Square	df	Square	F Value	P- value Prob > F
Model	25770.12	14	1840.723	6.344683	0.0005**
A-pH	22.42667	1	22.42667	0.077301	0.007**
B-Temperature	40.14507	1	40.14507	0.138374	0.071*
C-Incubation period	11118.95	1	11118.95	38.32529	0.0001**
D-yeast extract	301.1834	1	301.1834	1.038132	0.324NS
AB	6.943225	1	6.943225	0.023932	0.879NS
AC	6.0516	1	6.0516	0.020859	0.887NS
AD	5.1984	1	5.1984	0.017918	0.895NS
BC	4.9284	1	4.9284	0.016987	0.0498*
BD	152.5225	1	152.5225	0.525721	0.0479*
CD	10.46523	1	10.46523	0.036072	0.851NS
A2	8822.995	1	8822.995	30.41148	0.0001***

Table 4a. Contd.

B2	1877.148	1	1877.148	6.470237	0.0225*
C2	641.2591	1	641.2591	2.21032	0.1578NS
D2	6728.789	1	6728.789	23.19308	0.0002**
Residual	4351.808	15	290.1205		
Lack of Fit	4345.857	10	434.5857	365.1766	0.231NS
Pure Error	5.95035	5	1.19007		
Core Total	30121.92	29			

***, **, *Statistically significant at $p < 0.0001$, $p < 0.01$ and $p < 0.05$ NS, Non-significant.

Table 4b. FTIR of malachite green before removal showing peaks and their corresponding bonds and name of chemical groups.

Peak	Bond	Group
435.91 464.84	S-S	Sulfide groups
503.42	C-I	
547.78 563.21	C-Br	Aliphatic halogenated compounds
619.15	C-H, S-S	Alkene, Polysulfides
659.66	CH_3-S	Thio ethers
1128.36	C-N	Tertiary amine
1624.06	C=N	Imino group
2345.44	C≡C, C≡N	Alkynes,Amines
2866.22	C-H	Alkene
2931.8	C-H stretch	Methyl asymmetric stretch
3421.72	O-H stretch	Alcohol

and incubation period were tested on dye removal by the RSM model and the results are shown in the contours and 3D plots. The temperature and incubation period interaction was significant (F value-0.016., P-value 0.04) for the statistical optimization of the parameters and the perturbation was observed by the way of increased and decreased levels of the parameters using the RSM surface model. It indicated that the statistical optima for the potential dye removal were temperature of 32.16°C and incubation period of 20 days (Figure 3d).

Effect of temperature and yeast extract on dye removal

The interactions and individual effects of the temperature and processing time were tested on dye removal by the RSM model and the results are shown in the contours and 3D plots. The yeast extract and temperature interactions were significant (F value-0.54, P-0.042) for the statistical optimization of these parameters. The perturbation was observed by the way of increased and decreased levels of the parameters using the RSM surface model. It indicated that the statistical optima for the potential dye removal was at yeast extract of 5.62 $mg.l^{-1}$ and temperature of 27.03°C (Figure 3e), and other second order interactions were not significant with dye removal. The results are presented in the contours and 3D plots (Figure 4 A - C, F).

The optimal conditions for dye removal process were pH 6.97, temperature of 32.16°C, incubation period of 20 days and yeast extract of 5.62 $mg.l^{-1}$ (Figure 2d). Under these optimal conditions, the maximum dye removal of 84.59% was obtained and it was 9% higher than the normal method of removal of azo-dye.

