Decolorizing kinetics of Reactive Blue 13 Azo Dye by *Proteus mirabilis*

Olukanni O. David*, Osuntoki A. Akinniyi and Gbenle G. Olabode

Abstract

A dye decolorizing bacterium, *Proteus mirabilis*, isolated from soil was used in batch experiments for decolorization of the sulphonated dye, Reactive blue 13. Effects of initial substrate (dye) and bacterial cell mass concentrations on the specific decolorization rate and extent of dye decolorization were investigated. Aeration as against agitation increased the decolorization rate by 4-fold. The dependence of the decolorization rate on initial dye concentration essentially followed Monod-type kinetics and the maximal rate occurred with the dye concentration 382.98 mg/L. The decolorization rate by *Proteus mirabilis* was found to follow half (0.5498) and first (1.0977) order kinetics with respect to dye concentration and cell mass concentrations respectively. The specific decolorization rate estimated from the experimental data was 37.03 mg/L/h/g dry cell mass (DCM), and the value of the apparent K_m was 284.76 mg/L. This study showed that the strain of *Proteus mirabilis* has potential in wastewater color removal.

Key words: Kinetics, decolorization, *Proteus mirabilis*, aeration, azo dyes, Reactive Blue 13.

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1. Introduction

The structural and functional diversity of azo dyes have resulted in serious environmental pollution. Azo dyes are chromophogenic compounds, characterized by one or more azo groups (-N=N-). More than 2,000 azo dyes are currently being used to dye various materials such as textiles, leather, plastics, cosmetics and food[1]. Reactive azo dyes form the largest class of water-soluble synthetic dyes with the greatest variety of colors and structures, and are generally recalcitrant to oxidative decolorizations[2]. Although most of these dyes are not toxic to the environment, the color in water streams may reduce light penetration hence making aquatic life more difficult[3].

Methods that have been used in the treatment of textile effluents include physiochemical methods such as filtration, specific chemical oxidation, reverse osmosis, coagulation-flocculation, adsorption and photo-degradation etc[4-6]. Some of these methods are effective but with serious limitations such as sludge formation, formation of more toxic by-products and high cost of implementation[2,7-8]. Microbial decolorization, however has proved to be a relatively cheaper and eco-friendly alternative[9]

Over the years, microbial removal of colors from textile wastewaters has focused on anaerobic systems. Anaerobic color removal results in toxic end products, aromatic amines, which have been reported to be more toxic than the dyes themselves, mutagenic to humans and cannot be degraded further under the same condition[3, 10-11]. Although the aromatic amines can be degraded under aerobic conditions, treatments have received serious criticisms because of poor extend of decolorization[12]. Combined

anaerobic/aerobic treatment has recently been suggested[12]. This requires two separate phases: decolorization and detoxification; thus requiring longer period of treatment.

Recently, aerobic bacteria that can both decolorize azo dyes and degrade the aromatic amines formed from such decolorization have been reported[13-15]. In such researches, the effectiveness of such organisms was reported in terms of percentage decolorization and incubation times, with slight references made of the concentration of the dyes and the organism used. Furthermore, little attention has been given to the study on biodecolorization/biodegradation kinetics[16]. Kinetics studies could be more helpful in comparing the affinity of organisms to dye, pollutants or effluent. Such studies can also be used to optimize the applied substrates and biomass, thereby reducing the cost of biotreatment.

In this research, the dependence of decolorization ability of a newly isolated bacterium on dye concentration and biomass concentration was investigated. Its requirement for oxygen and the kinetic constants were also determined.

Materials and Methods

Reactive Blue 13 (RB13), a sulphonated monoazo dye used in the experiments, was collected from the technical department of a textile industry located in Lagos, Nigeria. The structure of the dye is shown in Figure 1. The dye has its absorption band maximum at 568 nm wavelength. Other chemicals used are of analytical grade; nutrient broth and nutrient agar were source from Lab M, UK. All the solutions were prepared using distilled water.

The organism used in this study was isolated from soil sample of wastes site at Redemption City, near Lagos. The organism was identified using 16S rDNA facilities of Laragen Inc. USA[17].

