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Decolorization of anthraquinone-based dye (Vat Brown R) by *Pseudomonas aeruginosa* NCH - Optimization and kinetic study

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The objective of this research work is related to the fact that the source of isolation and acclimatization process influences the microorganism's potential for the decolorization of various substances. Some of the widely used anthraquinone vat dyes decolorization by the pure bacterial strain is a significant aspect that will assist in the in-situ bioremediation of the ecosystem.The present study is to evaluate the enhanced decolorization of Vat Brown R by an isolated bacterium, *Pseudomonas aeruginosa* NCH, from textile dye wastewater under aerobic conditions. The effect of pH, temperature, and inoculum size was optimized using response surface methodology with the box-behnken experimental design. The strain NCH showed maximum decolorization efficacy under optimum conditions at pH9.76, temperature 34.69°C, and an inoculum size of 9.51% (*v/v*), respectively. A decolorization of 90.34% was observed with 100 mg $L^{-1}$of Vat Brown R within 18h under these conditions. Confirmatory experiments have verified the optimum combination of the three variables predicted by RSM. Kinetics study was carried out using various approaches: Michaelis-Menten (V_{max} = 29.1 mg L^{-1} h^{-1} and K_m = 25.2 mg L^{-1}), Lineweaver-Burk (V_{max} = 30.12 mg L^{-1} h^{-1} and K_m = 26.91 mg L^{-1}), and Eadie-hofstee model (V_{max} = 30.23 mg $L^{-1}h^{-1}$and K_m = 27.29 mg L^{-1}), and the results showed that the degradation followed a first-order reaction kinetics. The subsequent degradation of the dye and the formation of metabolites were studied using analytical techniques such as UV-vis spectroscopy and FT-IR analysis. UV-vis spectroscopy validated the detoxification of the dye and confirmed that *Pseudomonas* sp. NCH overcomes this decolorizing activity through biodegradation. This study investigated the highest decolorization efficiency of strain NCHused in the biodegradation of wastewaters containing anthraquinone dyes.

Keywords: Biodecolourization, Kinetics, Optimization, *Pseudomonas aeruginosa* NCH, Response surface methodology, Vat Brown R

The tremendous development in color manufacturing and textile industries has resulted in increased pollution of the water bodies released into the environment, making it a primary source of severe environmental pollution[1,2]. The textile effluent generates mostly a large quantity of unfixed dye (5 to 50%) that is often lost during the dyeing process and eventually enters the environment[1]. As the dyes are highly recalcitrant and toxic compounds with minimal degradation efficiency[3], they cause aesthetic problems in water bodies. They can cause environmental toxicity and severe human health risk by entering the food chain. Anthraquinone dyes are the most commonly used synthetic dyes,causing significant contamination of textile wastewater. They are characterized by the presence of chromophoric groups formed by the conjugation of C=O with C=C[4,5].These dyes are commonly used in textiles, paper, tannery, plastics, food, and many other industries. These dye molecules are highly stable due to the presence of fused aromatic rings, making them resistant to degradation by conventional treatment methods[6-8].

The treatment of textile wastewater was performed using various physicochemical techniques[9-11]. Such approaches are cost-intensive, and less efficient, generating secondary pollutants, and sludge, which iscomplex to degrade[12,13]. These drawbacks have encouraged research into new methods for the treatment of textile wastewaters, such as biological techniques using microorganisms that are cost-effective, and environmentally friendly, with less sludge formation[14-17]. It has been reported that fungi and bacteria are used to treat textile effluents. The use of bacterial culture is advantageous as they have a faster growth rate and need less time for decolorization.

In general, the effects of process variables on microbial decolorization are optimized by using the one-factor-at-a-time (OFAT) method by keeping one

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variable constant[18,19]. OFAT method resulted in an inaccurate experimental run,and the overall impact of the influencing parameters could not be identified. However, Response surface methodology (RSM) is a statistical design approach that can evaluate the linear, interactive, and quadratic effects of the variables on response. It predicts a mathematical model with a minimum experimental run and is used in optimizing the process variables for decolorization.