DISCUSSION

The present study reported for the first time that thraustochytrids could be able to degrade azo-dye. The laccase enzyme capable of degrading the dye was found to be present in the culture filtrate of thraustochytrids. Other dye-degrading microbes are also known for the pro-duction of

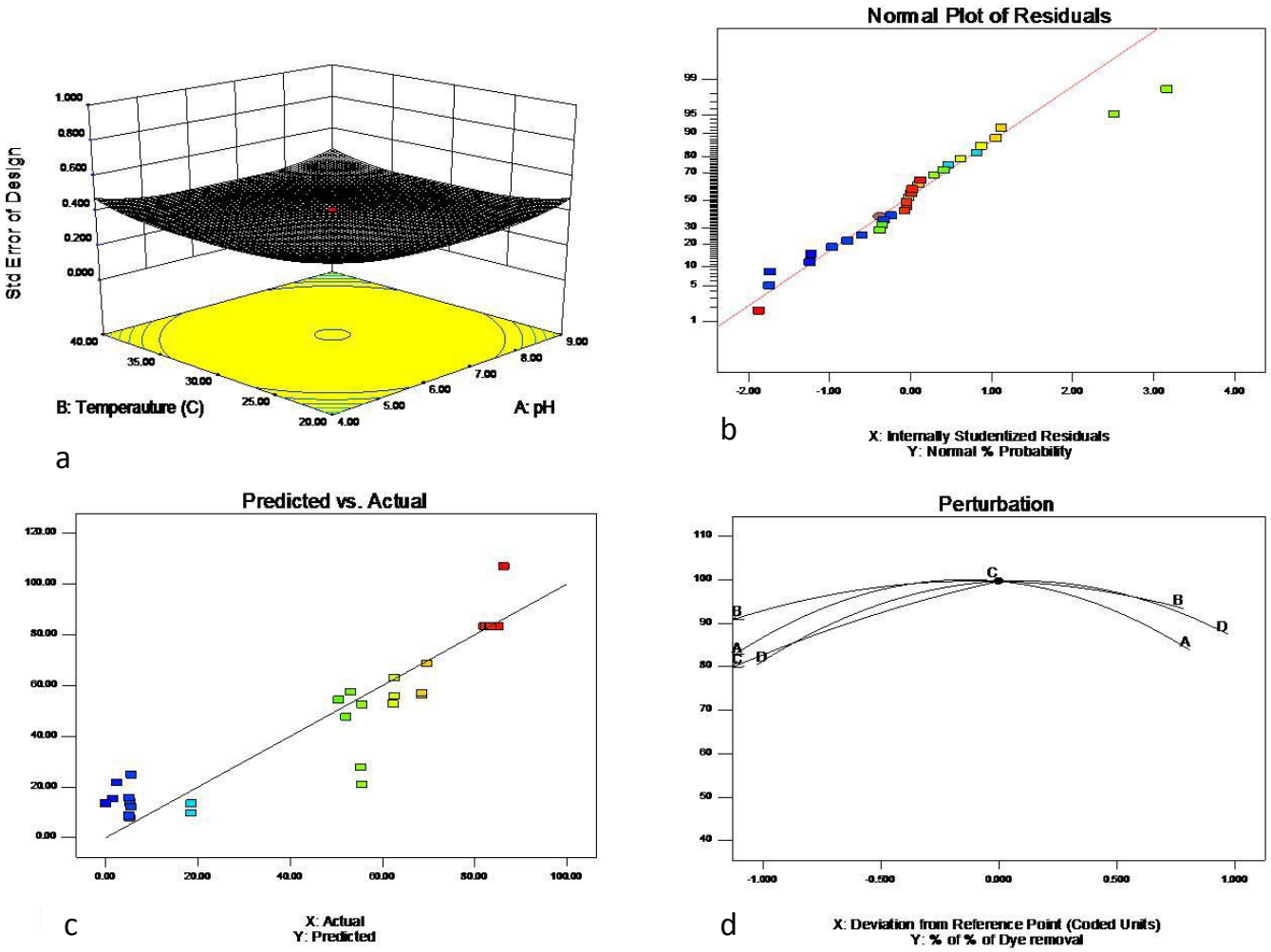

Figure 2. a. Three-dimensional standard error plot for dye removal by *Aplanochytrium* sp., **b.** Normal plot for the residuals and normal percentage of probability for the response of predicted and experimental values. **c.** Predicted and actual experimental response for the dye removal. **d.** perturbation plot for dye removal.

Table 4c. FTIR of malachite green dye after 24 days of removal by *Aplanochytrium* sp., showing peaks and their corresponding bonds and name of chemical groups.

