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Decolourization of Reactive Brilliant Blue KN-R by immobilized cells of *Aspergillus ficuum*

DONG Xin-jiao, CHEN Zhu

(Department of Biology & Environmental Science, Wenzhou Normal College, Wenzhou 325003, China. E-mail: xjdong@mail.wzptt.zj.cn)

Abstract: *Aspergillus ficuum* was immobilized with sodium alginate, and decolourization of Reactive Brilliant Blue KN-R was studied on immobilized and free *Aspergillus ficuum*. The optimal preparation condition of the strain immobilization was obtained by the orthogonal test, it is sodium alginate 3%, $CaCl_2$ 5%, wet mycelia 30 g/L, calcific time 8 h. It was found that the immobilized cells could effectively decolourize Reactive Brilliant Blue KN-R, the optimum temperature and pH were 33℃ and 5.0, respectively. The kinetics study of decolourization of immobilized cells showed that the decolourization of *Aspergillus ficuum* immobilized conformed to zero-order reaction model. The decolourization efficiency of immobilized cell compared with that of free cell in different physical conditions. Results showed that the decolourization of immobilized cells with mycelia had the best efficiency. The immobilized cells could be reused after the first decolourization.

Keywords: immobilized cell; Reactive Brilliant Blue KN-R; decolourization; *Aspergillus ficuum*

Introduction

Reactive Brilliant Blue KN-R, which is a kind of anthraquinone dye, is extensively used in many fields such as textile industry, print-and-dyeing industry, and so on. A large amount of Reactive Brilliant Blue KN-R, which is a difficult-decomposing organic compound, is discharged into environment every year. More and more researchers pay attention to removing of Brilliant Blue KN-R for its high solubility, difficult decolourization, high toxicity and complex components (Song, 1999; Xin, 2001; Wang, 1999a).

Cell immobilization technology has advantages in high cell density, high susceptibility to toxicity and reusability. Cell immobilization was extensively investigated and applied in treatment of industrial wastewater and decomposition of organic compound in the early of 1980s (Wang, 1999b; Anselmo, 1992; Menke, 1994). Many kinds of carriers for cell immobilization were developed recently, and calcium alginate was widely used in entrapment immobilization (Menke, 1994; Quan, 2001). Studies on biodegrading decolourization of anthraquinone dye whose production is only inferior to azo dye were few reported in China, and study on cell immobilization for decolourization of anthraquinone dye was not reported. In this paper, decolourization of anthraquinone dye by *Aspergillus ficuum* was investigated. The optimal preparation condition of the strain immobilization and the optimal condition of decolourization were obtained, and decolourization kinetics of immobilized cell was investigated. Our research could give base for biodegradation of dye wastewater.

1 Materials and methods

1.1 Materials

Strain: *Aspergillus ficuum* was selected, separated and preserved by the microbial lab.

Dye: Reactive Brilliant Blue KN-R, produced by Zhejiang Donggang Chemical Group Company.

Media: (1) growth medium: Czapack medium. (2) Medium for experiment of decolourization (nitrogen restricted) (1 L): KH_2PO_4 2.0 g, $MgSO_4$ 0.5 g, $CaCl_2$ 0.1 g, α-Ketoglutaric acid 1 g, vitamin B_1 1 mg, 0.1% tuwin 80 50 ml, arumonium tartrate 2 g, glucose 10.0 g, trace element 50 ml, pH 5.0. (3) Sabourand medium (1L): glucose 40 g, peptone 10 g, pH 5.0.

1.2 Methods

Preparation of spore suspension: strain preserved in slant at 4℃ was incubated at 33℃ for 5 days, then inoculated into the slant with solid growth medium to propagate. A large amount of black spore grew after 5 days. The spore was transferred into sterilized water, then the suspension was preserved at 4℃. The concentration of the cell was assayed by hematocyte counting plate.

Preparation of immobilized cell: The evenly grinded mycelia were mixed with appropriate volume of 3% sodium alginate

solution, then the drips of solution of 5% $CaCl_2$ were added by an injector (control by creeping bump) at an even rate, thus the regular-sized pellets were formed. The pellets were incubated at 33℃ for 24 h, washed by sterilized water for several times and preserved (Prakasham, 1999).

Establishment of reaction system: (1) system of dynamic culture: 10 ml solution of immobilized pellet embedding cells was added into 50 ml decolourization medium containing dye (the ultimate concentration of dye was 50 mg/L) and cultured at 33℃. (2) System of static culture: All the conditions were the same to those of system of dynamic culture except that the immobilized cells were cultured statically.

Determination of decolourization rate: The sample was filtered and centrifuged. The OD_{594} of the supernatant was assayed comparing with that of decolourization test medium without immobilized cell pellet, and then the decolourization rate was determined (Dong, 2001).