Maintenance of organism

The organism was screened for the ability to decolorize azo dyes using 60 mg/L dyes mixtures in nutrient broth. It was then maintained on nutrient agar slants at 4°C. The pure culture was grown in 250 ml Erlenmeyer flask containing 100 ml nutrient broth (g/L): beef extract 1 g, yeast extract 2 g, peptone 5 g and NaCl 5 g), at 37°C for 24 h. The 24 h culture was kept at 4°C, and used as the seed inoculum.

Biomass estimation

The dry cell mass of the strain was periodically estimated using the turbidity of its 24 h culture at 600 nm (OD_{600}) as described by Dong *et al* [18]:

$$OD_{600} = OD_{sample} - OD_{\sup ernan\tan t} \qquad (1).$$

A ml of the culture was dried to constant weight in three pre-weighed small vessels (1.5 ml eppendorf tubes). The average change in weight between the vessels and the vessels with cells was regarded as the DCM of the organism/ml. The relationship between the DCM of *Proteus mirabilis* and OD_{600} was found to be 1.0 OD_{600} = 10.7678 g DCM/L. Cell mass was constantly determined using this relationship.

Decolorization in Static Anoxic Condition

Three 250 ml Erlenmeyer flask containing 100 ml, of the dye solutions were inoculated aseptically with 0.2 g DCM of the organism, the flasks were plugged with sterile cotton wool, and incubated at 37°C.

Effect of Aeration on Decolorization

It was observed under static anoxic condition that the organism grew above solution (around the flask); this led to a prediction that the strain requires more oxygen for growth and probably decolorization activity. To investigate this, the experiment was repeated without plugging the flasks with cotton wool (aeration experiment); the abiotic control was not plugged also.

Effect of Agitation on Decolorization

The decolorization ability of the organism was also tested in shaking condition (agitation) to further substantiate the requirement of the organism for oxygen in a similar experiment with the flasks plugged with cotton wool. Nutrient broth-dye solution (60 mg/L) of RB 13 was inoculated with 0.2 g as in the static anoxic experiment. The reaction was, however, incubated in a rotary shaker (120 rpm) at 37°C.

In all experiments, 6 ml samples were withdrawn at 3 h intervals for 24 h. 3 ml of samples were centrifuged at 5000 rpm for 15 min to remove biomass. Decolorization was measured spectrophotometrically at the solution's pre-determined maximum absorbance (λ_{max}). Decolorization was recorded as %:

$$\%Decolorization = \frac{A_o - A_t}{A_o} \times 100 \qquad (2).$$

Where A_o is absorbance of the dyes solution and A_t is absorbance of the treated dyes solution at specific time, t.

Dependence of Specific Decolorization Rate on Initial Concentration of RB13

Experiments with different concentrations (30 – 2500 mg/L) of the reactive blue 13 were performed. An estimated 0.11 g DCM of the cell mass was added to 100 mL of each dye solution made with nutrient broth and decolorization was studied at the strain's optimal temperature of 37°C. The experiments were done in triplicates and mean values were used in the determination. Natural logarithm of the specific decolorization rate ($V_{dye,M}$) was plotted against that of dye concentration [RB13]. $V_{dye,M}$ was described as :

$$V_{dye,M} = \frac{(A_o - A_t)[RB13]}{A_o tM} \qquad (3).$$

Where $V_{dye,M}$ is the specific decolorization rate (mg dye/L/h/g DCM), , [RB13] is the initial concentration of RB13 (mg dye/L), t is incubation period (h) and Where M is cell mass concentration (g DCM/L).

The order of the decolorization with respect to concentration of RB13 was determined as the gradient of the equation:

$$\ln V_{dye,M} = a \ln[RB13] + k \qquad (4).$$

a is the order of the reaction and k is the rate constant.

