Journal of Hazardous Materials 185 (2011) 837–843

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Process optimization for efficient dye removal by *Aspergillus lentulus* FJ172995

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ARTICLE INFO

Article history:
Received 14 July 2010
Received in revised form 3 September 2010
Accepted 25 September 2010
Available online 7 October 2010

Keywords:
Biomass
Dye concentration
Dye removal
Glucose
RSM
Urea

ABSTRACT

Response surface methodology involving three variables with five level second order central composite experimental design was employed to optimize conditions for maximum dye removal by *Aspergillus lentulus* FJ172995. The interaction between three variables; glucose, urea and initial dye concentration was studied and modeled for two responses: dye removal and biomass production. The results indicate that urea is the main factor influencing dye removal whereas glucose plays a major role in biomass production. Also, initial dye concentration has depreciative effect on dye removal thereby suggesting that for the treatment of effluent containing higher concentrations of dye, nutrient input should be increased. A high dye removal efficiency (99.97%) and high uptake capacity (97.54 mg/g) was obtained in 24 h using optimum process variables.

1. Introduction

Recently, response surface methodology is being used for the optimization of various biological processes such as enzyme production [1,2], heavy metal removal from waste water [3–5], hydrogen production [6] and secondary metabolite production [7,8]. Utilization of this tool for the optimization of process parameters for dye removal from waste water has also gained momentum [9]. Sharma et al. [10] used RSM to optimize the temperature, pH and dye concentration for achieving maximal dye decolorization by *Aspergillus fumigatus fresenius* in growing mode. Li and Jia [11] optimized the process parameters, dye concentration and hydraulic retention time, for solid state fermentation incorporating rice hull-*Schizophyllum* sp. decolorization system involving dye removal through biosorption and biodegradation. Other studies include optimization of nutrient media for supporting growth of the microorganism and achieving maximal dye removal [12,13].

Recently, utilization of microbial cells in dye removal process have gained momentum and batch studies as well as bioreactor studies based on such technologies have been extensively reviewed [14–18]. Being economic and eco-friendly, biological processes are gaining popularity over conventional physical and chemical processes for the treatment of dye wastewater. Also, production of microbial biomass is less energy intensive than the production of activated charcoal which is the widely used adsorbent for such treatment purposes. In addition to this, biosorption capacity of the activated charcoal [19,20] has been found to be less than that of fungal biomass for methylene blue removal [21]. Dye removal process through growing cells requires nutrient inputs to support the growth of microorganisms. Thus it becomes important to optimize the critical nutrients (such as carbon and nitrogen) required for optimal biomass production and dye removal. Further, the requirement of these nutrients for complete dye removal shall be governed by the initial dye concentration. Hence, the optimal nutrient requirement must be worked out with respect to the dye concentration. In this regard, RSM is very useful tool that reduces the number of experimental trials needed to evaluate multiple parameters and their interaction. Sharma et al. [5] optimized the nutrient supplementation for Cr (VI) removal by *Aspergillus lentulus* and pointed out the vital requirement of yeast extract for the process. Nevertheless, as the cost of yeast extract is very high, its use as a nitrogen source for industrial waste water treatment cannot be commercially viable and alternate nitrogen sources need to be investigated.

Therefore, in the previous study by present authors [22], nitrogen source in the composite media, i.e. the yeast extract was replaced by urea and ammonium chloride combination to lower the cost of the media. In the present study, various ratios of ammonium chloride and urea were tested for achieving optimal removal for Acid Navy Blue dye. Further, the process parameters like carbon content, nitrogen content and initial dye concentration were optimized using response surface methodology for achieving efficient percentage dye removal by producing optimum biomass by

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doi:10.1016/j.jhazmat.2010.09.096

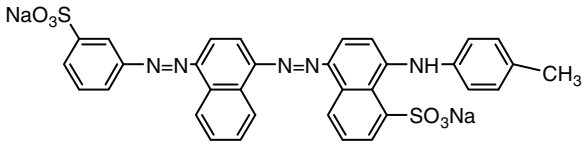

Fig. 1. Structure of Acid Navy Blue dye (C.I.: Acid Blue 120, λ_{max}: 561 nm).

the fungal isolate. The test dye Acid Navy Blue is extensively used in textile industries in India for the dyeing of woolen and silk fabrics. The test organism used in the present study has been isolated from the real textile effluent and is capable of removing dye even in extremes of pH, temperature and salt concentration [23].