2 Results and discussion

2.1 Determination of optimal preparation conditions of strain immobilization

There were many factors affecting the decolourization rate when immobilized cells were prepared. The main factors included the concentration of immobilized cell, $CaCl_2$, sodium alginate and the time of calciumization. The table of orthogonal test ($L_9(3^4)$) with these four factor and three levels was designed according to the optimal level of single factor attained in pre-experiment. The optimal preparation condition was determined on the decolourization rate. Nine groups of immobilized cells were prepared according to Table 1 at different factors and levels.

Table 1 Design of orthogonal test

Level	Factor A	Factor B	Factor C	Factor D
	$\rho_{mycelium}$, g/L	W_{CaCl_2}, %	$W_{Alginate}$, %	$t_{calciumization}$, h
1	10	2	2	4
2	20	3	3	6
3	30	5	4	8

The appropriate immobilized cells were added into 50 ml of 50 mg/L medium for test of decolourization, which simulating the dying wastewater. After the cells were cultured for 48 h at 33℃ and at a shaking rate of 150 r/min, the decolourization rate were determined on the absorbency of dye. The results are shown in Table 2. According to the orthogonal test, the optimal preparation conditions were determined as 30 g/L of mycelia (wet weight), 5% (w/w) of $CaCl_2$, 3% (w/w) of sodium alginate, 8 hours of calciumization.

Table 2 Results of orthogonal test

Factor	A	B	C	D	Scheme	Decolourization rate, %
1					$A_1B_1C_1D_1$	77.4
2					$A_1B_2C_2D_2$	72.1
3					$A_1B_3C_3D_3$	78.2
4					$A_2B_1C_2D_3$	77.2
5					$A_2B_2C_3D_1$	71.3
6					$A_2B_3C_1D_2$	70.9
7					$A_3B_1C_3D_2$	79.5
8					$A_3B_2C_1D_3$	87.0
9					$A_3B_3C_2D_1$	86.7
K_1	227.7	234.1	235.3	235.4		
K_2	219.4	230.4	236.0	222.5		
K_3	253.2	235.8	229.0	242.4		
k_1	75.9	78.0	78.4	78.5		
k_2	73.1	76.8	78.7	74.2		
k_3	84.4	78.6	76.3	80.8		
R	11.3	1.8	2.4	6.6		
Optimal level	A_3	B_3	C_2	D_3		
Optimal scheme	$A_3B_2C_2D_3$					

2.2 Test of optimal decolourization condition of Reactive Brilliant Blue KN-R by immobilized cell

2.2.1 Effect of culture time on decolourization rate

To determine effect of culture time on decolourization rate, 10 ml of immobilized cell was added into 50 ml medium(the dye concentration was 50 mg/L) for test of decolourization and cultured at 33℃ and a shaking rate of 150 r/min. The absorbency of the medium was measured at different time, and the decolourization rates were determined on the absorbencies of the samples. The same quantity of immobilized cell, free cell and twofold quantity of free cell were tested. The results(Fig. 1) indicated that the decolourization rate of the same quantity of immobilized cell was lower than that of free cell for the difficult of oxygen and mass transferring. However, the decolourization rate of the twofold quantity of immobilized cell was higher than that of free cell for the high cell concentration and cell propagation in pellet.

2.2.2 Effect of initial pH on decolourization rate

To determine effect of initial pH on decolourization rate, immobilized cells were added into decolourization medium containing 50 mg/L Reactive Brilliant Blue KN-R with initial pH 3.0, 4.0, 5.0, 6.0, 7.0 and 8.0, respectively and cultured dynamically at 33℃ for 48 hours, and then the decolourization rates were determined. The results (Fig. 2) indicated that immobilized cells were relatively susceptible to pH, and the optimal pH was 5.0.

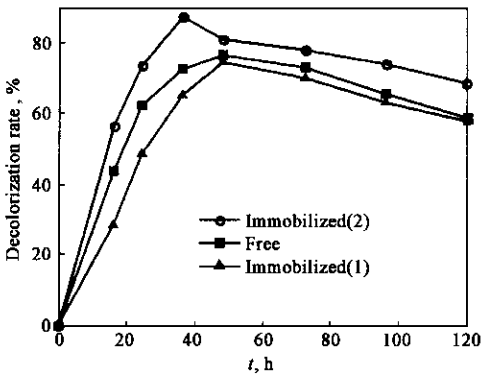

Fig.1 Effect of culture time on decolourization rate

2.2.3 Effect of temperature on decolourization rate

The effect of temperature on decolourization rate was investigated. The result showed that immobilized cells adapted to a wide range of temperature (Fig. 3). The decolourization rates were higher than 70% at the range from 20℃ to 35℃. The decolourization rate decreased observably when the temperature reached to 40℃, the possible reason was that high temperature would deactivate enzymes and affect the cell metabolism. The optimal temperature was 33℃.

