The fate and inhibition effect of Direct Red 28 in an anaerobic mixed culture

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Abstract

The fate of an azo dye namely Direct Red 28 (DR 28) was investigated with partially granulated anaerobic mixed culture using glucose (3000 mg l^{-1} COD) as carbon source and electron donor during batch incubation. The course of the decolorization process approximates to zero order kinetic with respect to dye concentration. The decolorization kinetic constant (K_1) values increased from 3.6 to 11.8 mg $l^{-1}h^{-1}$ as increases in dye concentrations from 200 to 3200 mg l^{-1} in DR 28. Monod, 0., 1., and 2. order reaction kinetics were tested in order to determine the most suitable substrate removal kinetic. Since the increasing dye concentrations significantly inhibited substrate degradation, different kinetics was tested in order to detect the type and the level of inhibition. Aromatic amine and volatile fatty acid accumulation was observed proportionally at a higher DR 28 concentration. A competitive kinetic model that describes the anaerobic co-metabolism of increasing DR 28 dye concentrations with glucose as co-substrate has been developed based on the experimental data.

Keywords-Azo dye; Direct Red 28; decolorization; competitive inhibition

Notations

The following symbols are used in this paper:

R= substrate utilization rate (mg $l^{-1}.h^{-1}$)

R_{max}= (k_{max} .X): maximum substrate removal rate (mg $l^{-1}.h^{-1}$)

k_{max} = maximum specific substrate utilization rate (h^{-1})

X= biomass concentration (mg l^{-1})

S= co-substrate concentration (mg l^{-1})

K_S= half saturation concentration (mg l^{-1})

K_M= half saturation concentration for Michaelis-Menten kinetic (mg l^{-1})

S_t= residual glucose-COD concentration at selected time (t) through co-substrate removal (mg l^{-1})

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S_0= glucose-COD concentration at the beginning of the batch incubation through co-substrate removal (mg l^{-1})

k_0= zero order rate constant through co-substrate removal (mg $l^{-1}h^{-1}$)

k_1=first order rate constant through co-substrate removal (h^{-1})

k_2= second order rate constant through co-substrate removal (l $mg^{-1}h^{-1}$)

t= time (h)

I_D= inhibitor (dye) concentration (mg l^{-1})

K_{ID}= constant of inhibition (mg l^{-1})

R_i=substrate removal rate at *i.* steps (mg $l^{-1}.h^{-1}$)

S_i=substrate concentration at *i.* steps (mg l^{-1})

$S_{(i-1)}$ and $S_{(i+1)}$= the substrate concentrations measured at time $t_{(i-1)}$ and $t_{(i+1)}$ (mg l^{-1})

i= number of measured substrate concentrations remaining in the reactor at certain time intervals during the batch study (is to n from zero)

n= number of data obtained during the time course of the batch study

C_t= residual dye concentration at selected time (t) of batch test through decolorization (mg dye l^{-1})

C_0= dye concentration at the beginning of the incubation through decolorization (mg l^{-1})

K_0= zero order rate constant through decolorization (mg $l^{-1}.h^{-1}$)

K_1= first order rate constant through decolorization (h^{-1})

K_2= second order rate constant through decolorization (l $mg^{-1}.h^{-1}$)

R^2= regression analysis coefficient.

A_t= light absorbance of dyes at λ_{max} for a selected time (t) of batch test;

A_0= the light absorbance for λ_{max} at the beginning of incubation.

1. Introduction

Azo dyes represent a major group of all the dyes produced world-wide (Carliell *et al*., 1995; Kalyuzhnyi & Sklyar, 2000). Approximately 10-15% of overall production is released into the environment, mainly via wastewater (Tan *et al*., 2000). This is very dangerous because some of the azo dyes or their breakdown products have a strong toxic, mutagenic or carcinogenic influence on living organisms. Therefore, the corresponding wastewaters should be treated before discharge (Kalyuzhnyi & Sklyar, 2000). However, a majority of azo dyes are quite resistant to biodegradation under aerobic conditions and easily pass through conventional aerobic wastewater treatment systems. On the other hand, azo dyes are readily decolorized by splitting the azo bond(s) in anaerobic environments. Therefore, anaerobic conditions are preferable for azo dye reduction. Azo dye reduction leads to the formation of aromatic amines. Aromatic amines are generally not

degraded and accumulate under anaerobic conditions (Fitz-Gerald & Bishop, 1995; O'Neill *et al*., 2000), with the exception of a few aromatic amines characterized by the presence of hydroxyl and/or carboxyl groups (Razo-Flores *et a*l., 1997).