2. Materials and methods

2.1. Microorganism and growth conditions

A fungal strain previously isolated from textile effluent procured from Baddi (Himachal Pradesh, India) and identified on molecular basis as *Aspergillus lentulus* FJ172995 was used in the study. The fungal isolate was maintained on Potato Dextrose Agar slants at 4 °C. Freshly revived culture was used for each experiment.

2.2. Dyes and chemicals

The dye used in the experiment, Acid Navy Blue (C.I. Name Acid Blue 120) was obtained from Department of Textile Technology, Indian Institute of Technology Delhi. Acid Navy Blue (Fig. 1) is anionic in nature belonging to the azo class and find extensive use in dyeing of woolen and silk fibers. Absorption maximum of the dye (Acid Navy Blue: 561 nm) was obtained by scanning the dye solution over the visible range. Standard curve was plotted by measuring absorbance at absorption maxima of the dye. The stock solution of 10,000 mg/l was prepared Acid Navy Blue in distilled water. All the other chemicals used were of analytical grade and were obtained from Merck and Qualigens.

2.3. Optimization study

Response surface methodology was used to design experiment to optimize two response variables: dye removal (%) and biomass produced (g/l). Three independent variables glucose (A), urea (B) and initial dye concentration (C) were chosen and each variable was coded at three levels between −1 and +1, and changed in ranges as shown in Table 1. The critical ranges of selected parameters were determined by the preliminary experiments [22]. The minimum and maximum concentration of glucose was set at 3 g/l and 7 g/l, the range for urea was set between 0.15 g/l and 0.45 g/l and the range for initial concentration of Acid Navy Blue dye was set between 100 mg/l and 300 mg/l. Fourteen experiments were augmented with six replications at the design center to evaluate the pure error and were carried in randomized order. The composition of rest of the media was K_2HPO_4, 0.5 g/l, NH_4Cl, 0.2 g/l and $MgSO_4$, 0.1 g/l; pH 6.5. The media was autoclaved for 20 min at 121 °C and inoculated with 5% spore suspension (6.25×10^6 spores/ml) of *A. lentulus* and incubated in a shaker at 30 °C and 180 rpm for 24 h.

Table 1
Independent variables and their levels in the experimental design.

Independent variables	Symbols	Code levels		
		−1	0	+1
Glucose (g/l)	*A*	3	5	7
Urea (g/l)	*B*	0.15	0.3	0.45
Dye concentration (mg/l)	*C*	100	200	300

At the end of this period, two responses, i.e. fungal biomass and residual dye concentration in the media were measured. The statistical software package Design-Expert, Stat-Ease Inc., Minneapolis, USA was used for regression analysis of experimental data and to plot response surface.

2.4. Analytical techniques

The concentration of test dye, Acid Navy Blue was determined by measuring the absorbance of samples through spectrophotometer (Systronics Visiscan 167) at 561 nm. A calibration plot between concentration and absorbance of the respective dye was used for determination of dye concentration. Dye removal (%) was calculated using the equation:

$$\text{Dye removal}\,(\%) = \left[\frac{(A_0 - A_t)}{A_0}\right] \times 100 \quad (1)$$

where A_0 is the initial absorbance, A_t is the absorbance at incubation time, t. Test samples were analyzed for absorbance after centrifuging (Sigma 4K15) the samples at 10,000 rpm for 10 min.

The dry cell weight of the biomass formed at the end of the incubation period was measured by filtering out the contents of the flasks through pre-dried and pre-weighed Whatman No.1 filter paper and drying it overnight at 60 °C to a constant weight.

3. Results

3.1. Determining the range for critical parameters

In a previous study [22], attempts were made to replace yeast extract with other nitrogen sources (urea, ammonium chloride, ammonium nitrate, sodium nitrate). However, it was observed that none of the sources at tested concentrations could match high decolorization rates displayed in presence of yeast extract. Hence, it was envisaged that optimization of a suitable combination and concentration of nitrogen source could provide results at par with the yeast extract. In the present study, the combination of urea and ammonium chloride has been used as nitrogen source. This was based on the fact that utilization of urea resulted in very high uptake capacity but lower decolorization rates while ammonium chloride depicted high decolorization rates but incomplete dye removal. To simplify, the amount of ammonium chloride in the media was fixed at 0.2 g/l and amount of urea was varied between 0.15 g/l and 0.45 g/l. This range for urea and ammonium chloride concentration was chosen in a way that it provides approximately the same amount of nitrogen as provided by the yeast extract to bring about complete dye removal.