Peak	Bond	Group
421.41		
441.7	S-S	Sulfide groups
495.71		
609.51	C-H, S-S	Alkene, Polysulfides
653.87	CH_3-S	Thio ethers
1635.64	C=N,N-H bending	Imino group
2065.76	Metal carbonyls	Metal carbonyls
2086.98	Metal carbonyls	Metal carbonyls
3417.86	O-H stretch	Alcohol

laccase (Claus et al., 2002; Tavares et al., 2008). When the substrate concentration like dye increases to a certain high value, the reaction rate reaches a plateau and keeps constant even if more substrate is used (Garcia et al., 2003). Statistical methods are used to predict the optimal conditions for the maximum dye removal (Li et al., 1996) and hence they were determined in the present work (Figure 3 A – F). Figure 3 A – F clearly show that the dye removal efficiency increased with increasing incubation period and dose of thraustochy-trids. The effect of initial pH on dye removal efficiency is illustrated in Figure 3 a-f. A similar observation was previously reported for removal of malachite green dye by using *Pithophora* sp., *Cosmarium* sp. and *Chlorella* sp. (Daneshvar et al., 2007b; Khataee et al., 2009a; Kumar et al., 2006). But no report has been so far available for the potential of Thraustochytrids in dye removal.

The dye removal was confirmed further by Fourier Transform Infra-Red (FT-IR). The FT-IR spectra of the degraded dye and the un-degraded dye are shown in Table 4a-c and Figure 4a and b. FT-IR spectra of un-degraded dye showed the specific peaks in a range