The process of anaerobic azo reduction has been intensively studied (Carliell et al., 1995; Razo-Flores *et al*., 1997; Beydilli *et al*., 1998). Most researchers have investigated the decolorization of azo dye in the anaerobic batch cultures. In a study performed by Işık and Sponza (2003), decolorization of Congo Red and Direct Black 38 was investigated using *Escherichia coli* and *Pseudomonas* sp. cultures in anaerobic, aerobic and microaerophilic condition. They reported that anaerobic conditions were more favorable than other conditions for decolorization. Beydilli et al., (1998) noted that 50 and 300 mg l^{-1} concentrations of Red 2 resulted in gas production higher than the control, whereas 500, 1000 and 2000 mg l^{-1} dye concentrations inhibited anaerobic culture activity and decreased total gas and methane production to 67% and 57% of control culture, respectively, at the concentration of 2000 mg l^{-1}.

Both zero order (Dubin & Wright, 1975; Brown, 1981) and first order (Carliell *et al*., 1995; Kalyuzhnyi & Sklyar, 2000; Beydilli *et al*., 1998; Wuhrmann *et al*., 1980; Weber, 1991), kinetics have been reported for the dye concentration through co-substrate degradation. Furthermore, the decolorization of 20 selected dyes by granular sludge was studied by van deer Zee *et al*. (2001). They found that decolorization reactions were followed by first-order kinetic. 97 and 96 % decolorizations were obtained in azo dyes Direct Black 19 and Reactive Black 5, respectively. Yoo (2002) found that the decolorization of C.I. Reactive Orange 96 shows a first order kinetic (0.12 mM min^{-1}) with respect to both dye (0.1 M) and sulfide concentrations (0.01M). However, Chang *et al*. (2001) used the Michaelis-Menten kinetic to describe the apparent correlation between the decolorization rate and dye concentration in a batch reactor containing immobilized *Pseudomonas luteola* and Reactive Red 22 dye. A first order reaction was obtained for both mesophilic and thermophilic decolorisation in azo dye Reactive Red 235 (Willetts & Ashbolt, 2000). The kinetics observed under thermophilic conditions (k= 0.0096 min^{-1}) were three times faster than under mesophilic conditions when the first order rate constants were compared.

Generally, the inhibition of azo dyes is known**,** but limited investigations have been performed to detect the type of inhibition and the level of inhibition on microorganisms**,** particularly on partially granulated mixed anaerobic cultures. Past studies showed that the kinetic constants decreased as the dye concentrations were increased through simultaneous color and substrate removals (van deer Zee *et al*. 2001; Brás, 2001). The anaerobic reduction studies with azo dyes performed until now showed that the methane productions and decolorization decreased and some intermediates such as aromatic amines and volatile fatty acids were accumulated through

methanogenesis of azo dyes (Razo-Flores *et al*., 1997; Wuhrmann et al., 1990; Knapp & Newby, 1995)**.** Moreover, the inhibition effects of azo dyes on microbial activity, on methane gas productions and on substrate removal rates and their inhibition kinetics were not extensively investigated. For example, Hu (2001) reported that the kinetic of azoreductase produced by *Pseudomonas luteola* can be explained with a competitive inhibition model using seven different azo-dyes at concentrations varying between 0 and 500 mg l^{-1}. Beydilli et al. (1998) studied the toxic effect of azo dyes on the inhibition of methane production in anaerobic mixed cultures. However, the inhibition of substrate removal rates was not clarified with linear or non-linear inhibition models.

The novel aspect of this study was to examine the inhibition effect of C.I. Direct Red (DR 28) azo dye, the level of inhibition, the effect of increasing dye concentrations on kinetic coefficients such as maximum substrate removal rate (R_{max}), half saturation constant (K_S), and dye inhibition constants (K_{ID}) through simultaneous anaerobic substrate and color removals. Furthermore, the kinetics of decolorization and substrate removals were studied, the methane gas productions, volatile fatty acids and aromatic amine concentrations were monitored throughout anaerobic experimental period.

