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Original Research

Response Surface Optimization of Vat Blue 4 Degradation Process Using *Pseudomonas aeruginosa* WYT

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Abstract

Vat Blue 4, one of the anthraquinone dyes, were widely used in printing and dyeing industry due to bright color and low price. However, low utilization rate and improper handling lead a large number of dyes discharged into environment with wastewater. But few research report the Vat Blue 4 degradation by bacteria, perhaps too complex structure to biodegraded. In this work, we isolate a new strain *Pseudomonas aeruginosa* WYT which could degrade Vat Blue 4, and the degradation rate is low and unstable. To overcome this shortcoming, single factor and response surface experiments were designed to optimize its culture conditions. The optimal conditions of *Pseudomonas aeruginosa* WYT degradation of VB4 were obtained through this: temperature 35.0ºC, initial biomass 0.06, pH 7.0, glucose 1.0 g/L, urea 1.0 g/L, VB4 50 mg/L and 2 mL/L trace salt. Under these optimized conditions, the maximum degradation rate increased from initial 85% to 97%.

Keywords: anthraquinone dyes, Vat Blue 4, biodegradation, response surface methodology

Introduction

Synthetic dyes are very diverse in chemical structure including azo, anthraquinone, triphenylmethane, phthalocyanine and heterocycle-containing dyes [1, 2]. And about 2-50% of these dyestuffs exist in the effluent due to the low utilization efficiency [3, 4]. These synthetic dyes discharged directly or processed incompletely discharged into the environment, may cause bleeding, skin ulceration, nausea and dermatitis [5-7]. The dyeing wastewater composition is complicated, characterized by strong color, high pH, high chemical oxygen demand (COD), low biodegradability and the changes of water quality in a wide range [8], belong to stubborn wastewater. Therefore, the deep treatment of printing and dyeing wastewater has caused widespread concern. Recently, many researchers reported the degradation processing of synthetic dyes, such as Fenton Oxidation [9], Photocatalytic Oxidation [10, 11], Ozone Oxidation [12, 13], Ultrasonic Catalytic Oxidation [14, 15] and Microwave Catalysis [16], etc. However, these methods are not only costly, but also inefficient and causing

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secondary pollution. Biodegradation of synthetic dyes in wastewater was considered as an efficient, low-cost and environmentally friendly method.

Anthraquinone dyes was the second used dye after azo dye due to its low price, easy access and good dyeing effect. The structure of anthraquinone dyes is complex and stable, and is more toxic to microorganisms compared to azo dyes [17]. Vat Blue 4 (VB4) is one of the most common anthraquinone dye used in textile dyeing industry to dyeing silk and cotton [18], which caused serious pollution to environmental water. Although few researchers using microorganism degradation of anthraquinone [19, 20], but few reported the degradation of VB4 by bacteria. Here, in the present investigation, an attempt was made to isolate efficient VB4-degrading strain and optimize culture condition to improve its biodegradability and stability. The aim of this study was to provide potential bacterial resources for biodegradation of dyeing wastewater.

Pseudomonas aeruginosa are prevalent in a variety of environments, such as the soil, surface of plants, and human body. More importantly, it occupies a large proportion in the wastewater treatment system. In this paper, *Pseudomonas aeruginosa* WYT was isolated to decolorization and degradation VB4 in trace carbon and nitrogen sources. Single factor experiments were used to determine the optimum range of several parameters, such as temperature, glucose concentration, urea concentration, inoculum size and trace salts solution. Then Plackett-Burman Design (PBD) was used for screening the factors that significantly impact VB4 degradation efficiency. Finally, response surface methodology with Central Composite Design (CCD) was subsequently applied to determine the effects of significant parameters, and their interactions effective wereexplore the optimum values.

Materials and Methods

Dyes, Media, Microbes and Growth Conditions

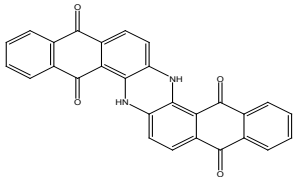
Vat Blue 4, indanthrene vat dye was discovered by R. Bohn in 1901 [21], its chemical name is 6,15-dihydroanthrazine-5,9,14,18-tetrone. VB4 was purchased from Tokyo Chemical Industry (TCI) in Shanghai and its molecular structure is given in Table 1.

VB4 decolorizing strains were isolate from activated sludge, obtained from a dyeing wastewater treatment plant in Xianyang, Shaanxi, China. After the sludge pretreated, the sample was incubated with basal medium (BM) once a week, which containing VB4 from 20 to 100 mg/L for a month to isolate VB4-degrading bacteria. Every domesticated need pipette 5 mL seed solution cultivated in 250 mL flasks containing 100 mL BM, and shaking bath at 30ºC, 150 rpm. The mixed bacterial suspension with degradation effect using spread plate method get monoclonal strains. Monoclonal strains were grown in Luria-Bertani medium and washed with saline 3 times, inoculated into the BM (containing 50 mg/L VB4) to prove the monoclonal strains degradation ability.

Biodegradation of VB4 was done in BM (g/L): KH_2PO_4 1.0, $MnSO_4 \cdot 7H_2O$ 0.02, $Na_2HPO_4 \cdot 12H_2O$ 2.0, $MgSO_4 \cdot 7H_2O$ 0.2, $FeSO_4 \cdot 7H_2O$ 0.01, $CaCl_2$ 0.02, NH_4Cl 0.5, Urea 1.0, Glucose 1.0. trace salt solution 2.0 mL. Trace salt solution(mg/L): $CoCl \cdot 6H_2O$ 4.0, $MnSO_4 \cdot H_2O$ 2.8, H_3BO_3 4.0, $CuSO_4 \cdot 5H_2O$ 0.02, $ZnSO_4 \cdot 7H_2O$ 28.0, MoO_3 4.0. Prior to autoclaving (121ºC, 20 min), using pH detectors (PHSJ-6) adjusted the initial pH to 7.0 with NaOH (1 mol/L) and HCl (1 mol/L). To provide sufficient oxygen for cell growth and VB4 degradation, each flask contained only 40% of its maximum volume of BM, during the optimization processes. All of the monoclonal strains were growing in LB medium and preserved at −20ºC with 30% glycerol.