The range for glucose was set between 3 g/l and 7 g/l because the preliminary studies suggested that more than 2 g/l glucose is required to bring about effective dye removal within 24 h and also excessive biomass formed at higher concentration (10 g/l) did not contribute towards dye removal but rather reduced the uptake capacity (mg dye/g biomass) of the biomass. The dye concentration for the optimization study was set between 100 mg/l and 300 mg/l as the fungal isolate taken in the present study is capable of removing Acid Navy Blue dye in this range effectively. Also, the concentration of the dye in industrial effluents is generally reported up to 300 mg/l [18].

3.2. Optimization of process parameters for dye removal and biomass production

Table 2 depicts the experimental results for dye removal % (Y_1) and fungal biomass (Y_2) using three factor CCD experimental design

Table 2
Experimental design and results of the central composite design.

Run	Factor			Response	
	A: Glucose (g/l)	B: Urea (g/l)	C: Dye concentration (mg/l)	Dye removal (%)	Biomass (g/l)
1	5.00	0.05	200.00	89.59	1.15
2	3.00	0.45	300.00	94.16	1.236
3	7.00	0.15	100.00	94.29	1.98
4	7.00	0.45	100.00	96.16	2.28
5	7.00	0.15	300.00	88.41	2.415
6	5.00	0.30	368.18	86.18	2.387
7	5.00	0.30	200.00	98.68	2.102
8	5.00	0.55	200.00	98.06	1.521
9	5.00	0.30	200.00	99.56	2.195
10	7.00	0.45	300.00	96.34	2.45
11	5.00	0.30	200.00	98.13	2.062
12	5.00	0.30	200.00	99.24	2.129
13	3.00	0.15	100.00	97.67	1.521
14	1.64	0.30	200.00	92.43	0.975
15	8.36	0.30	200.00	97.29	2.484
16	3.00	0.45	100.00	97.21	1.423
17	3.00	0.15	300.00	84.48	1.596
18	5.00	0.30	200.00	99.18	1.954
19	5.00	0.30	31.82	96.42	1.898
20	5.00	0.30	200.00	99.97	2.05

with six replicates and six axial points. The process of dye removal by *A. lentulus* is dependent on the biomass production which in turn is dependent on the various process inputs like nutrients glucose (*A*), urea (*B*) and initial dye concentration (C). The extent of influence of these factors for the process can be measured by their effect on biomass production and resultant dye removal. The responses Y_1 and Y_2 were fitted with second order polynomial equations.

The quadratic model equation for predicting the response function decolorization efficiency was expressed in terms of coded factors using second order polynomial equation:

$$Y_1(\text{dye removal}\%) = 99.11 + 0.72A + 2.44B - 2.87C - 1.38A^2 - 1.74B^2 - 2.64C^2 + 0.073AB + 1.32AC + 2.03BC \quad (2)$$

$$Y_2(\text{fungal biomass}) = 2.08 + 0.43A + 0.037B + 0.096C - 0.093A^2 - 0.23B^2 + 0.053C^2 + 0.099AB + 0.090AC - 0.066BC \quad (3)$$

The statistical significance of the model equation was evaluated by the *F*-test for analysis of variance (ANOVA). The ANOVA results for both the responses Y_1 and Y_2 are shown in Table 3, which indicates that the quadratic models could be used to navigate the design space. Table 3 shows that the Prob > *F*-values for dye removal (%) and biomass production are lower than 0.05 indicating that quadratic models were significant. The coefficient of determination (R^2) was found to be close to 1 (0.97 for Y_1 and 0.95 for Y_2). This also supported a high correlation between observed and predicted values. The "lack of fit tests" compares the residual error to the "Pure error" from replicated experimental design points. The *p*-values, greater than 0.05, for both the responses indicate that the lack of fit for the model was insignificant. Adequate precision measures the signal to noise ratio and a ratio greater than 4 is desirable. The adequate precision for Y_1 and Y_2 were 20.48 and 15.22, respectively. These high values of adequate precision demonstrated that models are significant for the process.

Table 3
ANOVA analysis for the two responses Y_1 [dye removal (%)] and Y_2 [biomass (g/l)].