2. Kinetic Approaches

2.1. Kinetic model based on Monod equation

Monod type kinetic models have been widely used to describe the apparent decolorization/biodegradation rate and co-substrate/dye removal (COD originated from glucose or dye) through simultaneous dye biodegradation and decolorization (Chang *et al*., 2001). The substrate removal rate in the Monod kinetic is commonly expressed by a deterministic model in a batch reactor (Grady *et al*., 1999);

$$-\frac{dS}{dt} = -R = -\frac{R_{max}.S}{K_S + S} \qquad (1)$$

via the method of partial fraction and integration of Eq.(1) in the assumption of X= constant ($R_{max}= k_{max} X$), it follows for $S=S_0$ at $t_0=0$ (Equation 2):

$$In\frac{S_0}{S_i}\frac{1}{t_i} = -\frac{1}{K_S}\frac{S_0 - S_i}{t_i} + \frac{R_{max}}{K_S} \qquad (2)$$

The rate of degradation of substrate is first order with respect to its concentration S, at low substrate concentrations ($S << K_S$) and at high concentrations ($S >> K_S$) (Equation 3);

$$-\frac{dS}{dt} = -k_1.S = -\frac{R_{max}.S}{K_S} \quad (3)$$

Substrate removal rate is constant regardless of the substrate concentration if $K_S << S$. In this situation the reaction order is zero (Equation 4).

$$-\frac{dS}{dt} = -k_0 = -R_{max} \quad (4)$$

2.2. Kinetic model for co-substrate degradation through decolorization of azo dye

Although there has been some success in applying Monod type kinetic to the dye decolorization process, some investigations showed that it proves difficult to apply them for their anaerobic systems. Therefore, the removal of co-substrate throughout decolorization of the azo dyes can follow zero, first and second order reaction kinetic which can be expressed by equations (5), (6) and (7).

$$S_t = S_0 - k_0.t \quad (5)$$

$$S_t = S_0.e^{-k_1.t} \quad (6)$$

$$\frac{1}{S_t} = \frac{1}{S_0} + k_2.t \quad (7)$$

Similarly, zero, first and second order kinetics were confirmed for azo dye decolorisation in batch anaerobic mixed cultures and were expressed as equations (8), (9) and (10).

$$C_t = C_0 - K_0.t \quad (8)$$

$$C_t = C_0.e^{-K_1.t} \quad (9)$$

$$\frac{1}{C_t} = \frac{1}{C_0} + K_2.t \quad (10)$$

2.3. Models for azo dye inhibition

Inhibition models are classified according to the effect of toxic compounds (in this study the azo dyes) on the reaction rate (R_{max}) and half saturation constant (K_S). That approach is used for adaptation of substrate removal rate to Monod kinetics.

In the presence of increasing concentrations of dye, the impact of dyes is explained by the modified Monod equations. Generally**,** the effect of dyes is related to R_{max} and K_S values. Depending on the type of azo dye and its concentration the variations in R_{max}, K_S values and the inhibitions were expressed by the following equations (Lehninger, 1977);

Competitive inhibition

$$-\frac{dS}{dt} = -R = -\frac{R_{max}.S}{K_S.\left(1+\frac{I_D}{K_{ID}}\right)+S} \qquad (11)$$

Non-competitive inhibition

$$-\frac{dS}{dt} = -R = -\frac{R_{max}}{\left(1+\frac{K_S}{S}\right).\left(1+\frac{I_D}{K_{ID}}\right)} \qquad (12)$$

Uncompetitive inhibition

$$-\frac{dS}{dt} = -R = -\frac{\frac{R_{max}.S}{(1+\frac{I_D}{K_{ID}})}}{\frac{K_S}{(1+\frac{I_D}{K_{ID}})}+S} \qquad (13)$$

If the equation (1) is linearized, in other words when 1/R is plotted against 1/S, a straight line is obtained (Lineweaver-Burk plot). This line will have a slope of K_S/ R_{max}, an intercept of $1/R_{max}$ on the 1/R axis, and an intercept of $-1/K_S$ on the 1/S axis. Such a double reciprocal plot has the advantage of allowing much more accurate determination of R_{max} and K_S. The double reciprocal plot can also give valuable information on inhibition. The possible effects of dyes on Lineweaver–Burk plot can be seen by the linearization of equations (11), (12) and (13). The possible inhibitions of increasing dye concentrations to the slope and to the intercepts and the type of inhibitions are given in equations (14), (15), (16) and (17).