The colony morphology of the isolated strain was observed on agar plates after incubated at 30ºC for 2 days. Physiological and biochemical characteristics of the VB4-degrading strains were examined using standard method [22]. PCR amplification was initiated with a pre-denaturation step at 98ºC for 2 min; denaturation at 98ºC for 2 s; annealing at 55ºC for 10 s, elongation at 72ºC for 20 s, this process followed by 35 cycles; and a final extension at 72ºC for 2 min. The quality of amplicons was checked by gel electrophoresis using 1% agarose, and sequenced by TSINGKE (Hang Zhou). Then the sequences were analyzed through the BLAST program. Phylogenetic tree was performed by the program MEGA (version 7.0), which bootstrap analysis of the neighbor-joining method based on 1000 resampling.

Table 1. Properties of the dye C.I. Vat Blue 4.

Chemical Structures	Molecular formula	Molecular weight	λmax (nm)	CAS
	$C_{28}H_{14}N_2O_4$	442.095	615	81-77-6

Effect of Different Degradation Parameters

The optimization of culture conditions is important for the development of any biotreatment processes for removing dyes contamination [23, 24]. To explore the effect of different factors on *Pseudomonas aeruginosa* WYT degradation of VB4, single colonies of VB4 discoloration were inoculated and grown in 50 mL flasks containing 10 mL of LB medium at 30ºC and 200 rpm. When the OD_{600} of the culture reached about 4.0, the cells were harvested by centrifugation at 5000×g and 4ºC for 5 min and then washed 3 times with saline. Degradation media in 250 mL flasks containing 100 mL BM and 50 mg/L VB4 was kept for 24 h under shaking condition (175 rpm) at 30ºC and pH 7.0. In the first experimental set using One-Factor-at-a-Time (OFAT) approach, various factors comprising temperature (20, 25, 30, 35 and 40ºC), initial biomass (λ_{600} = 0.00, 0.02, 0.04, 0.06, 0.08, 0.1), pH (4, 5, 6, 7, 8, 9, 10), glucose concentration (0.0, 0.5, 1, 1.5, 2 and 2.5 g/L), urea concentration (0.0, 0.5, 1, 1.5, 2 and 2.5 g/L) and initial VB4 concentration (50,100, 200, 300, 400, 500 mg/L). Moreover, all of these parameters (including temperature, initial biomass, pH, glucose and urea concentration) were accurate to 2 digits after the decimal point. In each experiment, one factor was changed, with the other factors remaining constant. The effects of these factors were evaluated by measuring the VB4 degradation rate and biomass after 24 h of culture, where each experiment was triplicated.

Plackett-Burman (PB) Design for Main Effect Factors Selection

The PB experimental design was used to evaluate the relative importance of various factors for VB4 degradation. This design assumes that there are no interactions between the different factors, in the range of variables under consideration. The theoretical linear model of the PB design is shown as follows:

$$Y = a_0 + c_0 CP + \sum b_i c_i + \varepsilon \quad (1)$$

Where Y is the estimated target function, a_0 represents the intercept, b_i is the regression coefficients while ε is the experimental error. The CP indicates whether the experiment was a center point (0) or a non-center point (±1). The c_0 term is the gross curvature effect and indicates the degree to which the center points differ from the non-center points. A big coefficient, both plus or minus, indicates that this factor has a great influence on the response value, while the coefficient close to 0 indicates that the factor has little or no effect on the response value [25, 26]. Here, it was used for screening the factors that would accelerate VB4 degradation. Seven external influence factors (temperature, initial biomass, pH, glucose concentration, urea concentration, dyes concentration and trace salt) were chosen as factors based on previously single factor experiments. These factors, along with their low and high factor levels, are provided in Table S1. The rows in Table S2 show 12 different trials and each column represents a different variable with replicated three times. The statistical analyses were performed by use of multiple regressions and ANOVA with the Minitab 17.

Response Surface Methodology for Optimizing the Degradation Conditions

The response surface methodology with CCD is regularly used to statistically evaluate the main and interactive effects of variables and to optimize the parameters of biotreatment processes [27]. In this experiment, VB4 decolorization conditions were optimized by CCD. The standard polynomial regression method is used to fit the experimental data of CCD design, and a two-order polynomial is obtained:

$$Y = \beta_0 + \sum_{i=1}^{n} \beta_i \chi_i + \sum_{i=1}^{n} \beta_{ii} \chi_i^2 \sum \sum_{i<j} \beta_{ij} \chi_i \chi_j + \varepsilon \quad (2)$$

where Y is the predicted response (dye removal, %); χ_i and χ_j represent independent variables; χ_0 is the compensation term while ε is the experimental error. The coefficient β_i, β_{ii} and β_{ij} represents the linear, quadratic and interaction coefficient, respectively [28]. Based on the PB experiment, selected A-temperature (27, 30, 35, 40, 43ºC), B-pH (5.32, 6.00, 7.00, 8.00, 8.68) and C-urea concentration (0.4, 0.6, 0.9, 1.2, 1.4 mg/L) as the dependent variable to improve the VB4 decolorization rate. Table S3 shows the definition and levels of each variable. A total of 20 experiments were carried out according to a 20 full factorial CCD, 1.681 was fixed as a value. The matrix of CCD and the results (experimental and predicted) for the response variable are given in Table 2. Experimental design and analysis were performed with Design-Export Software (8.0).

Biomass and Color Removal Studies

Biomass is one of the important factors affected dye decolorization rate [29]. In the process of VB4 degradation, the absorbance of dyes will seriously affect the measurement of biomass in the spectrophotometer (λ = 600 nm). In this research, the dry weight of cells is selected as an indicator of biomass. The measurement of the dry weight of the cells is by centrifuging all the cultures, then drying to the constant weight with a vacuum freeze-drying instrument and weighing the simple with balance. As to the measurement of degradation rate, need withdrawn the culture solution after cultured, and then centrifuged at 10,000×g for 10 min to analyze VB4 decolorization by comparing the absorbance values recorded on UV–VIS (Mapada, UV-3100, China) spectrophotometer at 615 nm. To investigate the extent of decolorization, VB4 was treated with *Pseudomonas aeruginosa* WYT

Table 2. Operational variables for CCD matrix and observed and predicted responses.