Source	Sum of squares	*DF*	Mean square	*F* value	Prob > *F*
For Y1					
Model	193.20	6	32.20	29.78	<0.0001
Residual	3.31	6	0.55		
Lack of fit	8.70	5	1.74	4.13	0.0729
Pure error	2.11	5	0.42		
R^2 = 0.9733					
Adeq precision = 20.479					
For Y2					
Model	1.13	6	0.19	10.37	0.0008
Residual	0.10	6	0.017		
Lack of fit	0.15	5	0.030	4.47	0.0630
Pure error	0.033	5	6.637E−03		
R^2 = 0.9545					
Adeq precision = 15.219					

Perturbation plot shows the comparative effects of glucose, urea and dye concentration on removal of Acid Navy Blue dye (Fig. 2a) and biomass production (Fig. 2b) by *A. lentulus*. Perturbation plot for percentage dye removal (Fig. 2a) shows a steep curvature in urea and dye concentration curve showing sensitivity of the dye removal process towards these two factors as compared to glucose. Also the pattern of deviation from the reference point shows that in the range studied, increase in urea increases dye removal and increase in dye concentration decreases percentage dye removal. Perturbation plot for biomass production (Fig. 2b) shows that glucose followed by urea is the governing factor in biomass formation. Glucose was the sole carbon source provided in the media for growth of the fungus. Therefore, the entire energy requirement for the fungal metabolism and growth was met by glucose thus affecting biomass production with higher degree as compared to urea which was the nitrogen source. Nitrogen was also supplemented to the fungus through ammonium chloride in the media.

Three dimensional curves were plotted to study the interaction between the three variables; initial glucose, urea and dye concentration in the media on dye removal (Fig. 3a–c) by *A. lentulus*. The process of dye removal was significantly affected by the concentration of urea. It increased from 88.4% to 96.3% as the amount of urea was increased from 0.15 g/l to 0.45 g/l at 7 g/l glucose and 300 mg/l dye concentration as is evident from the rising ridge of the response surface curve along the axis for urea. Dye removal also increased when the amount of glucose was increased in the studied range but to a lesser extent. It increased from 94.2% to 96.3% when amount of glucose was increased from 3 g/l to 7 g/l, while urea and dye concentration were at their maximum (0.45 g/l and 300 mg/l, respectively). However, maximum dye removal was observed with 5 g/l glucose concentration which is evident from the curvature of the ridge representing glucose at highest urea concentration (Fig. 3a). Also it is evident from the graphs that initial dye concentration had a negative impact on percentage dye removal which decreased by 13% (from 97.7% to 84.5%) with increase in dye concentration from 100 mg/l to 300 mg/l while the nutrient inputs being the minimal (glucose 3 g/l and urea 0.15 g/l). This reduction in percentage dye removal with increase in dye concentration (from 100 mg/l to 300 mg/l) was reduced to 0.2% (from 96.16% to 96.34%) when the nutrient input was maximized (glucose 7 g/l and urea 0.45 g/l).

Effect of these three variables; glucose, urea and dye concentration on biomass production is given in Fig. 4(a–c). The slope of the ridge along the glucose axis shows its significant and positive effect on biomass production (Fig. 4a and b). When glucose concentration was increased from 3 g/l to 7 g/l, the biomass production increased from 1.2 g/l to 2.0 g/l. Urea came out to be second most important variable affecting biomass production. Biomass production reached its maximum value (2.4 g/l) when the amount of urea was increased to 0.3 g/l and glucose too was at its maximum (Fig. 4a). Further increase in urea to 0.45 g/l resulted in slight reduction in biomass production. Increase in dye concentration; however did not have significant effect on biomass production as is evident from the near stationary region obtained in Fig. 4b and c for dye concentration.