Dependence of Specific Decolorization Rate on Initial Cell Concentration

Effect of biomass on the rate of decolorization was investigated in a 250 ml flask containing 100 ml, 100 mg/L RB13. Different flasks were inoculated with different concentration of inoculums ranging from 0.4 – 3.02 g/L. Aeration experiment set-up was used and % decolorization were determines after 3 h. ln $V_{dye,M}$ was plotted against ln M and the order of reaction was determined using an equation similar to equation 4:

$$\ln V_{dye,M} = b \ln M + \ln k \qquad (5).$$

where b is the order of the reaction and k is the rate constant.

Determination of Maximum Decolorization Rate

To determine the maximum decolorization rate and maximum concentration tolerance of the organism, experiments with different initial concentrations of R13 was conducted from 25 to 2500 mg/L of the dye using the aeration set-up, the % decolorization was also determined after 3 h. Lineweaver – burk model was used to determine the maximum specific decolorization rate, while the tolerance level was estimated from Michaelis – Meten plot.

Results

Kinetic studies are important to determine the relationship or dependence of decolorization on dye concentration, substrate concentration and cell mass concentration. It also gives clear evidence of how effective a particular organism can remediate an effluent.

The results of the 16S rDNA sequence showed that the organism was a strain of *Proteus mirabilis*[17], it sequence has been deposited in GenBank with ascension number GU945541. During static anoxic condition, the organism showed 97.50±1.75% decolorization of the dye within 24 h; but it achieved almost the same fit (94.97±1.58% decolorization) within 6 h on aeration. Agitation seemed to increase the lag phase of microbial growth from about 1 h to 3 h (Figure 2). This resulted in poor decolorization of the dye (22.67±2.6%) under this condition, even at 24 h of incubation.

Figures 3 and 4 showed the dependence of specific decolorization rate on the concentration of RB13 and cell mass concentrations respectively. From figure 3, it was observed that the rate of decolorization increased with increase in concentration of the dye until an apparent dye concentration (382.98 mg/L) was reached, after which the rate of decolorization decreased steadily. Similarly, in figure 4, the dependence of the rate on biomass showed that the rate of decolorization increase with increase in biomass until a biomass concentration was reached at which increase in biomass did not increase the rate of decolorization. From the gradients of the data fittings for figure 3 and 4, the values of the orders of reactions were found to be 0.5498 and 1.0977 with respect to dye concentrations and cell mass concentrations respectively.

When the double reciprocal graph of the dependence of the specific rate of decolorization on dye concentration was plotted, the specific decolorization rate estimated from the experimental data was 36.23 mg/L/h, the value of the apparent K_m was 278.83 mg/L, (Figure 5) and the inhibitory concentration was about 400 mg/L (Figure 3). It was also observed that the dependence of specific decolorization rate on dye concentration (Figure 5) could be interpreted by Lineweaver-Burk model.

$$\frac{1}{V_{dye,M}} = \frac{k_m}{V_{dye,M\ max}} \times \frac{1}{[RB13]} + \frac{1}{V_{dye,M\ max}} \qquad (6).$$

Discussion

The reduction in the incubation time for the complete decolorization of the dye from 24 h (in static anoxic condition) to about 6 h during aeration indicated that the rate

of decolorization was increased about 4-fold on aeration. It also showed that the organism has a high capability to decolorize 100 mg/L RB 13. Kalme *et. al.* [13] has suggested competition between azo dyes and oxygen for reduced electron as the reason for low decolorization of azo dyes under shaking condition, this could be a good explanation for the poor decolorization during agitation. It is then logical to propose that aeration increased the dissolved oxygen required for the organism growth while agitation increases the molecular oxygen that competes with the azo dyes for reduced electrons. In a previous study, Yu *et al*.[19] showed that the decolorization rate of certain *Pseudomonas* strains followed first order kinetics with respect to cell mass and half order kinetics with respect to dye concentration. This compares effectively with the findings of this research with deduced values of 1.0977 and 0.5498 respectively. The possibilities of interpreting the dependence of the specific rate of decolorization on dye concentration suggested the involvement of enzyme(s) in the decolorization.