Run order	Variables			Dye removal (%)	
	A-Temperature (°C)	B-pH	C-Urea concentration (mg/L)	Observed	Predicted
1	35	7	1.4	69.04	64.25
2	30	8	0.6	55.67	52.30
3	35	7	0.9	99.67	97.24
4	30	6	0.6	24.00	20.96
5	40	8	1.2	50.26	46.21
6	35	7	0.9	93.04	97.24
7	43	7	0.9	33.35	33.13
8	35	7	0.9	97.93	97.24
9	35	7	0.9	98.04	97.24
10	40	8	0.6	67.51	67.44
11	30	6	1.2	61.33	60.40
12	35	5.32	0.9	19.78	22.49
13	40	6	1.2	24.84	27.22
14	40	6	0.6	15.09	10.30
15	35	7	0.9	99.34	97.24
16	27	7	0.9	46.66	45.22
17	35	8.68	0.9	71.76	69.87
18	35	7	0.4	37.63	43.86
19	35	7	0.9	95.69	97.24
20	30	8	1.2	55.87	59.66

at different time intervals (0 , 4 , 8 , 12 , 16 , 20 and 24 h) and scanned from 300 to 900 nm wavelengths using a UV–vis spectrophotometer. VB4 decolorization rate was taken as the optimization target. It was calculated as the following formula [30]:

$$\text{VB4 Degradation rates (\%)} = \frac{\text{Initial absorbance} - \text{Absorbance after degradation}}{\text{Initial absorbance}} \quad (3)$$

Results and Discussions

Isolation and Identify of VB4 Degradation Strains

In recent years, the research of VB4 degrading were only microflora [21] and no one using single strain, perhaps the compound is too complex and toxicity to biodegrade. The sludge around drainage outlet were long-term scoured by dyeing wastewater select as the sample. Five strains (I, II, III, IV, V) were isolated as the most VB4 degraders, which decolorization rate arrive 85 %, 70 %, 35 %, 95 % and 90 % respectively. Fig. 1a) is the degradation effect of five strains on VB4 at 24 h, 30ºC. Strains that do not produce the pigment is screened to remove the color of the dye waste water, otherwise the pigment produced by the strain will lead second pollution. The monoclonal of five strains as shown in Fig. 1b).

In order to screen out the dominant strains for the degradation of VB4 in the environment, we using 16S rRNA amplicon sequencing analysis the flora structure of the sludge, find that the proportion of the strain *Pseudomonas aeruginosa* WYT(V) in the process of VB4 degradation rate is higher. Therefore, this strain was selected as the research object to degradation of VB4. The strain *Pseudomonas aeruginosa* WYT colonies grown on LB plate are light green, smooth on the surface, trim on the edge and Gram-negative. To further identify the strain *Pseudomonas aeruginosa* WYT and others, the nucleotide sequences of 16S rRNA were analyzed and compared with those of related strains in NCBI (National Center for Biotechnology Information). Fig. 2 showed the phylogenetic relationship between the strain *Pseudomonas aeruginosa* WYT and other related microorganisms found in the GenBank database. Phylogenetic analysis showed that strain *Pseudomonas aeruginosa* WYT was most closely related to members of the genus

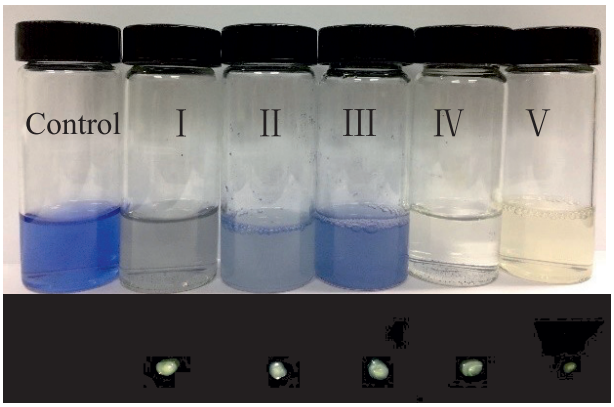


Fig. 1. The five strains isolated of VB4 degradation. a) Effect of five strains degradation on VB4 within 24 h. b) The monoclonal of five strains.

Pseudomonas, especially to the species *Pseudomonas aeruginosa* S25 (DQ095913.1), displaying a sequence similarity of 99.0%. Consequently, the phenotypic and phylogenetic characteristics of isolate stain belonged to the genus *Pseudomonas aeruginosa*. The other four strains were *Stenotrophomonas* sp. (I), *Pandoraea* sp. (II), *Paracoccus* sp. (III), and *Serratia marcescens* (IV) respectively.

Effect of Temperature

The anthraquinone dyes degradation process is significantly influenced by operational parameters (both intrinsic and extrinsic factors). In general, the influences of each factors have been investigated by numerous searchers to improve the efficiency of bacteria degradation dyes [19, 31, 32]. In previous studies, there is few used *Pseudomonas aeruginosa* degradation VB4, and the optimal condition was unknown. In this paper, we refer to the conditions of bacteria degradation of anthraquinone dyes, and explore the effects of each single factors on *Pseudomonas aeruginosa* WYT degradation of VB4.