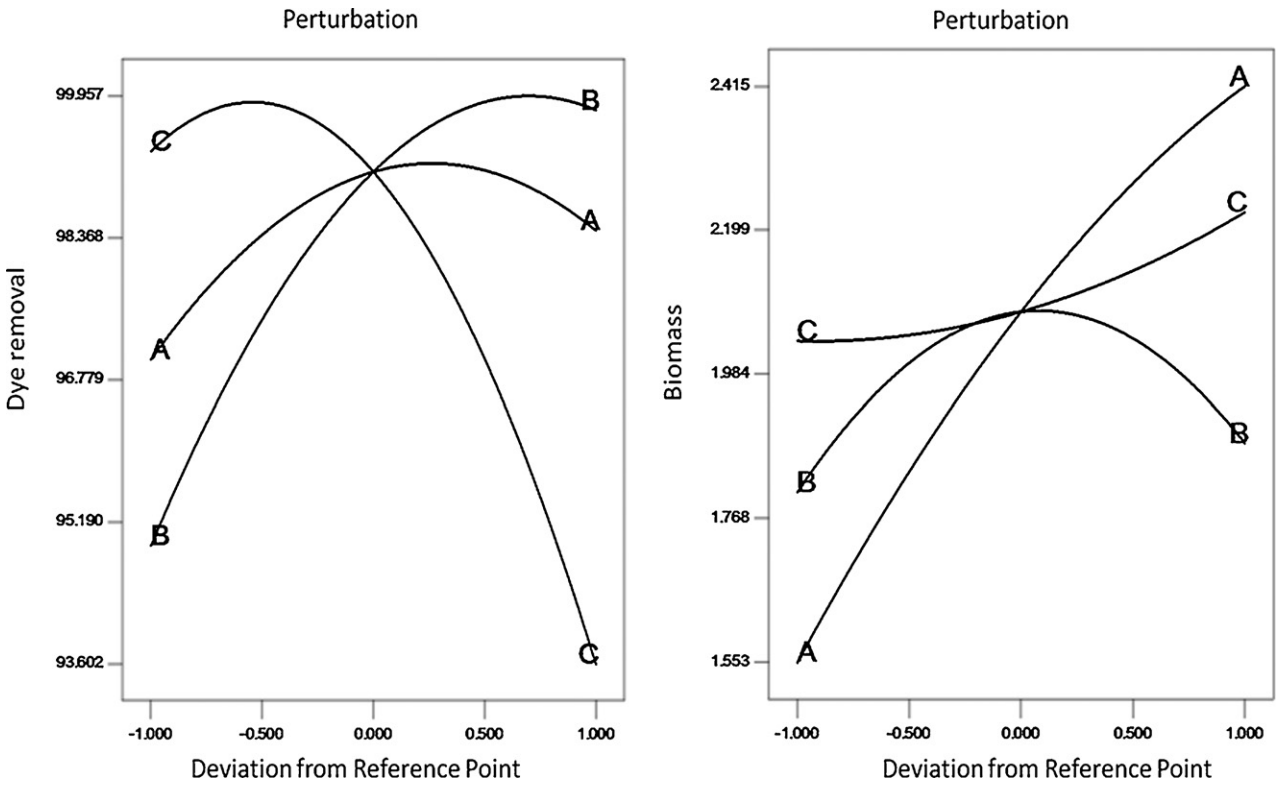

Fig. 2. Perturbation plots for (a) Acid Navy Blue dye removal and (b) biomass production by *Aspergillus lentulus*; (A) glucose, (B) urea, (C) dye concentration.

A near stationary region is defined as a region where the surface slopes (or gradient along the variable axis) are small compared to the estimate of experimental error.

3.3. Validation of results

Table 4 shows the dye removal performance of *A. lentulus* under different conditions in terms of maximum dye removal (%), time required for maximum removal and uptake capacity (mg dye/g biomass). An ideal situation would be to get complete dye removal in minimum time and with highest uptake capacity. While biomass grown in presence of yeast extract display complete dye removal within 20 h, its uptake capacity was very low (49.7 mg/g). Among the alternate sources biomass grown in the presence of urea alone shows complete dye removal and highest uptake capacity (98.9 mg/g) but time required is 40 h. Biomass grown in the presence of ammonium chloride showed incomplete removal even after 65 h. The unoptimized combination of urea and ammonium chloride also required 40 h for 96% dye removal and yielded low uptake capacity (68.23 mg/g). When optimized media (containing half the amount of glucose as unoptimized one and optimum amount of urea and ammonium chloride) was used, 99.97% dye removal was obtained in 24 h with a very high uptake capacity (97.5 mg/g). These results obtained with optimized media clearly reflect the importance of the optimization study in lowering the process cost (by reducing the nutrient input) on one hand and achieving more efficient dye removal (with minimization of dye laden sludge) on the other hand.

3.4. Cost analysis of the optimized media

The conventional composite media containing yeast extract (2.5 g/l) and glucose (10 g/l) was modified and optimized by replacing yeast extract by urea (0.3 g/l) and ammonium chloride (0.2 g/l). The amount of glucose in the optimized modified media was also reduced to 5 g/l. Table 5 depicts the cost of constituents of these two media in Indian national rupee (INR). It is observed that the cost of the optimized modified media was reduced by 85% when compared to composite media. This steep difference is caused mostly due to the replacement of yeast extract with two low cost alternatives urea and ammonium chloride. Thus this replacement and optimization can be exploited further for supporting microbial growth during treatment of dye wastewater at industrial scale.