Vat Brown R is the most extensively used anthraquinone dye for cotton and silk dyeing. The objective of this study is to isolate a novel bacterial strain to decolorize Vat Brown R dye. The effect of the process parameters (pH, temperature, and inoculum size) was analyzed on the dye decolorization efficacy. A Box-behnken design (BBD), together with RSM, has been used subsequently to achieve optimal process parameters, validated through observed responses. No literature on decolorization, parameter optimization, and metabolites identification of Vat Brown R using pure bacterial strain is available. The metabolites derived throughout degradation have been estimated using UV-visible spectroscopy and FT-IR analysis.

Materials and Methods

Dyes and Media

Vat Brown R dye (98% pure), procured from Sigma-Aldrich, India, was used in this study. Hi-Media Laboratories (India) supplied nutrient agar and nutrient broth. $NaNO_3$, K_2HPO_4, $MgSO_4$, KCl, Yeast Extract, and glucose (all are 99% pure) were purchased from Merck (India). The composition of liquid mineral-base medium included: $NaNO_3$ (0.3%), KCl (0.05%), $MgSO_4$ (0.05%), K_2HPO_4 (0.1%), and Yeast Extract (0.02%) with glucose (1%). The solid mineral-base medium was prepared by adding 2.5% (*w/v*) agar into the media[20]. The alkaline pH of the media was maintained by using sterilized NaOH (1 M).

Isolation and Identification

Textile wastewater sample was collected from local textile processing units outlet and dye contaminated soil from Odisha, India, in sterile plastic bags. It was used to isolate Vat Brown R decolorizing bacteria by enrichment culture technique with a 50 mg L^{-1} dye concentration in the liquid mineral-base medium[21]. The concentration of dye was subsequently increased from 50 mg L^{-1} to 500 mg L^{-1} at each incubation step. Isolates showing higher decolorization efficacy were selected for further studiesand were preserved at 4°C in glycerol stocks. Vat Brown R dye decolorizing bacteria were characterized by Bergey's Manual of Systematic Bacteriology, based on their biochemical and morphological characteristics [22]. The 16S rRNA gene sequence was used to perform the NCBI Genbank database search for the BLASTn alignment. The distance matrix and the phylogenetic tree were generated using the RDP database and MEGA 10 software. The isolated strain's partial 16S rRNA sequence was deposited with NCBI, GenBank.

Biodecolorization assays

The decolorization studies were performed in 250 mL Erlenmeyer flasks containing 100 mL of liquid mineral-base medium. The medium was inoculated with a 24 h grown *P. aeruginosa* NCH culture. A dye concentration of 100 mg L^{-1} was added aseptically to the medium and incubated at 37°C under shaking conditions. A 2 mL aliquots were withdrawn at the regular interval and centrifuged at 8000 rpm for 10 min. The supernatant was used to determine the decolorization efficiency of Vat Brown R by monitoring spectrophotometrically at 610 nm.

Response surface methodology optimization of process variables

RSM is an assembly of statistical and mathematical designs suitable for modeling and evaluating the effect of process variables on the response[23]. The correlation between multiple experimental design variables and the response function can be determined. RSM was applied to determine the optimal ranges of three input variables *viz*. pH, temperature (°C), and inoculum size (% (*v/v*)) by ''Design-Expert,'' version 7.0 (statistical software), Stat-Ease, Minneapolis, USA. The variables range was obtained from the experiment results of decolorization assays (Table 1).

A total of 17 experimental runs involving 12 factorial points and 5 center points were obtained using a box-behnken design matrix (BBD). Table 1 shows the ranges of independent variables along with the coded and actual levels.

Table 1 — Independent variables and their coded levels were used for the optimization of Vat Brown R by strain NCH.

Independent variables	Symbols	Coded and Actual Levels		
Coded level		Low (−1)	Middle (0)	High (+1)
pH	A	8	10	12
Temperature (°C)	B	30	35	40
Inoculum Size (% *v/v*)	C	5	10	15

The response function (Y) predicted by using a second-order polynomial regression equation is expressed by Eq. (1):

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i x_i + \sum_{i=1}^{k} \beta_{ii} x_i^2 + \sum_{i=1}^{k-1} \sum_{j=2}^{k} \beta_{ij} x_i x_j \quad \ldots (1)$$

where Y indicates the obtained response (% decolorization), β_0 represents a constant term for the response with x_i value zero for each variable. β_i gives the linear effect of the input factor, x_i, β_{ii}gives the square impact of the input factor, x_i, and β_{ij} gives the quadratic effect of the input factor $x_i$and x_j that indicates the variables affecting the characteristics of the process[24]. To know the individual and the interactive effects of the variables, the 3-D surface graphs, and their corresponding contour plots were used.