Temperature plays a crucial role in bacterial growth, reproduction, and enzyme stability. Some studies had shown that the optimum temperature range for discoloration of dyes is consistent with the bacterial growth temperature range of 25-37ºC [33-35]. Therefore, optimum temperatures of *Pseudomonas aeruginosa* WYT decolorization VB4 were cultured in 100 mL BM, which was supplemented 50 mg/L VB4 as a carbon source and grown at different temperatures (20-40ºC) for 24 h at 175 rpm in a shaker incubator. The final degradation rate and biomass as shown in Fig. 3a), which similar to others

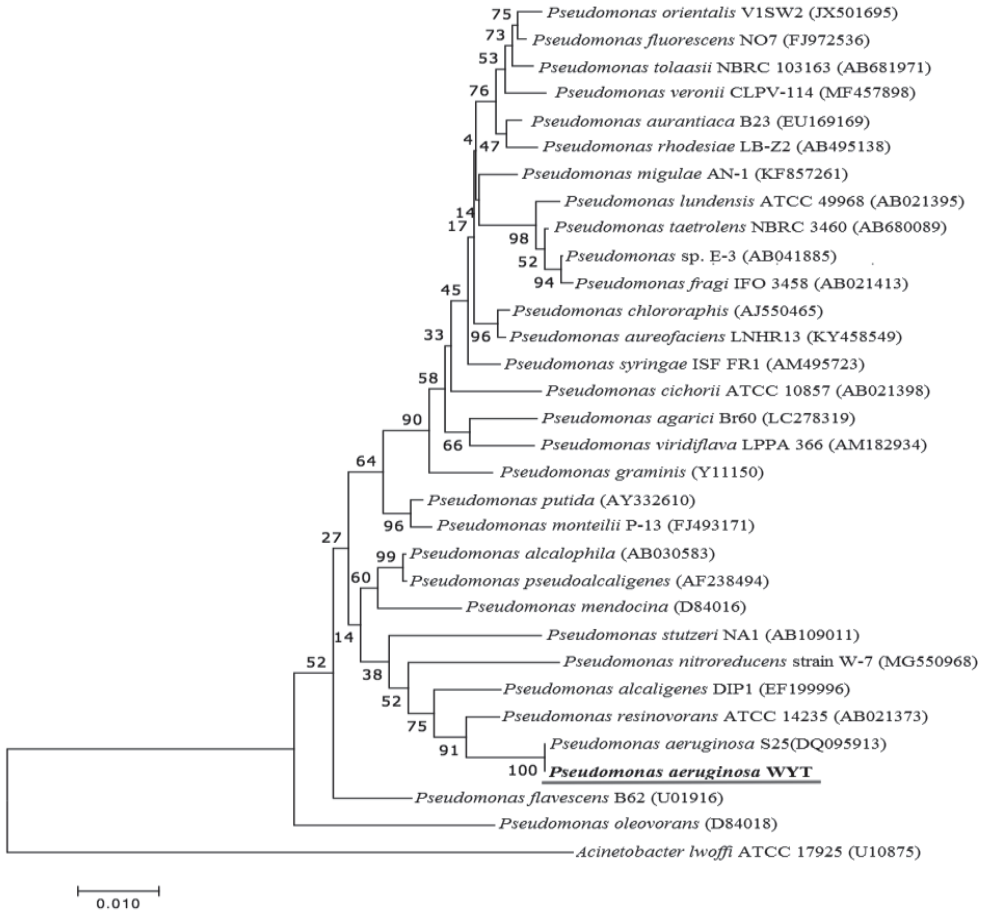


Fig. 2. The unrooted tree of *Pseudomonas aeruginosa* WYT.