4. Discussion

The RSM studies performed shows that in the range studied, process of dye removal is effected significantly by the amount of urea which is a nitrogen source followed by glucose, a carbon source. On the contrary, biomass production by the fungal isolate is favored largely by the amount of glucose present. This supports our previous observations during OVAT (one variable at a time) analysis of nutrient requirement by *A. lentulus* during dye removal. It was observed that glucose is responsible for the formation and initiation of the fungal pellets and that the size of the pellet increases with increase in glucose concentration. Alternatively, mycelial extensions of the pellet are well formed in the presence of nitrogen source such as urea. This aids in better dye uptake and higher dye removal. Similar observation was made by Rojek et al. [24], while investigating the decolorization of natural organic matter by *Phanerochaete chrysosporium*. They reported the increase in decolorization process with the addition of nitrogen source (ammonium chloride) while glucose was needed to initiate growth and dye removal process. Due to this observation, they regarded this process as non-enzymatic but metabolically dependent as there was increase in utilization of glucose with increase in dye removal at higher nitrogen concentrations. Mohana et al. [12] observed that with an increase in concentration of glucose (from 0.5 g/l to 1.5 g/l), there was an increase in dye decolorization but higher concentrations (above

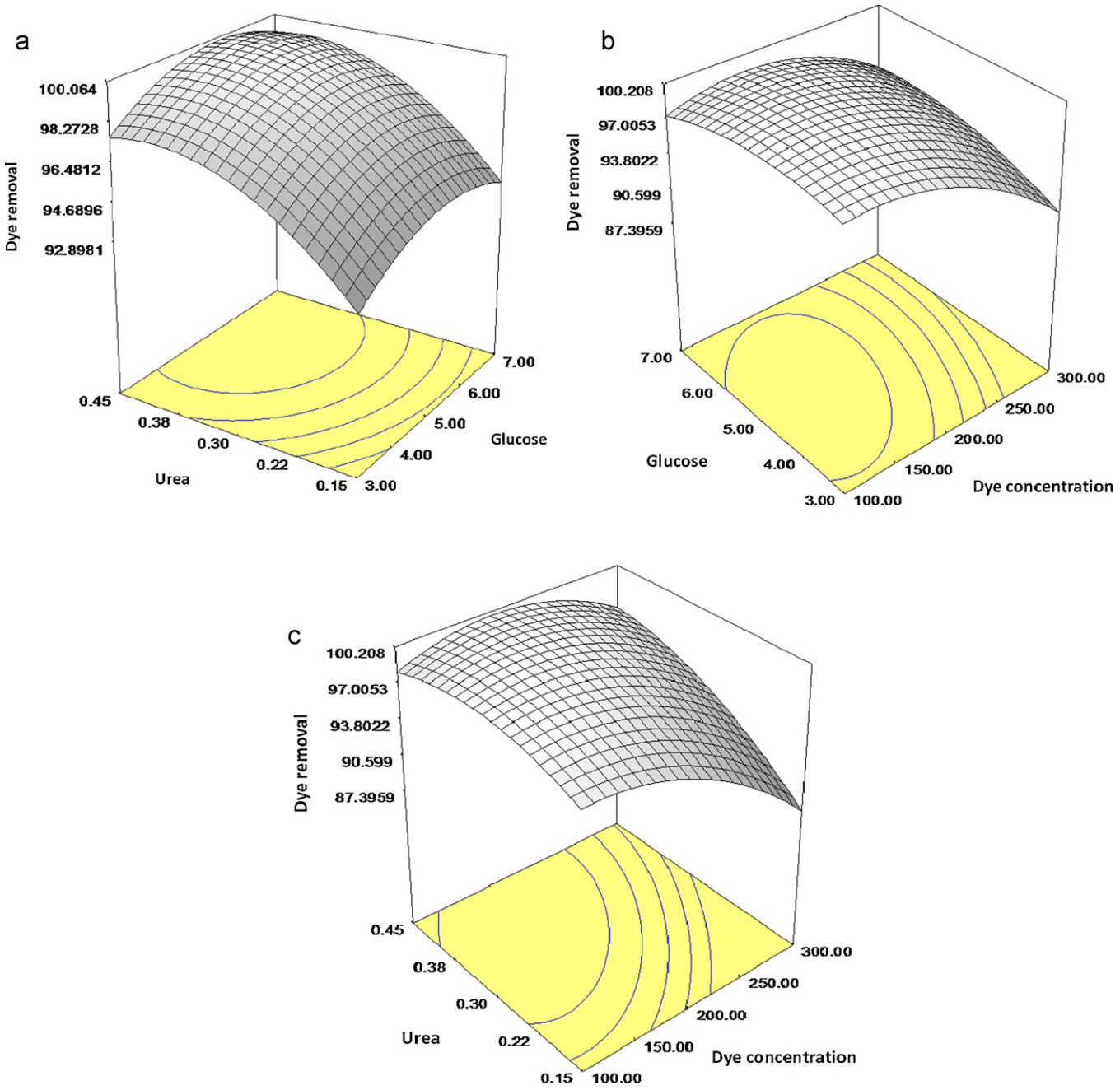

Fig. 3. Response surface curves for Acid Navy Blue dye removal by *A. lentulus* showing interaction between (a) glucose and urea, (b) glucose and dye concentration, (c) urea and dye concentration.