	Slope	**Intercept on ordinate**	
No dyes	$\frac{K_S}{R_{max}}$	$\frac{1}{R_{max}}$	(14)

Competitive	$\frac{K_S}{R_{max}}\left(1+\frac{I_D}{K_{ID}}\right)$	$\frac{1}{R_{max}}$	(15)
Non competitive	$\frac{K_S}{R_{max}}\left(1+\frac{I_D}{K_{ID}}\right)$	$\frac{1}{R_{max}}\left(1+\frac{I_D}{K_{ID}}\right)$	(16)
Uncompetitive	$\frac{K_S}{R_{max}}$	$\frac{1}{R_{max}}.\left(1+\frac{I_D}{K_{ID}}\right)$	(17)

2.4. Determination of kinetic coefficients

0^{th}, 1^{st} and 2^{nd} and Monod kinetics were applied to the residual COD values obtained from the removal of co-substrate throughout the batch incubation period in order to detect the substrate degradation kinetic. Experimental data obtained from the batch tests were plotted in form In (S_0/S) /t versus $(S_0-S)/t$, S versus time, ln S versus time, and 1/S versus time using equations (2), (5), (6) and (7), respectively.

The residual dye concentrations which remained in the batch fed anaerobic reactors were plotted in form C versus time, ln C versus time, and ln C/C_o versus time using equations (8), (9) and (10), respectively, in order to determine the decolorization kinetic.

Since the Monod kinetic could not be applied to the data obtained in this study, the reaction rate of the first order reaction kinetic was equalized to the reaction rate of the first order Monod kinetic, in equation (18), to detect the K_S values through simultaneous decolorization and biodegradation in order to determine the type of inhibition and the inhibition coefficient.

$$-k_1 . S_i = -\frac{R_{max} . S_i}{K_S + S_i} \qquad (18)$$

The substrate removal rates (R_i) throughout t times were obtained for every dye concentration using a finite difference, non-linear technique. The residual substrates (COD) were measured at certain intervals (S_i) throughout the times (t) in batch tests. The values of dS/dt, for substrate removal rates (R_i) were calculated using equation (19) for given t_i times. For purposes of illustration, the intervals of S_i and t_i shown on graph are enlarged as shown in Fig. 1. The values used for S_{i-1}, S_{i+1} were the actual experimental values during the batch time of the experiment. That is, no attempt was made to construct tangents to the curve through the data; the slopes, dS/dt, were the secant values.

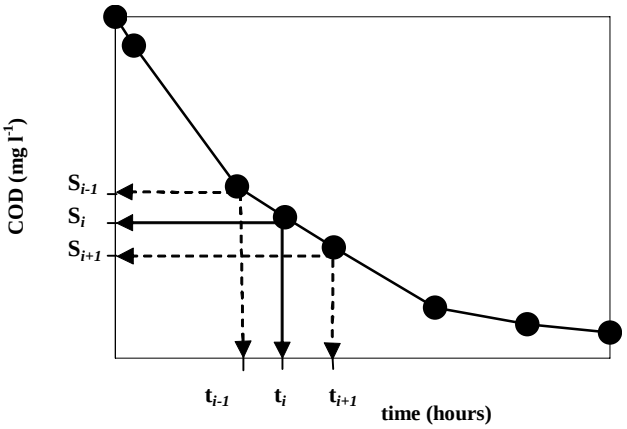

Figure 1. Determination of substrate variations versus time

$$\frac{dS}{dt} = R_i = \frac{S_{(i+1)} - S_{(i\ 1)}}{t_{(i+1)} - t_{(i\ 1)}} \qquad (19)$$