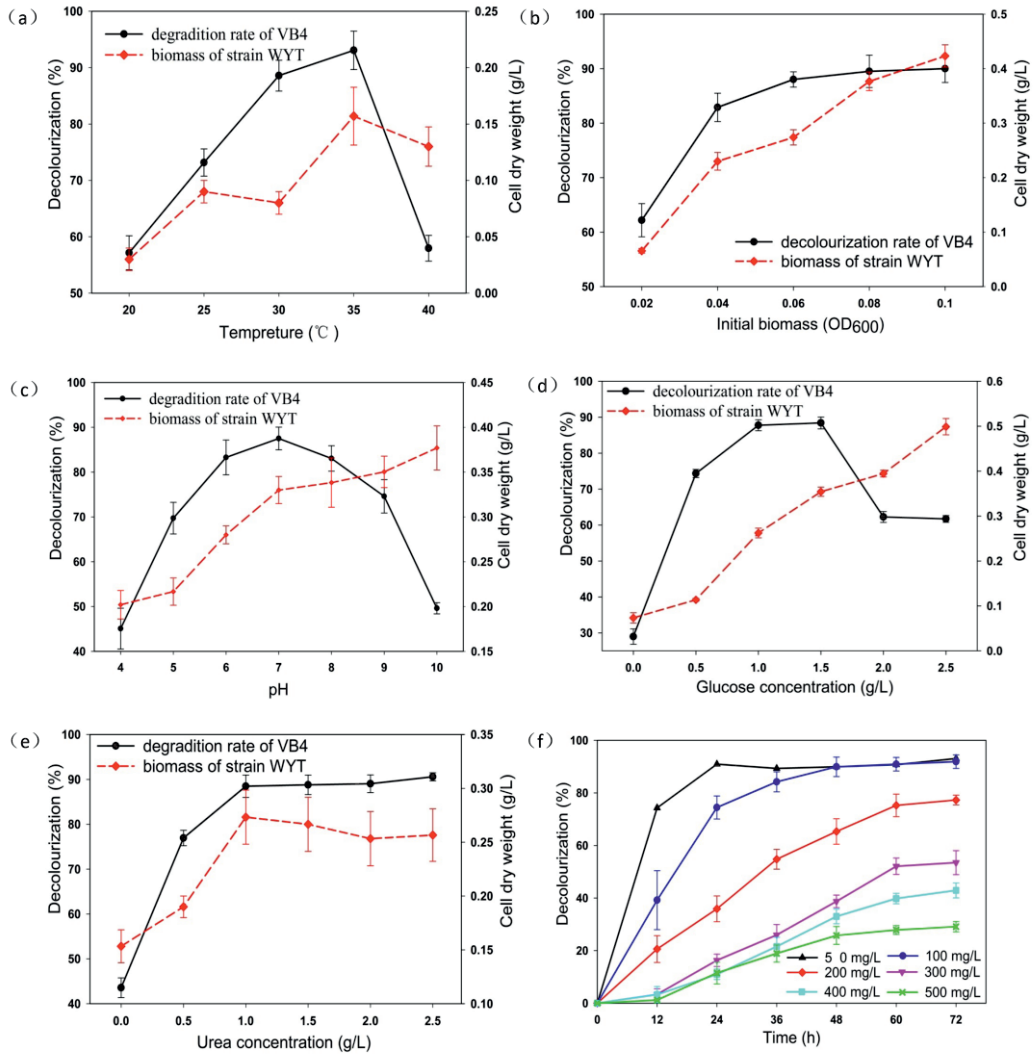


Fig. 3. Single factor experiments demonstrating the effect of six factors (temperature, the initial biomass, pH, glucose concentration, urea concentration, and and initial VB4 concentration) on VB4 degradation. The broken lines represent biomass of strain *Pseudomonas aeruginosa* WYT and VB4 degradation, respectively. a) Effect of different temperature (20, 25, 30, 35 and 40 °C) on VB4 degradation and final biomass. b) Effect of initial biomass (λ_{600}=0.00, 0.02, 0.04, 0.06, 0.08, 0.1) on VB4 degradation and final biomass. c) Effect of different pH (4, 5, 6, 7, 8, 9, 10) on VB4 degradation and final biomass. d) Effect of glucose concentration (0.0, 0.5, 1, 1.5, 2 and 2.5 g/L) on VB4 degradation and final biomass. e) Effect of urea concentration (0.0, 0.5, 1, 1.5, 2 and 2.5 g/L) on VB4 degradation and final biomass. Effect of initial dye concentration (50,100, 200, 300, 400, 500 m g/L) on degradation rate and final biomass.

research result: *Pseudomonas aeruginosa* has a high degradation rate in the range of 30-37ºC [36, 37]. The maximal dye decolorization (93.07%) was reached when cultivating temperature was set at 35ºC. However, the decolorizing activity were sharply decrease while increased temperature to 40ºC, loss of cell viability or deactivation of the enzymes perhaps responsible for the decolorization [31, 38].

Effect of Initial Biomass

To verify the effect of different initial biomass (0.02-0.06) on dyes degradation, we inoculated the *Pseudomonas aeruginosa* WYT in BM, which containing 50 mg/L VB4 was placed in 30ºC thermostat shaker, and the decolorization rate and final biomass was measured and calculated after 24 h. The result was shown in Fig. 3b), with the increase of inoculation amount, the decolorization rate of VB4 first rise and then stabilized. Decolorization rate was the lowest and only 62.18% when initial biomass was 0.02 ($OD_{600\ nm}$). It may be that the initial biomass of *Pseudomonas aeruginosa* WYT were too little to resist the toxicity of the dye, which leads to the minimum of final biomass. However, final decolorization rate changes are not obvious, when the initial biomass of *Pseudomonas aeruginosa* WYT were 0.04, 0.06, 0.08, 0.1 ($OD_{600\ nm}$). This result indicating that the initial biomass within a wide range of decolorization effect is not obvious, which is beneficial to the application of this strain in the actual dyeing wastewater treatment [39, 40].

Effect of pH

Initial pH of wastewater is an important environmental factor influencing degradation process, because it not only effect biosorbent site but also the solution chemistry of the dyes [41, 42]. Previous studies reported that various pH conditions affected adsorption rate significantly, which is the first step of bacteria degradation dyes [42]. *Pseudomonas aeruginosa* is well known for its successful adaptation to several harsh environmental [43-45] and its natural growth habitats can span a pH range from 4.5 to 9.5 [46]. But any pH higher or lower than 7 led to decrease in decolorization activity as well as the growth of the bacterial culture [47]. To verify the tolerance of *Pseudomonas aeruginosa* WYT to different pH. Optimization studies with regard to pH of the medium (4-10) versus dye decolorization showed maximum (90.07%) decolorization of VB4 within 24 hours at pH 7.0 (Fig. 3c). Results indicate that different pH of the medium affect the dye degradation rate and biomass significantly. At low pH values, the decolorization rate of VB4 and biomass were the lowest. When pH is 9-10, the autoclaving produced much sediment in the alkaline culture medium, and the sediment adsorbed dye causes the error of biomass measurement.

Effect of Glucose and Urea

Strains isolated in laboratory usually do not work very well in the actual environment, because the carbon and nitrogen source are enriched in the experiment [48]. Suitable carbon source and concentration are benefit to bacteria decolorization and degradation of dyes. The carbon sources usually used in the laboratory including: L-rhamnose, creatinine, glucose, sucrose, D-mannose, D-galactose, D-arabinose, and D-maltose, etc. Nitrogen sources are mainly: ammonium chloride, peptone, yeast powder, ammonium nitrate, potassium nitrate, and ammonium sulfate, etc. Although more bacteria of anthraquinone degradation were isolation by predecessors, but the experiment was carried out in the nutritive broth [20, 31, 49]. Therefore, in this experiment, simple and low price of glucose and urea selected as a source of carbon and nitrogen, wishing could be applied to the actual processing of printing and dyeing wastewater

Fig. 3d) showed the effect of glucose concentration on degradation efficiency and the growth rate of strain *Pseudomonas aeruginosa* WYT. It was displayed that over 87% of VB4 (50 mg/L) was decolorized within 24 h when the glucose concentration was 1-1.5 g/L. Meanwhile, the decolorization rates were all less than 80% within 24 h when the concentrations of glucose were 0.0-0.5 g/L and 2.0-2.5 g/L. It was suggested that neither inadequate nor excessive amounts of glucose would be benefit for decolorization of VB4 by *Pseudomonas aeruginosa* WYT. Perhaps the carbon catabolite repression (the Crabtree effect), wherein higher external glucose concentration limits tricarboxylic acid (TCA) cycle activity of the microbe [50]. The biomass increased when the glucose concentration was 1.5-2.5 g/L, but degradation rate decreased rapidly, *Pseudomonas aeruginosa* WYT perhaps produced much pyocyanin in excessive glucose conditions. Fig. 3e) showed the effect of urea concentration on degradation efficiency and the growth rate of strain *Pseudomonas aeruginosa* WYT. The urea concentration in the range of 0.0-2.5 g/L, VB4 degradation rate consistent with *Pseudomonas aeruginosa* WYT growth trend.