The high inhibition concentration of about 400 mg/L indicated that the organism has a potential for practical application in the decolorization of textile effluent, since most effluent have been reported to have dye concentration of about 60 mg/L [6]. The results of Pasti-Grigsby *et al*.[20] that 300mg/L was toxic to white rot fungus *Phanerochaette chrysosporium* also confirmed that this strain of *Proteus mirabulis* effectively decolorized the dye. The Michaelis constant (K_m) of 278.83 mg/L, maximum specific decolorization rate of 36.23 mg/L/h and the inhibitory concentration of 400 mg/L, suggested that the organism has good toxic tolerance for the sulphonated azo dye. However a much higher specific decolorization rate of 81.2 mg/L/h and the inhibitory

concentration of >1000 mg/l has been reported for the azo dye Direct Fast Scarlet 4BS[21], but immobilized microbial consortium was used and not a single strain as in this study.

In conclusion, the decolorization of the sulphonated dye, RB13, by a strain of *Proteus mirabilis* was study. The rate of the decolorization increased on aeration but decreased during agitation. The rate also depended on dye concentration (half order kinetics) and the cell mass concentration (first order kinetics). The dependence of the rate on the initial dye concentration followed Monod type kinetics. This newly isolated strain of *Proteus mirabilis* has potential in color effluent bioremediation.

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List of figures

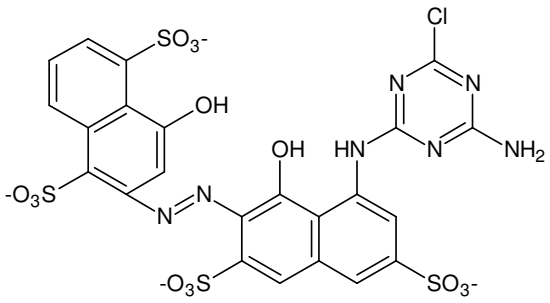

Figure 1

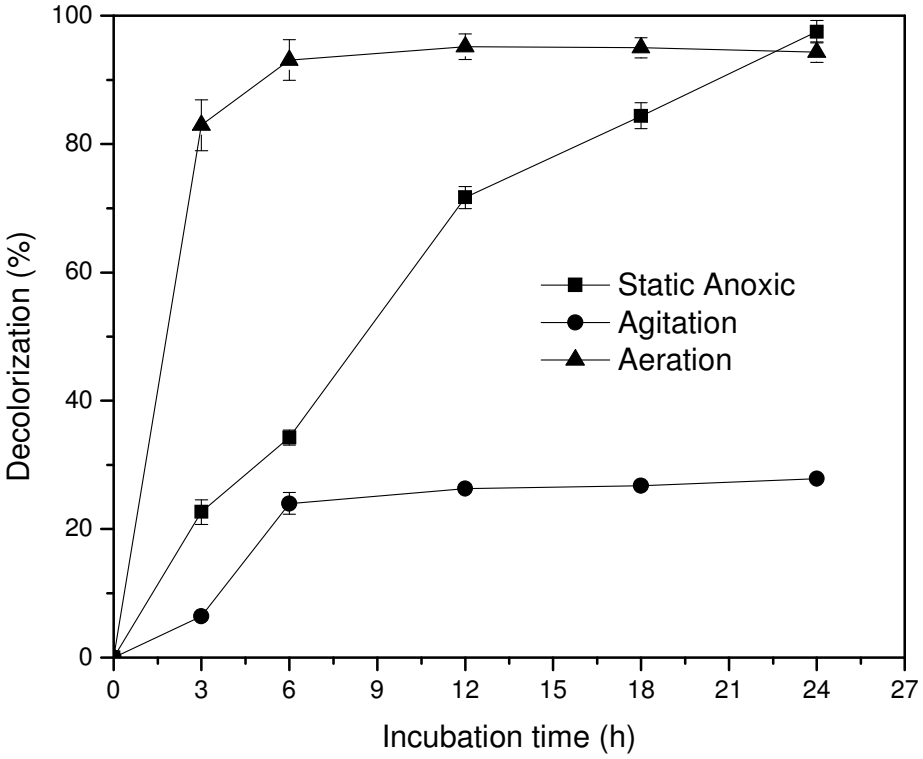
Decolorization (%)
Incubation time (h)
Static Anoxic
Agitation
Aeration
0
20
40
60
80
100
0
3
6
9
12
15
18
21
24
27

Figure 2.