1.5 g/l) were found to be inhibitory. Maximum decolorization was achieved at certain concentration of the two variables (glucose and yeast extract), beyond which their effect got reversed. Srinivasan and Murthy [13] optimized the process of dye removal process with respect to three variables glucose, ammonium chloride and dye concentrations. They found that glucose showed a positive main effect and dye concentration showed a negative main effect on percentage dye removal. Also, they reported

Table 4
Effect of different nutritional conditions on removal of Acid Navy Blue (initial concentration 200 mg/l) by *A. lentulus*.

	Uptake capacity (mg/g)	Max removal (%)	Time for max removal (h)	Reference
Yeast extract (10 mM N)	49.69	99.98	20	[14]
Urea (10 mM N)	98.92	99.98	40	[14]
Ammonium chloride (10 mM N)	85.11	96.37	65	[14]
Unoptimized media[a]	68.23	96.18	40	Present study
Optimized media[b]	97.56	99.97	24	Present study

[a] Unoptimized media: glucose 10 g/l, urea 0.2 g/l, NH_4Cl 0.2 g/l, K_2HPO_4 0.5 g/l, $MgSO_4$ 0.1 g/l.
[b] Optimized media: glucose 5 g/l, urea 0.3 g/l, NH_4Cl 0.2 g/l, K_2HPO_4 0.5 g/l, $MgSO_4$ 0.1 g/l.