At low substrate concentrations and high K_S values the substrate was removed according to first order in Monod kinetic under anaerobic conditions. Considering this and taking into account the first order substrate removal kinetic, K_S can be calculated by rearranging equation (18) and written in equation (20).

$$K_S = \frac{R_{max}}{k_1} - \frac{\sum_{i=0}^{n} S_i}{(n-1)} \qquad (20)$$

3. Material and methods

3.1. Lab-scale batch anaerobic reactors, synthetic media, seed and dyes used throughout the experiments

The studies were performed in 115ml dark glass serum bottles sealed with 5mm rubber septum kept in place by a screw cap. Each serum bottle contained 4.0 ml of partially granulated anaerobic sludge obtained from the UASB reactor treating the wastewaters of PAK MAYA yeast factory in Izmir, 3000 mg COD l^{-1} of glucose and the necessary volume of macro and micro nutrients (Vanderbilt mineral medium) as described in Table 1. This mineral medium was used in all batch investigations and consisted of the following inorganic composition (in mg l^{-1}): NH_4Cl, 400; $MgSO_4.7H_2O$, 400; KCl, 400; $Na_2S.9H_2O$, 300; $(NH_4)_2HPO_4$, 80; $CaCl_2.2H_2O$, 50; $FeCl_3.4H_2O$, 40; $CoCl_2.6H_2O$, 10; KI, 10; $(NaPO_3)_6$, 10; L-cysteine, 10; $AlCl_3.6H_2O$, 0.5;

$MnCl_2.4H_2O$, 0.5; $CuCl_2$, 0.5; $ZnCl_2$, 0.5; NH_4VO_3, 0.5; $NaMoO_4.2H_2O$, 0.5; H_3BO_3, 0.5; $NiCl_2.6H_2O$, 0.5; $NaWO_4.2H_2O$, 0.5; Na_2SeO_3, 0.5 (Speece, 1996).

Table 1. Protocol for simultaneous degradation, decolourization and kinetics throughout batch tests

Stock	Seed control (ml)	Dye containing bottles (ml)	Resulting concentrations (mg l^{-1})
Sludge, 75 g l^{-1}	4	4	4000
Glucose, 30 g COD l^{-1}	-	7.5	3000
Vanderbilt min. med.	7.5	7.5	Desired composition
$NaHCO_3$, 50 g l^{-1}	7.5	7.5	5000
Nathioglycollate, 50 g l^{-1}	1.0	1.0	(w/w)%0.067
Dye, 10 g l^{-1}	-	0; 1.5; 3; 6; 12; 24	0; 200; 400; 800; 1600;3200
Total volume	75	75	

The anaerobic conditions were maintained by adding 667 mg l^{-1} of sodium thioglycollate (% 0.067) that is proposed between (w/w) % 0.01-0.2 for anaerobic conditions. The alkalinity and neutral pH were adjusted by addition of 5000 mg l^{-1} $NaHCO_3$. 3000 mg l^{-1} of glucose–COD was used as co-substrate providing reducing equivalents with electron fission. All incubations were carried out in a temperature controlled incubator at 35ºC. The bottles were vigorously shaken at certain intervals and the liquid samples were taken from the supernatants with a syringe for analysis.

3.2. Experimental procedure

Throughout the substrate removal and decolorization experiments all tests were conducted in duplicate in order to check the accuracy of the results. Throughout the batch tests, a control without dye and a seed blank sample were used to detect and compare the methane production in order to correct the methane production in batch serum bottles containing all concentrations of the dye. Serum bottles containing dyes and controls were duplicated throughout the tests to detect the accuracy of the kinetic and the absorbance data through simultaneous decolorization and degradation of azo dyes. Throughout these tests the data necessary for substrate kinetic was detected from both the COD values and the methane gas. The methane gas to COD conversion was carried out as follows: 0.395 ml methane gas was produced from the removal of 1 mg COD l^{-1}.

C.I. Direct Red 28, known as Congo Red, is a banned azo dye, containing carcinogenic aromatic amine (benzidine), and was used in dissolved form. Fig. 2 shows the structure and the chemical properties of the dyes.