Analytical methods

The residual dye before and after treatment was quantified using UV-vis spectroscopy (Shimadzu UV-1800) in the visible range. The absorbance was taken at a wavelength of 610 nm. Aliquots (2 mL) were withdrawn from this decolorizing sample at a regular interval, and the cell was harvested by centrifugation at 8000 RPM for 10 min.

Fourier Transform Infrared Spectroscopy (FT-IR) was carried out using the Thermo Fisher Scientific, Nicolet IS10 in ATR mode (with the 16-scan speed in the mid-IR range of 400 to 4000 cm^{-1}) to analyze the biodegradation of the pure form of the dye (before degradation) and its metabolites were obtained (after degradation).

Results and Discussion

Isolation and Identification of Vat Brown R decolorizing strain

A total of eight isolates, obtained from enrichment culture were inoculated in liquid mineral-base medium containing 50 mg $L^{-1}$of Vat Brown R. The dye concentration was increased subsequently from 50 mg L^{-1} to 500 mg L^{-1} at each step of incubation. Isolates showing high tolerance and degradation efficacy (≥ 80%) were selected for further study. Strain NCH was able to tolerate up to 500 mg L^{-1} Vat Brown R dye by degrading ≥ 95% of 100 mg L^{-1} within 18 h. The strain NCH was found to be a gram-negative, rod-shaped, non-spore-forming bacterium. The bacterium formed bluish-green, concave, irregular-shaped colonies and demonstrated positive results for oxidase, catalase, and citrate test. On 16S rRNA sequencing and phylogenetic analysis, this NCH strain was identified to be *Pseudomonas aeruginosa* (Fig. 1). The sequence of the strain was deposited in the gene bank with accession numberMN587986.

Optimization of process parameters to achieve maximum decolorization of Vat Brown R by *Pseudomonas aeruginosa* NCH

The BBD with three variables (pH, temperature, and inoculum size) was selected for the optimization of the decolorization process. The optimal operating variables were estimated from a second-order polynomial function. Table 2 describes the design matrix of independent parameters, along with the predicted and observed values of the response. The observed values were analyzed for removal of Vat Brown R and represented as decolorization percentage. A reduced model was considered, which includedstatistically significant independent variables (P <0.05). The second-order polynomial equation (in coded units) was derived by implementing multiple regression analysis, which might correspond to the dye decolorization and the parameters studied.

$$Y = +89.69 - 6.69\,A - 4.03\,B - 2.41\,C + 6.88\,AC + 2.10\,BC - 30.65\,A^2 - 33.30\,B^2 - 17.31\,C^2 \quad \ldots (2)$$

where Y: predicted response (decolorization), A: pH (coded values), B: temperature (coded values), and C: inoculum size (coded values) have been obtained from a second-order polynomial equation.

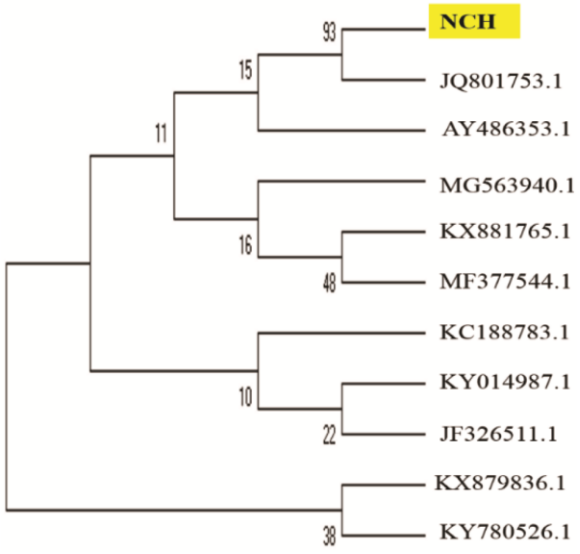

Fig. 1 — Phylogenetic tree showing the evolutionary relationship of 'NCH'strain with 10 taxa using the Neighbor-Joining method

Table 2 — Experimental design matrix, along with the observed and predicted values.