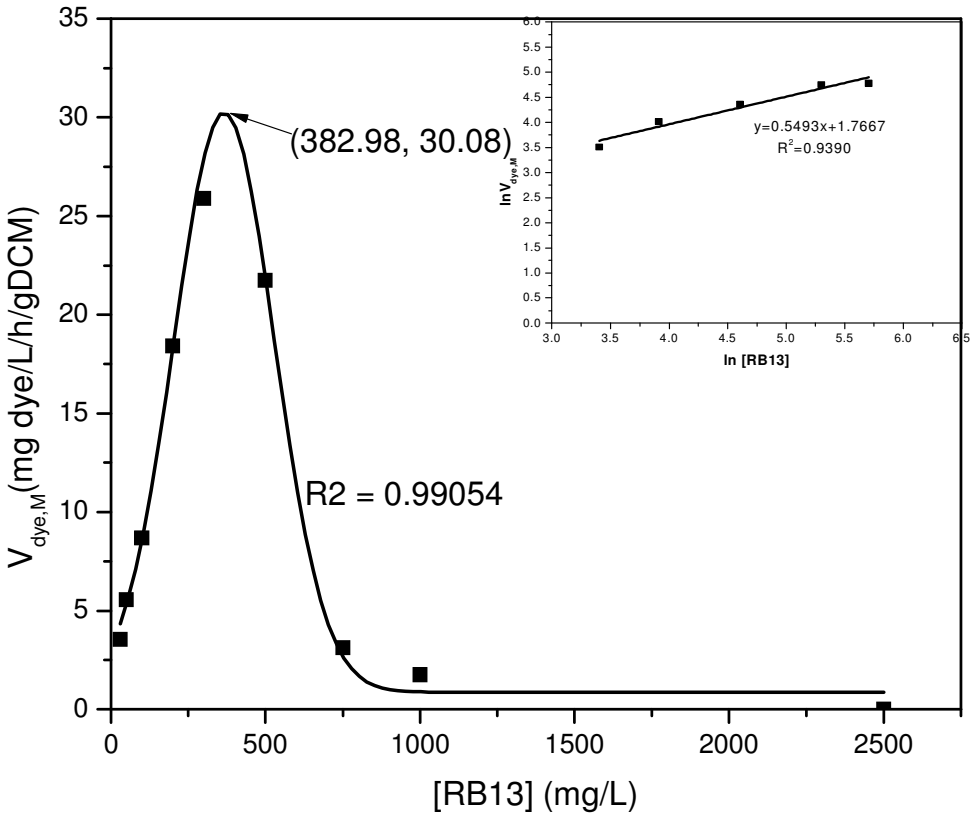
(382.98, 30.08)
R2 = 0.99054
$V_{dye,M}$(mg dye/L/h/gDCM)
[RB13] (mg/L)
y=0.5493x+1.7667
R^2=0.9390
ln $V_{dye,M}$
ln [RB13]

Figure 3.