Effect of Dye Concentration

The concentration of dye substrate can influence the efficiency of dye removal through a combination of factors, if the concentration is too low, the related enzymes secreted from the degradation bacteria may not be able to effectively identify the dye, but if the dyes concentration is high, may be toxic to bacteria or block the enzyme active site and reduces the degradation efficiency [51-53]. In this study, the effect of different concentrations of VB4 dye on decolorization was observed by taking strong concentration of 50, 100, 200, 300, 400, and 500 mg/L levels. The final decolorization rate was 93.1, 91.9, 77.3, 53.5, 42.9 and 29.1% respectively, results indicate that increase of dye concentration, the final decolorization rate decreased significantly (Fig. 3f). Higher concentrations of VB4 (beyond 100 mg/L) repress the growth and the extent of decolorization (lower decolorization rate). Similar results have been also reported in the literature for other anthraquinone dyes [19, 31, 54, 55].

Plackett-Burman Design for Screening Significant Impact Factors

Environmental conditions (both external and internal) are important for cell growth and the activity of most enzymes, which affect the dyes degradation efficiency [19, 31, 56]. To explore the effect of various environmental conditions on VB4 degradation, a two-level Plackett-Burman design was implemented to screen the significant parameters among seven parameters: temperature, initial biomass, pH, dye concentration, trace salt, glucose and urea concentration. Table S2 were the Plackett-Burman design for factors optimization and measured response. All analytes were subjected to t-test in turn and from the obtained results. The result showed that three of the seven factors had a significant effect on VB4 degradation rate (p-value<0.05) and one of these three factors had very significant (p-value<0.01) effects. Statistically significant influence to VB4 degradation parameters were temperature, pH and urea, selected and subjected to further optimization by Steepest Ascent Experiment. Trace salt is negligible (p-value = 0.344), maybe its amount was too little to affect the degradation rate. Initial biomass has also no significant (p-value = 0.283) effect

on the degradation efficiency of the VB4, indicates that in actual dyeing wastewater treatment, the decolorization and degradation rate can be improved by changing the external environment, rather than adding more bacterial inoculum. The analysis of variance for the regression model were shown in Table S4, and its uncoded values regression relation for the prediction of output is presented in Equation (4).

$$\begin{aligned}&\text{VB4 Degradation rates (\%)}\\&= 59.53 - 5.81 \times \text{temperature} + 2.31 \times \text{initial biomass}\\&+ 12.89 \times \text{pH} + 3.75 \times \text{glucose} - 7.92\\&\times \text{urea} - 2.96 \times \text{dyes} - 2.00 \times \text{trace salt}\end{aligned} \quad (4)$$

Based on the results from the Plackett-Burman test, step length and rising paths of external conditions (temperature, pH and urea concentration), were set as shown in Table S5. Parameters of pH are gradually rising in the Steepest Ascent Experiment, because of its positive correlation of the decolorization degradation rate, while temperature and urea are opposite. The degradation rate of VB4 by *Pseudomonas aeruginosa* WYT first increased and then decreased. The optimal trial conditions were obtained for the fourth group with a maximum degradation rate of 96.37%, and were chosen as the center point in the following optimization for the CCD.

Response Surface Methodology for Optimizing the Degradation Conditions

Response surface methodology is an important branch of experimental design used in the development of an adequate functional relationship between the response and number of associated control variables [57, 58]. The objectives of conditions optimization, including the growth of strains and improved the degradation rate, which often be accomplished directly using response surface methodology. Many researchers used response surface methodology to optimize the degradation process of dyes, and obtained the optimal degradation conditions finally [29, 59, 60]. Response surface methodology is a simple and easy mathematical technique useful for developing, improving, and optimizing processes.

Central Composite Design belongs to response surface methodology, and is an independent quadratic design in that it does not contain an embedded factorial or fractional factorial design and is commonly used [61]. Temperature (26.6-43.4ºC), pH (5.32-6.86) and urea concentration (0.4-1.4 g/L) were chosen as the independent variables based on the preliminary experiments, and degradation rate was chosen as the response value. Totally, twenty experimental runs with six replications at the center point of cubic domain were conducted to improve the VB4 degradation rate. The design of each factor levels and 20 sets of actual conditions was shown in Table S3 and Table 2, respectively. These experimental results were modeled with a second-order polynomial equation to explain the dependence of VB4 degradation rate on the three factors. The mathematical regression model using the coded factors is given as Equation (5).

$$Y = 97.24 - 4.51A + 14.10B + 6.07C + 6.45AB - 5.63AC - 8.02BC - 20.03A^2 - 18.09B^2 - 15.30C^2 \quad (5)$$

Table 3. ANOVA for response surface quadratic model.

Error source	SS	DF	MS	F-value	p-value
A-Temperature	277.47	1	277.47	15.49	0.0028**
B-pH	2713.46	1	2713.46	151.53	<0.0001***
C-Urea	502.44	1	502.44	28.06	0.0003**
A*B	333.21	1	333.21	18.61	0.0015**
A*C	253.46	1	253.46	14.15	0.0037**
B*C	514.08	1	514.08	28.71	0.0003***
A^2	5761.66	1	5761.66	321.74	<0.0001***
B^2	4703.24	1	4703.24	262.64	<0.0001***
C^2	3364.57	1	3364.57	187.88	<0.0001***
Residual	179.08	10	17.91		
Lake of fit	147.61	5	29.52	4.69	0.0575 not significant
Pure error	31.46	5	6.29		
Cor total	16374.38	19			

SS = Standard Square; DF = Degrees of Freedom; MS = Mean Square; $R^2 = 0.9891$; Adj $R^2 = 0.9792$; Pred $R^2 = 0.9275$
*Significant (P<0.05). **Very significant (P<0.01) ***Most significant (P<0.0001).