Runs	pH	Temperature	Inoculum size	Decolourization (%)	
	A	B	C	Observed	Predicted
1	+1	0	+1	38.75	39.51
2	0	0	0	89.40	89.69
3	−1	0	−1	58.46	57.70
4	0	−1	+1	40.11	38.60
5	−1	−1	0	35.24	36.02
6	+1	0	−1	31.29	30.57
7	+1	−1	0	22.76	23.50
8	0	+1	−1	33.86	35.37
9	0	0	0	90.40	89.69
10	0	0	0	89.22	89.69
11	0	0	0	90.70	89.69
12	−1	0	+1	38.41	39.13
13	+1	+1	0	15.38	14.60
14	0	0	0	88.75	89.69
15	0	−1	−1	47.63	47.61
16	−1	+1	0	29.57	28.83
17	0	+1	+1	34.72	34.74

Table 3 — ANOVA of the quadratic regression model

Factors	Sum of Squares	Degree of freedom	Mean Square	F- Value	*P*-value Prob> F	Remarks
Model	11688.27	8	1461.03	934.38	< 0.0001	Significant
A-pH	357.78	1	357.78	228.81	< 0.0001	
B-Temperature	129.69	1	129.69	82.94	< 0.0001	
C-Inoculum Size	46.32	1	46.32	29.62	0.0006	
AC	189.20	1	189.20	121.00	< 0.0001	
BC	17.56	1	17.56	11.23	0.0101	
A^2	3956.62	1	3956.62	2530.40	< 0.0001	
B^2	4669.57	1	4669.57	2986.36	< 0.0001	
C^2	1261.92	1	1261.92	807.04	< 0.0001	
Residual	12.51	8	1.56			
Lack of Fit	9.80	4	2.45	3.61	0.1207	not significant
Pure Error	2.71	4	0.68			
Correlation Total	11700.78	16				

R^2 = 0.9989, Pred. R^2 = 0.9894, CV = 2.43%, PRESS = 124.19

Table 3 illustrates the ANOVA (analysis of variance) of the quadratic regression model, showing that the predictability of the model is at a 95% confidence interval. The F-value of 934.38 with a low p-value shows the model's statistical significance. The p-value below 0.05 suggests that the parameters of the model are significant (i.e., A, B, C, AC, BC, A^2, B^2, and C^2 in this study), while values above 0.10 are not significant. The ''Lack of Fit F-value'' of 3.61 infers its non-significance compared to that of the pure error. There may be a 12.07% probability that this higher ''Lack of Fit F-value'' may be attributed to noise, interpreted as a good indicator in which the model can fit. The predicted R^2 and adjusted R^2 are in good agreement with the values of 0.9894 and 0.9979, respectively.

The R^2 (coefficient of regression) of 0.9989 nearer to 1, signifies that the model could interpret 99% of the uncertainty in the response and fits well with the actual values. The signal-to-noise ratio is determined by the ''Adeq Precision''. It is desirable to have a ratio higher than 4. In this case, the ratio is 82.070 suggesting an appropriate signal for navigating the design space. ANOVA analysis determined the acceptability of the predicted experimental data.

The interactions between the variables, 3-D plots, and contour plots were studied. The response surface plots were presented in (Fig. 2A-D) together with the contour plots showing the response behavior by changing two variables simultaneously. The contour plots with elliptical shapes showed significant interactions between the variables[23,24].