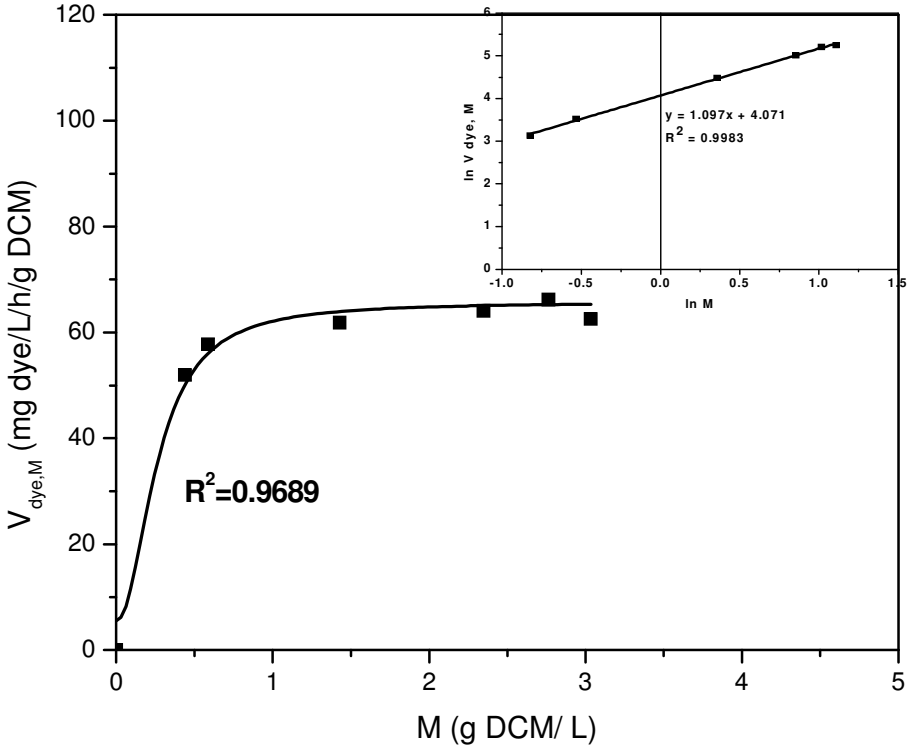
120
100
80
60
40
20
0
0
1
2
3
4
5
M (g DCM/ L)
V dye,M (mg dye/L/h/g DCM)
R2=0.9689
ln V dye, M
ln M
y = 1.097x + 4.071
R2 = 0.9983

Figure 4.

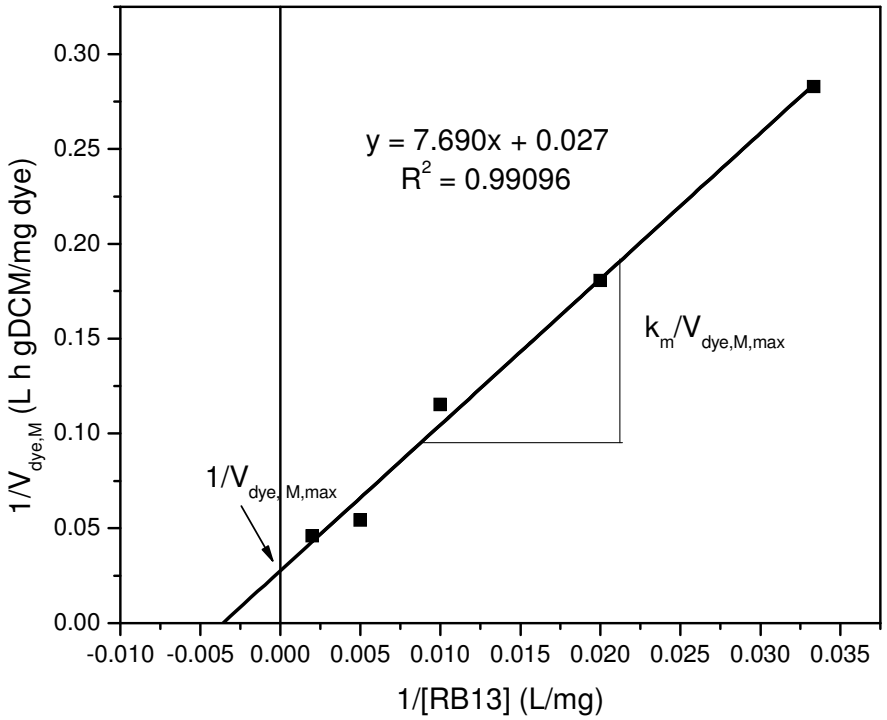
y = 7.690x + 0.027
R2 = 0.99096
km/Vdye,M,max
1/Vdye, M,max
1/Vdye,M (L h gDCM/mg dye)
1/[RB13] (L/mg)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
-0.010
-0.005
0.000
0.005
0.010
0.015
0.020
0.025
0.030
0.035

Figure 5.