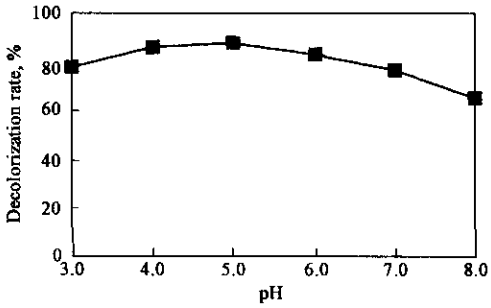

Fig.2 Effect of pH on decolourization rate

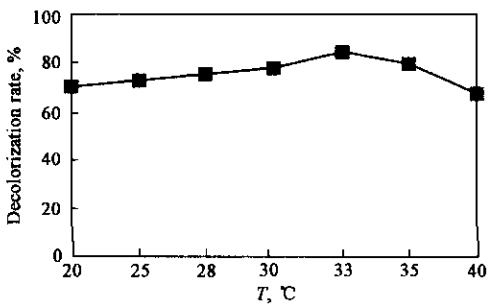

Fig.3 Effect of temperature on decolourization rate

2.3 Comparison of two systems of dynamic and static cultures

The data in Table 3 show that the two systems of dynamic and static cultures had evidently different effects on Reactive Brilliant Blue KN-R decolourization rate, which indicated the dynamic culture had better effect on decolourization rate than static culture. The possible reason was that the dynamic culture had better oxygen and nutrition transferring. The phenomena were not the same as that of *Phanerochaete Chrysosporium* (Li, 2001).

Table 3 Efficiencies of decolourization for Reactive Brilliant Blue KN-R in shaking and static cultures

Culture	Dye concentration, mg/L	Decolourization rate, %							
		6 h	12 h	18 h	24 h	30 h	36 h	42 h	48 h
Static	50	15.3	19.4	20.1	20.4	20.6	21.2	21.6	26.5
Shaking	50	27.0	29.2	39.3	51.3	57.9	71.4	84.1	87.8

2.4 Kinetics of Reactive Brilliant Blue KN-R decolourization by immobilized cell and free cell

The curve of Reactive Brilliant Blue KN-R decolourization by immobilized cell is shown in Fig. 4. The general biochemical reaction rate could be shown as

$$\gamma = \frac{\gamma_m \cdot c}{k + c}. \tag{1}$$

Where γ denoted reaction rate, γ_m denoted maximal reaction rate, c denoted concentration of substrate, k denoted constant of semi-saturation reaction rate. When $c \ll k$, Equation (1) could be shown as:

$$\gamma = \frac{\gamma_m \cdot c}{k}. \tag{2}$$

The decolourization conformed to first-order reaction model, and the constant of decolourization rate was

$$k_1 = \frac{\gamma_m}{k}.$$

When $c \gg k$, Equation (1) could be shown as:

$$\gamma = \gamma_m. \tag{3}$$

The decolourization conformed to zero-order reaction model, and the constant of decolourization rate was

$$k_0 = \gamma_m.$$

According to Equation (2) and Equation (3), the equation of the concentration of substrate and the time could be shown as:

First-order reaction: $\ln c = a + k_1 t$,

Zero-order reaction: $c = b + k_0 t$.

The data in Fig. 4 was simulated as equation of first-order reaction and zero-order reaction, respectively. The results indicated that when concentrations of Reactive Brilliant Blue KN-R were 10, 20, 30, 50 and 100 mg/L, the curves of decolourization conformed to zero-order reaction model are shown in Fig. 5. The kinetics equation of each curve is shown in Table 4.

Fig. 5 shows that when the initial concentrations of Reactive Brilliant Blue KN-R were 10, 20, 30, 50 and 100 mg/L, the Reactive Brilliant Blue KN-R decolourization of immobilized cells conformed to zero-order reaction model and Reactive Brilliant Blue KN-R could be decolourized at a constant rate. The constants of decolourization rate varied when different initial concentrations of Reactive Brilliant Blue KN-R were tested. The constant of decolourization rate augmented with the increasing of the concentration of Reactive Brilliant Blue KN-R at the range from 10 to 100 mg/L, because the amount of substrate was not enough for the tested quantity of immobilized cells. The reason was that the dye to be decolourized could not provide enough carbon and nitrogen source for immobilized cells. The higher concentration of dye would provide more energy for the growth and propagation of immobilized cells at this range, so the decolourization rate increased. When the concentration of dye reached 200 mg/L, the decolourization process did not conformed to zero-order reaction model because the metabolism of immobilized cells was inhibited by high concentration of dye. The decolourization rate of free cells was higher than that of immobilized cells because immobilized cells had the difficult of oxygen and mass transferring. But the rates were not appreciably different, which indicated that the strain immobilization by calcium alginate was an effective method.