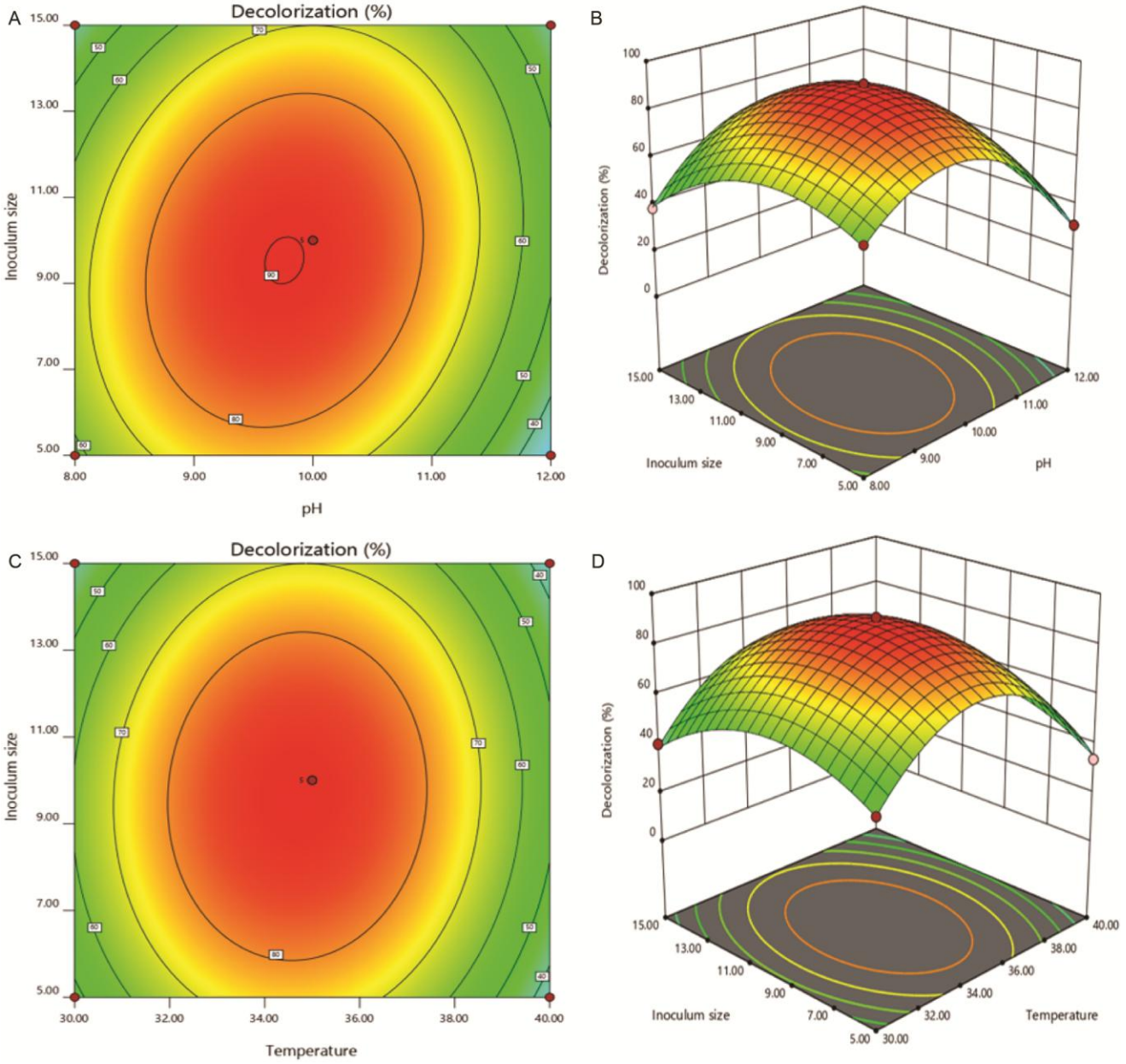

Fig.2 (A-D) — Three-dimensional response surface plot and its corresponding contours for the effect of pH, temperature, and inoculum size on decolorization of Vat Brown R by *P. aeruginosa* NCH

Figure 2A & B illustrates the 3-D and contour plots, depictingthe interaction betweeninoculum size and temperature. Decolorization percentage increases with an increase in temperature (35 to 40°C) and inoculum size (7 to 9% (*v/v*)). Reduced decolorization percentage was found for less inoculum size as compared to high inoculum size. Higher inoculum size contributes to the optimum use of the free dye molecules, resulting in increased decolorization efficiency. Figure 2A & B reveals a synergic interaction between the inoculum size and the temperature.

Figure 2C & D shows the interaction between inoculum size and pH. The decolorization percentage increased with an increase in inoculum size from 7% to 9% (*v/v*) and pH from 9 to 10.5. Further,the increase in both the parameters resulted in a decrease in decolorization percentage. The 3-D and the contour plots represent the synergistic effect between the process variables towards the use of higher inoculum size and pH.