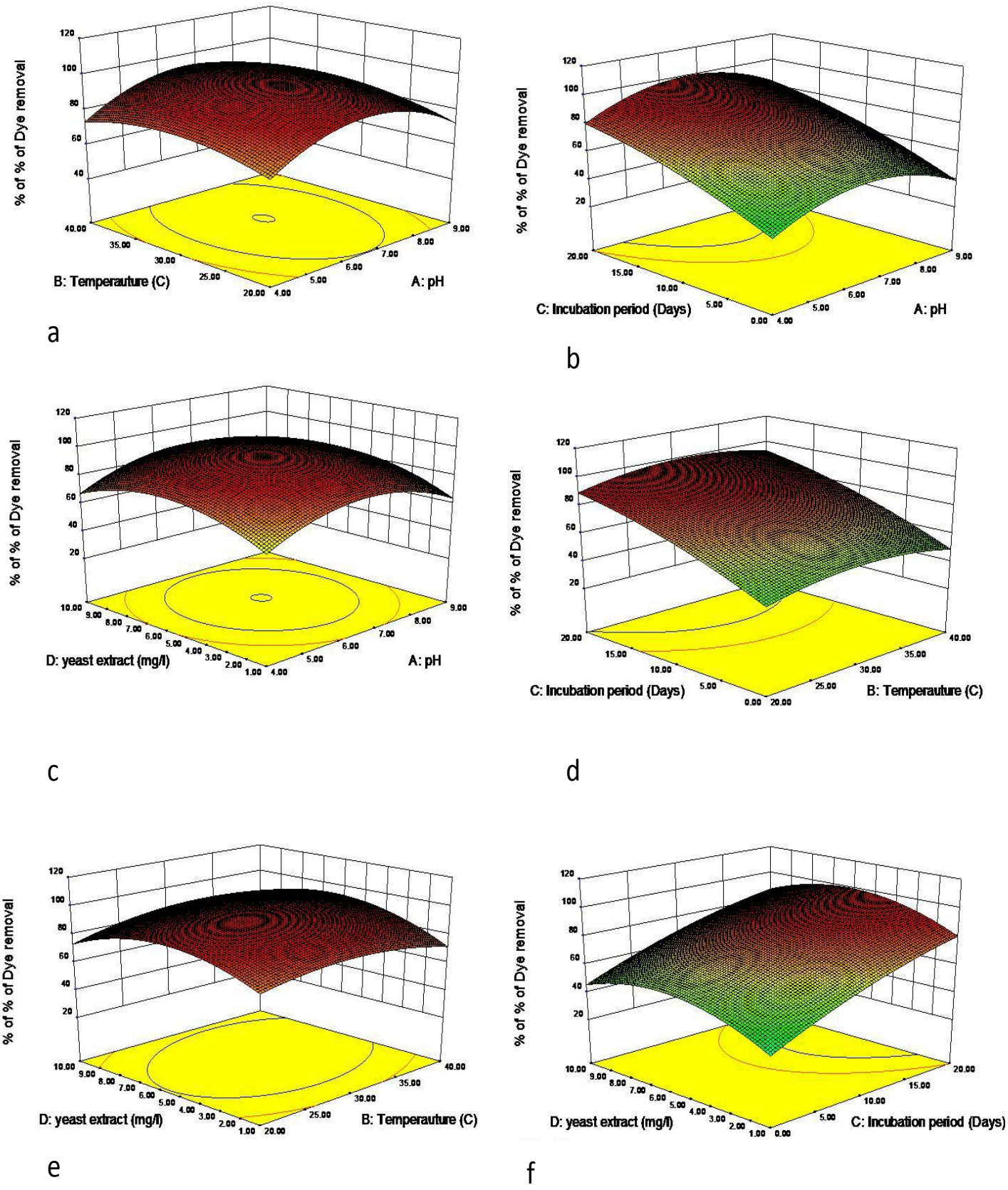

Figure 3. Three-dimensional response surface plot for (a) effect of temperature and pH, (b) effect of incubation period and adsorbent dosage (mg/l), (c) effect of ferric ammonium citrate and pH, (d) effect of incubation period and temperature, (e) effect of ferric ammonium citrate and temperature and (f) effect of ferric ammonium citrate and incubation period, on response of dye removal by *Aplanochytrium* sp.

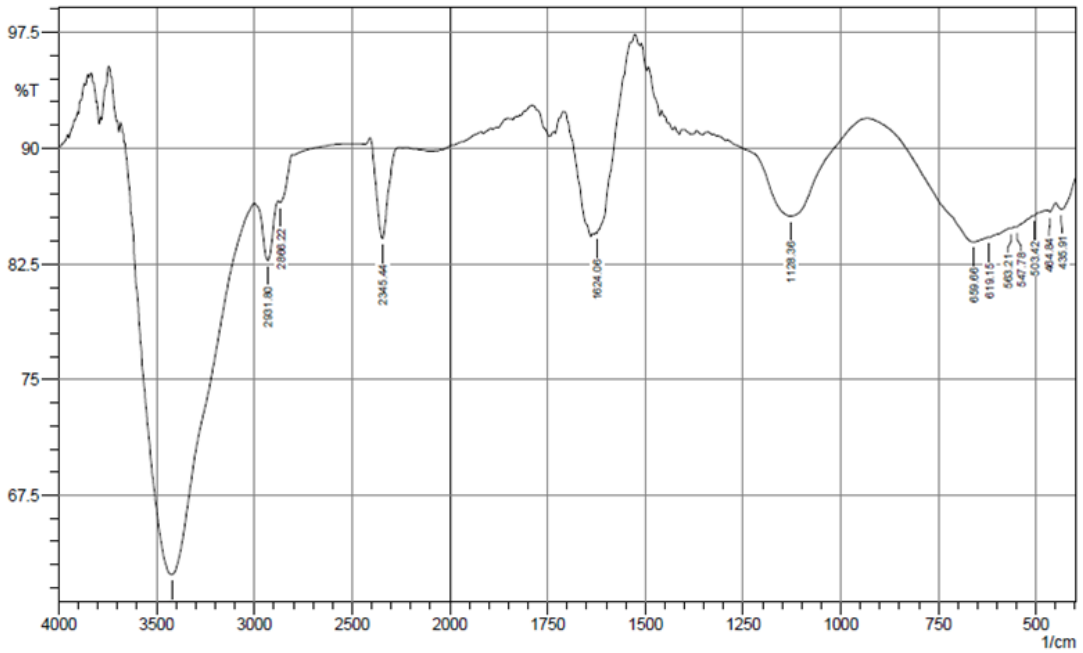

Figure 4a. FTIR spectra of malachite green before removal.