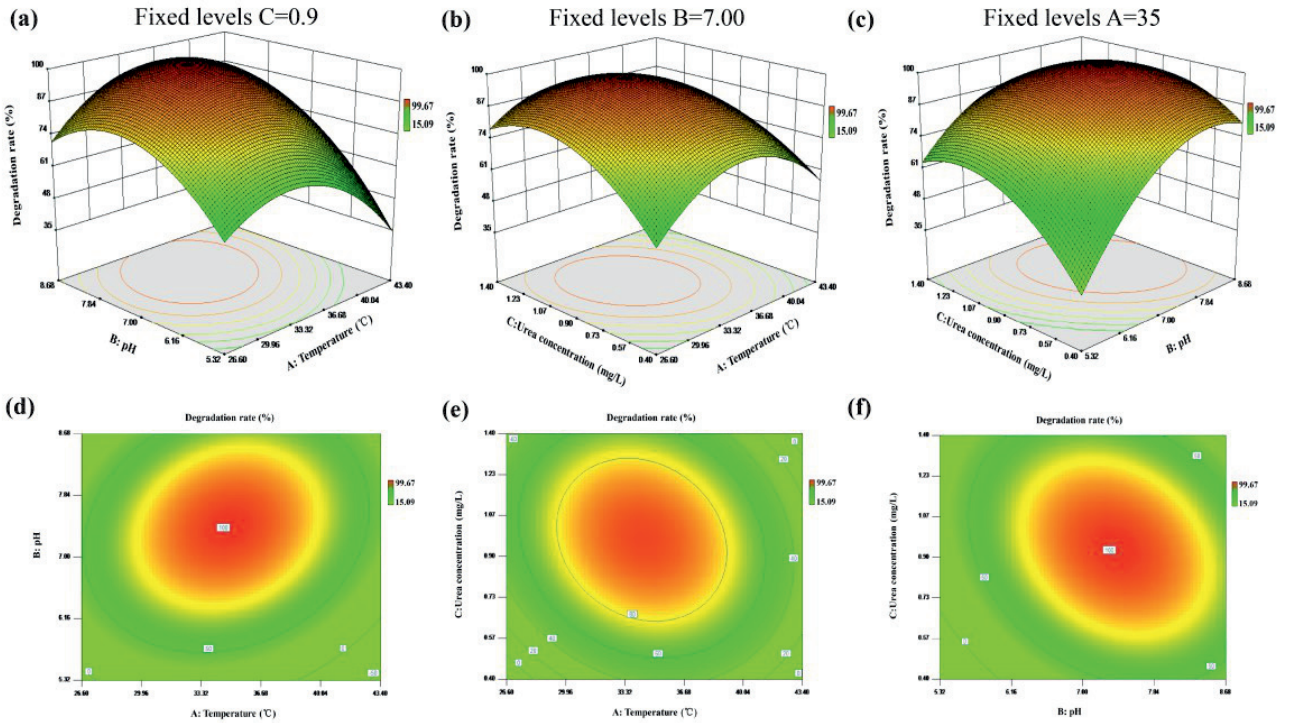


Fig. 4. Response surface and contour plot of the VB4 degradation rate (Y). Response surface plot for VB4 degradation rate as a function of a) A: temperature and B: pH, urea concentration = 0.9 mg/L b) A: temperature and C: urea concentration, Ph = 7.0 c) B: pH and C: urea concentration, temperature = 35ºC.

where Y is predicted response of VB4 degradation rate (%), the temperature (A), initial pH (B) and concentrations of urea (C) are coded variables. Positive coefficients of the linear independent variables indicate that high pH (B) and urea concentration (C) within the studied range favored the process of degradation. Conversely, negative coefficients indicate an antagonistic [59]. Analyzing the measured responses by the Design-Expert software, result indicates that the quadratic models are statistically recommended for the responses for the further analysis.

Further response surface methodology steps, using variance analysis the CCD experimental, results of linear and quadratic order response surface model given in Table 3. The regression coefficients and the interaction between each independent factor can be considered statistically significant for p-values below 0.05, with 95% of confidence interval [61, 62]. Four factors of pH (B), quadratic temperature (A^2), pH (B^2) and urea concentration (C^2) were the most decisive variables (p-value<0.0001) affecting the VB4 decolorization by *Pseudomonas aeruginosa* WYT, and others were very significant (p-value<0.01). Lack of fit is an indicator to demonstrate the repeatable and reasonable of experimental design, it also could compare the residual error and pure error from replicated design points [63]. The lack of fit for response value (0.0575) was not found as significant considering p-value, showed that model was valid for present work. On the other hand, ANOVA analysis (Table 3) was also employed to determine the regression coefficient (R^2 = 0.9891) and adjusted regression coefficient (Adj R^2 = 0.9792). It was indicating that 98.91% of variation in the decolorization process was attributed to the experimental variables studied, and the model could not explain only 1.09%.

Using response surface methodology reveal the independent factors (temperature, initial pH and urea concentrations) and their interactions on the VB4 degradation. Both a three-dimensional response surfaces and contour map were generated to directly showed interactions among three factors (Fig. 4). All contour lines (Fig. 4b, d, f) were loosely arranged, indicating that the interaction between two factors was not significant, which was consistent with the results of variance analysis [29]. The three-dimensional response surfaces pointed downwards, and there was an extremum in the selected scope, which was the optimal value of response.

Fig. 4a) showed the interaction between temperature and initial pH, VB4 decolorization rate increase quickly with increase of both temperature and pH, but then drop rapidly. By comparing the three response surfaces pictures, it was shown that initial pH (B) and urea concentrations (C) had remarkable effected on VB4 degradation, which relatively steeper in both axes (Fig. 4c). The predicted maximum VB4 degradation rate by *Pseudomonas aeruginosa* WYT was 97.24% in 24 h. The optimum decolorization and degradation conditions were 35.37ºC of temperature, 7.35 of pH and 0.95 mg/L urea concentration.