Model Validation and Confirmation

The decolorization studies were designed using

Table 4 — Model validation and confirmation were conducted at optimum conditions

Solution	pH	Temperature (°C)	Inoculum size (%) (*v/v*)	Predicted response (%)	Observed response (%)	Desirability
1	9.80	39.60	8.27	89.74	79.32	0.995
2	10.03	31.50	10.32	91.23	82.44	0.995
3	9.76	34.68	9.51	90.34	89.45	0.995 (selected)

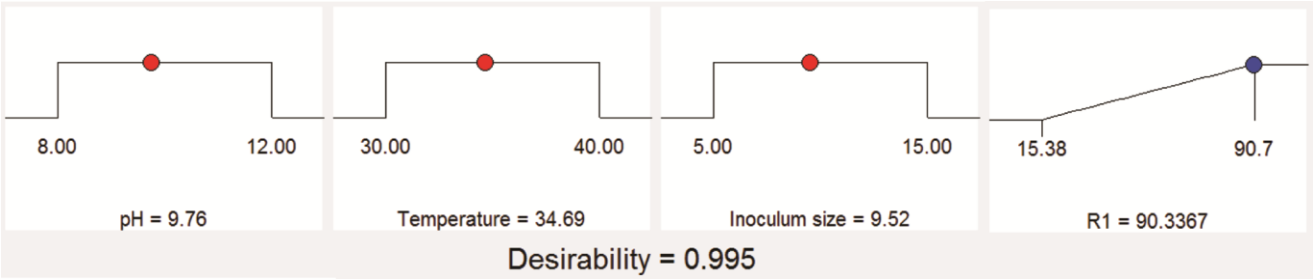

Fig. 3 — Optimized parameters for decolorization of Vat Brown R by *P. aeruginosa* NCH

selected optimum parameters to evaluate the fitness of the model. The model validation was performed through three confirmatory experiments to assess the adequacy of the predicted response, as shown in (Table 4). For further studies, one of the solutions was selected to validate the experimental data. The optimum parameters for Vat Brown R decolorization were: pH: 9.76, temperature 34.68°C, and inoculum size 9.51% (*v/v*) with the desirability of 0.995 (Fig. 3). A maximum decolorization efficiency of 90.34% was achieved within 18 h of incubation. Thus, the model developed was considered to be accurate and reliable.

Degradation kinetics for determination of K_m and V_{max}

Figure 4 demonstrates Vat Brown R decolorization at different dye concentrations (25 mg L^{-1} to 500 mg L^{-1}). This study was conducted with 100 mg L^{-1} of Vat Brown R under optimized conditions. A decolorization efficiency of 90.34% was observed with pH 9.76, a temperature of 34.68°C, and an inoculum size of 9.51% (*v/v*). It has been observed that the decolorization rate decreases as the initial dye concentration increases, which may be attributed to the efficacy of the enzymes in identifying the dye molecule as a substrate[25,26].

Michaelis-Menten model, Lineweaver-Burk model, and Eadie-Hofstee model were attempted in this study to fit the data obtained from batch studies using *pseudomonas aeruginosa* for dye degradation.

Michaelis-Menten model

The Michaelis-Menten model can be used to represent the kinetic reactions, as the decolorizing enzyme is associated with Vat Brown R degradation. This model fits the experimental data quite well, as shown in (Fig. 5A). It is possible to access the equation coefficient

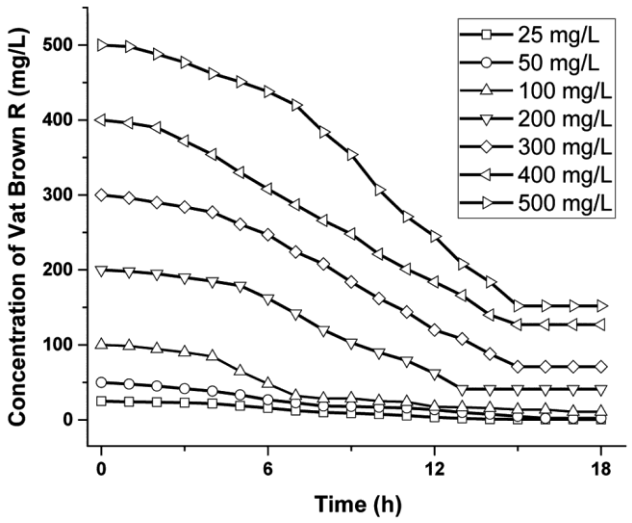

Fig. 4 — Decolorization pattern of Vat Brown R at different concentrations

by determining the sum of squared deviations and minimizing it. The results obtained are: ½V_{max} = 14.55mg L^{-1} h^{-1}, K_m = 25.2 mg L^{-1}, V_{max} = 29.1 mg L^{-1} h^{-1}.