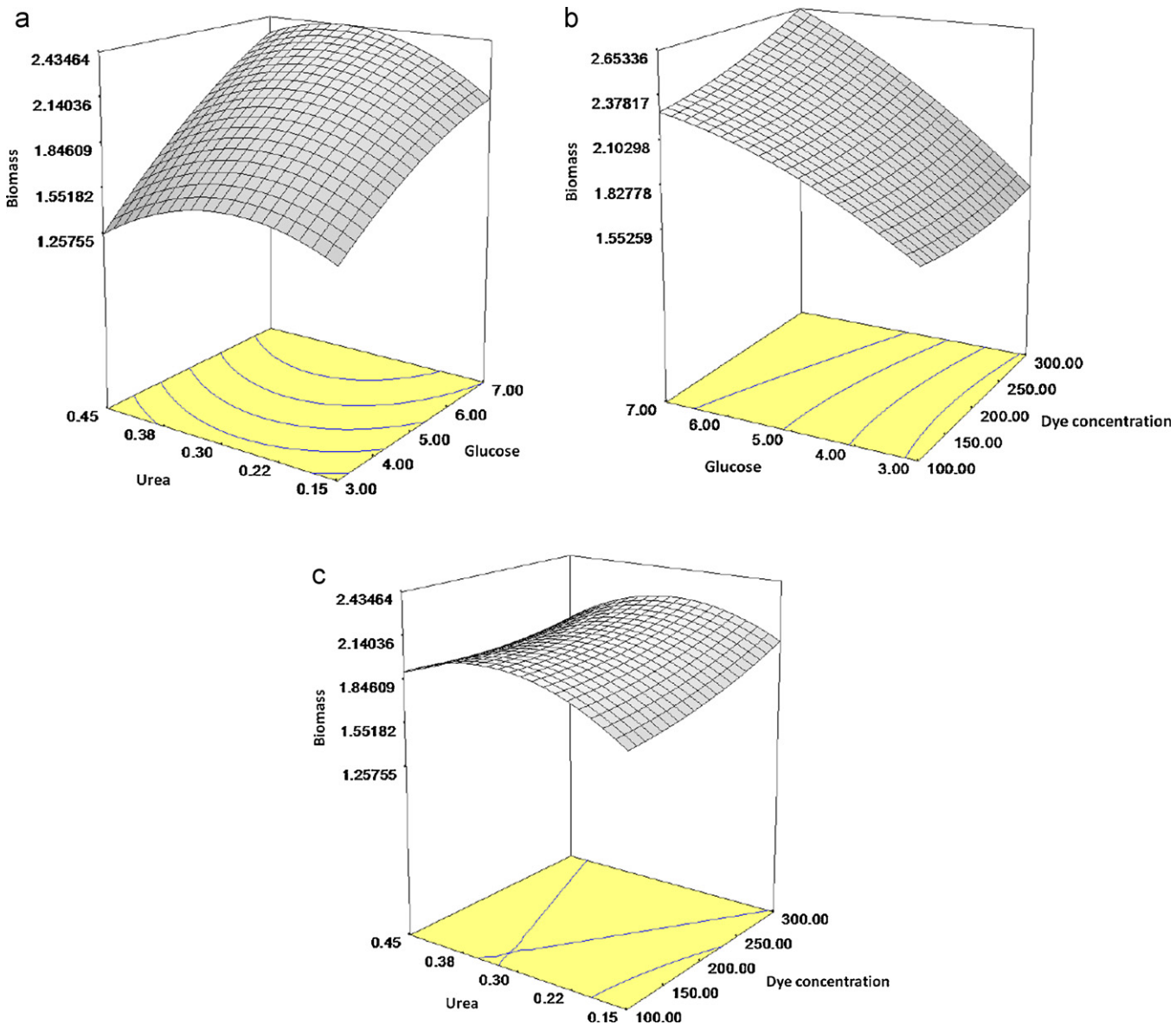

Fig. 4. Response surface curves for biomass production by *A. lentulus* showing interaction between (a) urea and glucose, (b) glucose and dye concentration, (c) urea and dye concentration.

decrease in dye removal efficiency from 94.2% at 0.32 g/l concentration of ammonium chloride to 89% at 3.6 g/l ammonium chloride.

Hence the nutrient requirement for optimum pollutant removal seems to depend on the nature of microbial species employed. However, comparing the present results with that observed recently [5] on the effect of media composition on Cr (VI) removal using the same strain (*A. lentulus*), it can be inferred that the nutrient requirement is not only strain specific but also pollutant specific. In case of Cr (VI), both pollutant removal as well biomass growth are influenced by the single parameter, i.e. yeast extract, while glucose plays only a supportive role in the studied range. On the other hand, in case of the dye, biomass growth is highly influenced by glucose while dye removal is influenced by urea concentration. Also, the amount of nutrients required for Cr (VI) removal are higher than that for dye removal, making the latter

Table 5
Cost comparison of the conventional composite media with optimized modified media for the treatment of one liter dye waste water.

Conventional composite media			Optimized modified media		
Constituent	Amount consumed (g)	Cost (INR)	Constituent	Amount consumed (g)	Cost (INR)
Glucose	10	2.56	Glucose	5	1.28
Yeast extract	2.5	8.75	Urea	0.3	0.08
Ammonium nitrate	0.5	0.13	Ammonium chloride	0.2	0.05
Di-potassium hydrogen phosphate	0.5	0.05	Di-potassium hydrogen phosphate	0.5	0.05
Magnesium sulphate	0.1	0.24	Magnesium sulphate	0.1	0.24
Total cost/l dye wastewater		11.73			1.70

process more economical. This behavior can be explained on the basis of relative toxicity of the pollutants as Cr (VI) is highly toxic while the dye used here is non-toxic. These results indicate that nutrient supplementation for pollutant removal must be regulated depending upon the nature and concentration of the pollutant in waste streams.

5. Conclusions

From the optimization studies it is concluded that the process of dye removal by *A. lentulus* is affected by process variables such as nutrient (urea and glucose) concentration and dye concentration. Glucose influence the process by affecting the biomass production and urea affects the process by influencing the dye uptake. The optimum conditions for obtaining 99.97% dye removal are glucose 5 g/l, urea 0.3 g/l and initial dye concentration 200 mg/l. Under these optimum conditions, *A. lentulus* is able to bring about complete dye removal within minimum time and with maximum dye uptake capacity. To conclude, the simple optimized media developed in this study is able to perform better than complex media based on yeast extract and higher concentration of glucose, thereby signifying substantial reduction in the process cost.

Acknowledgements

Financial assistance from Department of Science & Technology, Government of India and CSIR Senior Research Fellowship to one of the authors (PK), are gratefully acknowledged. Authors would also like to thank Dr. Shweta Sharma for her kind help rendered during the manuscript preparation. Mr. Sabal Singh (IIT Delhi, India) is thankfully acknowledged for his assistance in experimental work.

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