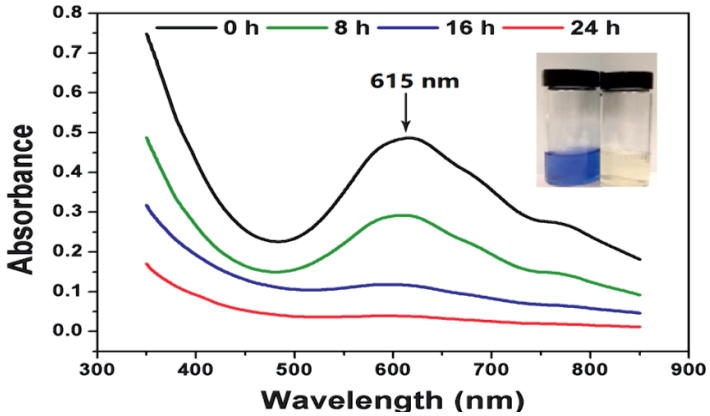


Fig. 5. UV-vis spectra of Vat Blue 4 and its intermediates during the decolorization by WYT.

UV-vis Revealed the Degradation Efficiency in Optimized Parameter

To investigate the possible decolorization mechanism of Vat Blue 4, UV–vis spectra (300-900 nm) of supernatants at different time intervals as shown in Fig. 3. It's exhibited that intensity at 615 nm (characteristic absorbance wavelength) remarkably decreased and nearly reached zero after 24 h, where its decolorization rate reached 97.21%. The great changes occurring in UV-vis spectra indicated that the molecular structure of dye changed evidently after decolorization [64]. The blue color of VB4 was caused by chromophore of anthraquinone was cleaved during the reaction (Fig. 5), which proved that the disappearance of characteristic peak (615) of VB4 due to degradation.

Conclusions

In this study, we focused on isolation and purification of VB4 degradation strains from activated sludge of dyeing wastewater treatment. Five strains were obtained, and there were *Stenotrophomonas* sp., *Pandoraea* sp., *Paracoccus* sp., *Serratia marcescens* and *Pseudomonas aeruginosa* WYT respectively. *Pseudomonas aeruginosa* WYT was selected to degradation VB4, and then using single factor experiment and response surface methodology optimum the degradation process. Furthermore, through three group of response surfaces and contour map display the interactions among three factors. Finally, the optimal degradation conditions of VB4 were obtained: culture temperature 35.0ºC, initial biomass 0.06, pH 7.0, glucose 1.0 g/L, urea 1.0 g/L, VB4 was 50 mg/L and 2 mL/L trace salt. Under these optimal condition, 50 mg/L VB4 were degradation about 97% in 24 h. In this study, VB4 was first degraded by single strain (*Pseudomonas aeruginosa* WYT), expected to provide reference for actual printing and dyeing wastewater treatment.

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Conflict of Interests

The authors declare no conflicts of interest.

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Supporting Information

Table S1. Plackett–Burman design for factors optimization, positive (+1) and 4 negative (−1) levels of independent variables used in trials.

Factor setting	Temperature ºC	Initial biomass OD_600	pH	Glucose mg/L	Urea mg/L	Dyes mg/L	Trace salt mL/L
High (+1)	40	0.08	8	1.5	1.5	80	4
Low (-1)	30	0.04	6	0.5	0.5	20	2
Center	35	0.06	7	1	1	50	3

Table S2. Plackett-Burman design for factors optimization and measured response.

Trial	Temperature ºC	Initial biomass OD_{600}	pH	Glucose mg/L	Urea mg/L	Dyes mg/L	Trace salt mL	Degradation rate %
1	40	0.08	6	1.5	1.5	20	2	53.43
2	40	0.08	8	1.5	0.5	80	2	81.88
3	40	0.04	8	0.5	1.5	80	4	44.38
4	40	0.04	8	0.5	1.5	20	2	68.49
5	30	0.04	8	1.5	0.5	80	2	80.62
6	30	0.08	8	0.5	0.5	20	4	90.41
7	40	0.04	6	1.5	0.5	20	4	56.16
8	30	0.04	6	0.5	0.5	20	2	54.79
9	30	0.08	8	1.5	1.5	20	4	68.75
10	40	0.08	6	0.5	0.5	80	4	40.88
11	30	0.08	6	0.5	1.5	80	2	35.75
12	30	0.04	8	1.5	1.5	80	4	38.88

Table S3. Independent variables levels used for CCD matrix.

Independent variables	Variable level				
	Lowest	Low	Center	High	Highest
Defination (units)	-1.68	-1	0	1	1.68
A-Temperature (ºC)	26.6	30	35	40	43.4
B-pH	5.32	6	7	8	8.68
C-Urea concentration (mg/L)	0.4	0.6	0.9	1.2	1.4

Table S4. Analysis of variance (ANOVA) for the regression model.

Error source	DF	SS	MS	F-value	P-value	Rank
Temperature	1	404.43	404.43	9.67	0.036*	3
Initial biomass	1	64.23	64.23	1.54	0.283	6
pH	1	1993.03	1993.03	47.66	0.002 **	1
Glucose	1	168.89	168.89	4.04	0.115	4
Urea	1	753.24	753.24	18.01	0.013*	2
Dye	1	105.07	105.07	2.51	0.188	5
Trace salt	1	48.00	48.00	1.15	0.344	7
Regression	7	3536.90	3536.90			
Error	4	167.26	41.82			
Total	11	3704.16				
$R^2 = 0.955$						

DF, degree of freedom; SS, sum of squares; MS, mean square.
* Significant ($P<0.05$).
** Very significant ($P<0.01$).

Table S5. The design and results of steepest ascent experiment for VB4 degradation efficiency.

Trial	Temperature ºC	Initial biomass OD_{600}	pH	Glucose mg/L	Urea mg/L	Dyes mg/L	Trace salt mL	Degradation rate %
1	45	0.06	4	1.00	1.8	50	2	21.37
2	40		5		1.5			40.35
3	35		6		1.2			70.28
4	30		7		0.9			96.37
5	25		8		0.6			94.66
6	20		9		0.3			90.24