Lineweaver-Burkmodel

The Lineweaver-Burk equation was obtained through a double-reciprocal approach by modifying the Michaelis-Menten equation. A 1/V *vs.* 1/S plot gives 1/V_{max} as intercept when 1/S reaches zero and −1/K_m as intercept when V reaches zero (Fig. 5B). By estimating the sum of squared deviations and minimizing them, it is possible to obtain another set of K_m and V_{max}. The values obtained are: K_m = 26.91 mg $L^{-1}$and V_{max} = 30.12 mg L^{-1} h^{-1}.

Eadie-Hofstee model

The use of the Eadie-Hofstee plot was the other

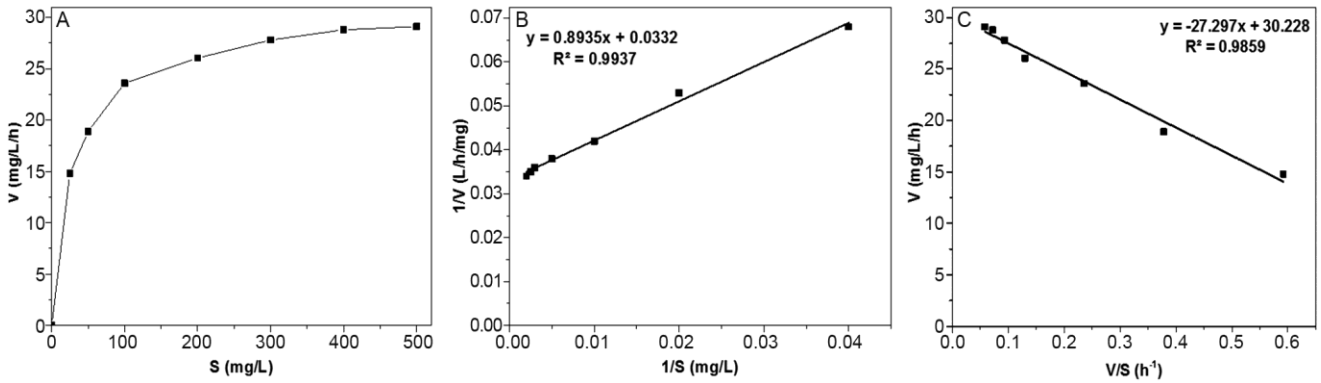

Fig. 5 — Degradation kinetics of Vat Brown R: (A) Michaelis-menten model; (B) Lineweaver-burk model; and (C) Eadie-hofstee model

Table 5 — The correlation of Km and Vmax values was obtained using three different kinetics models

Model	K_m (mg L^{-1})	V_{max} (mg L^{-1} h^{-1})
Michaelis-Menten	25.2	29.1
Lineweaver-Burk	26.91	30.12
Eadie-Hofstee	27.29	30.23

way of determining K_m and V_{max}. By rearranging the Michelis-Menten equation, V *vs.* V/S was plotted, which gives a straight line with V_{max} as intercept and $-K_m$ as a slope (Fig. 5C). By determining the sum of squared deviations and minimizing them, another set of K_m and $V_{max\ was}$ obtained with the values of 27.29 mg L^{-1} and 30.23 mg L^{-1} h^{-1}, respectively.

Table 5 displays a comparison of K_m and V_{max} values using the above three models. It can be concluded from the results that the decolorization of Vat Brown R by NCH is an irreversible process with first-order reaction kinetics.

Biodegradation analysis of Vat Brown R

A batch study with 100 mg L^{-1} of Vat Brown R was carried out to depict the possible mechanism involved in decolorization. UV-vis spectral analysis (200-800 nm) of the supernatant at different time intervals was performed. A λ_{max} (maximum absorbance) of 610 nm was observed remarkably with a gradual decrease in absorbance as well as dye concentration. Figure 6 illustrates the change in UV-Vis spectra of the dye before and after decolorizing cultivation with *P. aeruginosa*. The disappearance of the significant absorbance peak and color in the pellet leads to the conclusion that *P. Aeruginosa*decolorizes Vat Brown R dye efficiently. The decolorization might be due to biodegradation associated with bioabsorption[27-30].