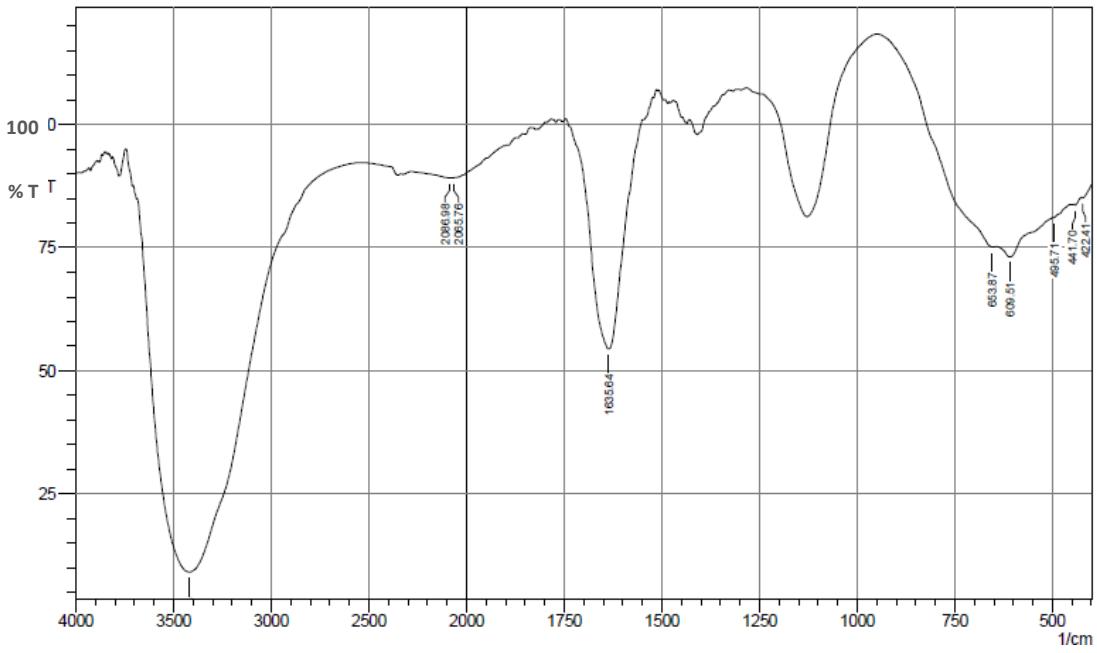

Figure 4b. FTIR spectra of malachite green after removal with *Aplanochytrium* sp.

between 1500 and 500 cm^{-1} for the mono and para-di substituted benzene rings. The peaks between 400 and 550 cm^{-1} represent the presence of sulfide groups and bromine groups. Also, the peak at 1128.36 cm^{-1} for the C-N stretching vibrations and peak at 2931cm^{-1} for C-H stretching of asymmetric-CH_3 group gives the perception of structure of malachite green. The O-H stretching (3421.72) confirmed the alcoholic groups. The peaks at 1624 cm^{-1} for the C=N stretching. The peaks of the degraded dye were compared with un-degraded dye. There were 13 peaks in un-degraded dye as against only 9 peaks in degraded dye.

The FT-IR spectra of degraded dye showed peak at 1635 cm^{-1} for C=N- stretch and N-H bends represents the formation of primary and secondary amines. The sharp peaks of un-degraded dye at 2300 to 2900 cm^{-1} for di substituted benzene derivatives indicate the aromatic nature of amines which were completely degraded by *Aplanochytrium* sp. This prompted the proposal of the possible mechanism for the removal of aromatic amines in the dye. The triple bonded compounds of degraded dye in the malachite green might have reacted with carbonyl compounds present in *Aplanochytrium* sp. The addition of carbon into triple bonded compounds might have cleaved the bonds to form double bonded and single bonded compounds.

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