Table 4 Reactive Brilliant Blue KN-R decolourization by immobilized and free cells

Initial concentration, mg/L	Kinetics equation	k_0, h^{-1}	r^2
10	$C = -0.10t + 8.1$	0.10	0.9345
20	$C = -0.23t + 17.1$	0.23	0.9700
30	$C = -0.38t + 27.19$	0.38	0.9872
50 (free)	$C = -0.73t + 44.47$	0.73	0.9518
50	$C = -0.60t + 45.05$	0.60	0.9629
100	$C = -0.98t + 91.97$	0.98	0.9606

2.5 Test of decolourization of immobilized cells in different physical conditions

Immobilized cells in three different physical conditions were tested to determine which had the best efficiency of Reactive Brilliant Blue KN-R decolourization. The three physical conditions included: spores in the spore suspension; cells with mycelia cultured in nitrogen-restricted medium for 5 days; cells with mycelia cultured in Sabourand medium for 5 days. The results showed that the cells in each of these three physical conditions had different efficiency of decolourization. Spores immobilized by calcium alginate had the relatively worse efficiency of decolourization with 51.3% in 48 hours; while cells with

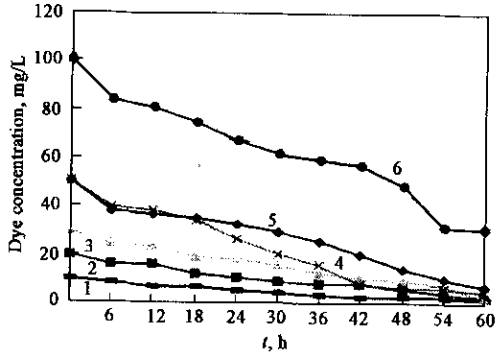

Fig. 4 Reactive Brilliant Blue KN-R decolourization by immobilized and free cells(first-order)
1. 10 mg/L; 2. 20 mg/L; 3. 30 mg/L; 4. 50 mg/L (free); 5. 50 mg/L; 6. 100 mg/L

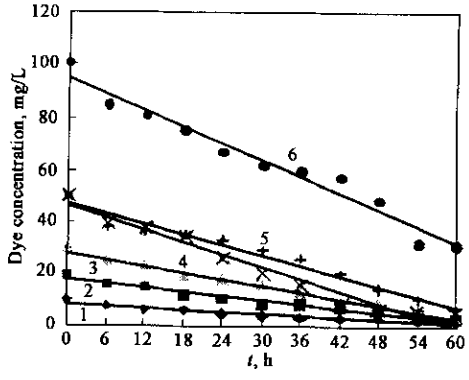

Fig. 5 Reactive Brilliant Blue KN-R decolourization by immobilized and free cells(zero-order)
(Nos. 1 – 6 are the same as Fig. 5)

mycelia cultured in Sabourand medium and in nitrogen-restricted medium had better efficiency of decolourization with respective 80.1% and 92.2%. It was concluded that immobilized cells cultured in nutrition-restricted medium had the best efficiency of decolourization, because cells in the physical condition of second metabolism needed the nutrition restricted. The physical condition of cell would determine the decolourization rate in the immobilization technology of fungi.

2.6 Test of re-decolourization of immobilized pellets

Immobilized pellets that had decolourized in 50 ml of 50 mg/L medium for 48 hours were taken out and added into another same medium containing dye, the efficiency of decolourization were detected after decolourizing in the same condition. The results showed that the efficiencies of decolourization for the first time, second time and third time were respectively 91.1%, 87.8% and 86.4%. Analysis of LSR method showed that the differences of efficiency of decolourization between the third time and the second time and between the second time and the first time were not appreciable; and the difference between the third time and the first time was appreciable at the level of $\alpha = 0.05$. It was concluded that reusable immobilized cells would influence the efficiency of decolourization but still have relatively high efficiency of decolourization, and have extensive potentiality for industry.

3 Conclusions

The optimal preparation condition of the strain immobilization by the orthogonal test: sodium alginate 3%, $CaCl_2$ 5%, wet mycelia 30 g/L, calcific time 8 h.

The optimal pH and temperature for decolourization by immobilized cells were 5.0 and 33℃, respectively.

Immobilized *Aspergillus ficuum* had the better efficiency of decolourization in dynamic culture than in static culture.

The decolourization of *Aspergillus ficuum* immobilized conformed to zero-order reaction model when the concentrations of Reactive Brilliant Blue KN-R were 10, 20, 30, 50 and 100 mg/L. The constant of decolourization augments with the increasing of the initial concentration of Reactive Brilliant Blue KN-R.

The physical conditions of immobilized cells had directly effect on efficiency of decolourization.

Immobilized cells could be reused and still have relatively high efficiency of decolourization.

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