Figure 7A and B) illustrates the FT-IR spectrum

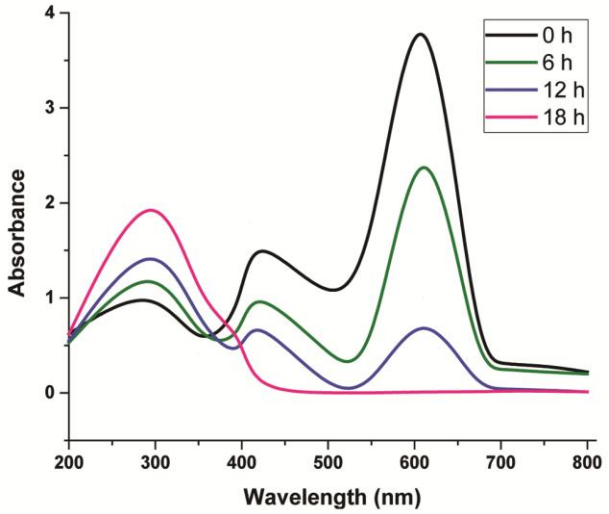

Fig. 6 — UV-vis spectrum for decolorization of Vat Brown R (100 mg L^{-1}) by *P. aeruginosa* NCH

of Vat Brown R dye (100 mg L^{-1}) before and after decolorization. The spectrum of Vat Brown R dye shows the appearance of the peak around 3508.3 cm^{-1} and 3086 cm^{-1} are of -OH stretching vibration, with H-bonded and =C-H stretching vibration of the aromatic group, respectively. The peak at 2865.7 $cm^{-1}$is due to the C-H symmetrical stretching vibration of the alkane group. Similarly, the peak near 1639.7 $cm^{-1}$and 1323.4 $cm^{-1}$are of C=C stretching of the alkenes group and C-N stretching of the aromatic amines. After decolorization, the spectrum of Vat Brown R metabolites shows the appearance of the new peaks near 1340.2 cm^{-1} and 1640.6 cm^{-1} are of C-N stretching vibration for aromatic amines and C=C stretching vibration of the alkenes group. The peaks around 3207 cm^{-1} and 3434.1 cm^{-1} are of -OH stretching vibration, and N-H stretching vibration of the amines group, respectively. The formation of

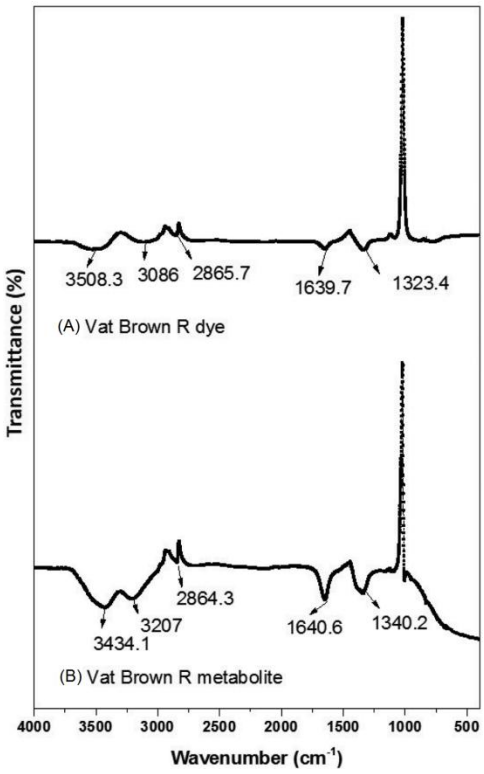

Fig. 7 — The FT-IR spectrum of Vat Green 1 (100 mg/L) before and after decolorization by PMS

various metabolites thus provides evidence of the Vat Brown R mineralization.

Conclusion

In this study, *Pseudomonas aeruginosa* NCH was isolated and found to decolorize textile wastewater containing Vat Brown R. Under aerobic conditions, this isolated strain showed an enhanced decolorization efficiency within 18 h of incubation. The strain NCHwas employed for the optimization experiments using the Box-Behnken design of response surface methodology to achieve maximum decolorization efficiency. Different models were used for the kinetic studies of the textile wastewater decolorization process. Vat Brown R degradation followed first-order reaction kinetics. The UV-vis spectroscopy and FT-IR analysis confirm the decolorization of Vat Brown R by *Pseudomonas aeruginosa* NCH. This approach thus proves to be significant for the treatment of wastewater containing anthraquinone dyes using strain NCH.The overall results of this research work suggested that bacterial strains isolated from dye-contaminated textile wastewater can be used for the degradation of organic contaminants in the environment.

Conflict of interest

All authors declare no conflict of interest.

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