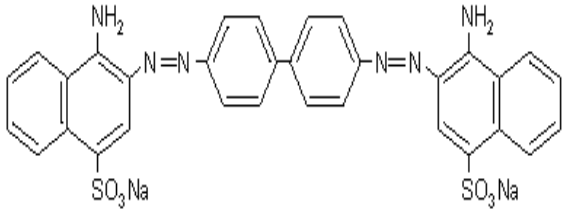

Figure 2. The structure of DR 28 used in batch assays (Direct Red 28 (Congo Red), Color Index number: 22120, λ_{max}: 497 nm, COD of 1000 mg l^{-1} of Direct Red 28 solution: 746 mg l^{-1})

3.3. Analytical procedure

Total suspended solids (TSS) in granulated sludge were measured by the filtration technique using membrane filters with pores sized 0.45 μm (APHA-AWWA, 1992). Methane production was monitored during the assay by using a sodium hydroxide solution (3%, w/v) displacement system. The strength of the solution was sufficient to remove the carbondioxide from the total gas (Razo-Flores et al., 1997). Total volatile fatty acid (TVFA) concentrations and bicarbonate alkalinity in the samples were measured using the titrimetric Anderson and Yang method with a computer program (Anderson and Yang, 1992). The total aromatic amines were determined colorimetrically at 440 nm after reacting with 4-dimethylamino benzaldehyde-HCl according to the method described by Oren et al. (1991). Total aromatic amines (TAA) released from anaerobic and chemical reduction were quantified using benzidine as standard at absorbance maxima of 440 nm. The chemical reduction of azo dyes was carried out with sodium dithionite. The reduction process was as follows: 0.06 g of dye sample was heated at boiling point with 1 M NaOH for 1 h and after 30 min. 0.6 g sodium dithionite was added. 1000 mg l^{-1} of dye was reduced according to the method described by Pielesz (1999). (See Fig. 3).

Color measurements were carried out in 5 ml samples removed from the supernatants of the serum bottles to detect the decolorization efficiencies once every 2-3 hours. It was measured by an Optima Photomech 301-D UV-VIS spectrophotometer at wavelength of 497 nm in which maximum absorbance spectra is obtained for DR 28. The samples were centrifuged at 7000 rpm for 10 min and the absorbance values of supernatants were recorded for color measurements. Color removal ratio was determined according to equation (21).

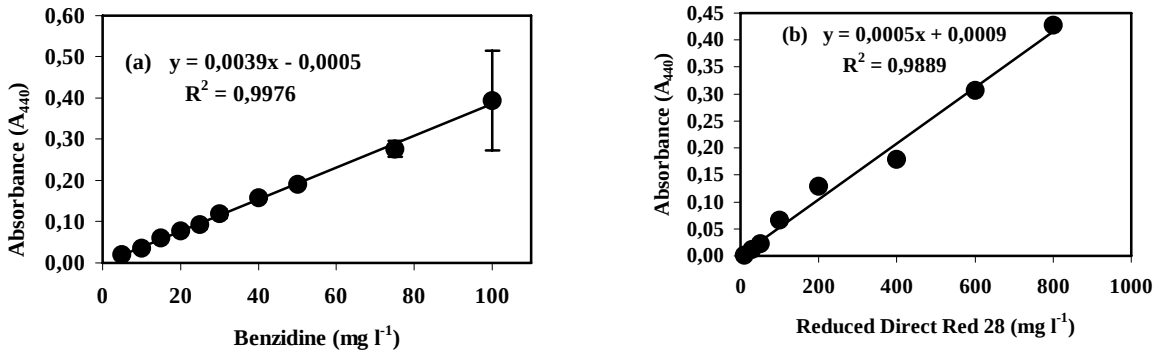

Figure 3. The calibration curves of benzidine, and DR 28 versus absorbance at 440 nm.

$$\text{Color removal}\,(\%) = \frac{(A_0 \quad A_t)}{A_0}.100 \qquad (21)$$

The decolorization of the azo dye with partially granulated anaerobic mixed cultures was measured as the decrease of light absorbance at their maximum wavelengths. Concentrations of dyes were determined from the measured light absorbance at maximum wavelength after determining the best fit with higher correlation coefficient (R^2>0.95) for absorbance versus concentration during incubation.

4. Results

4.1. Co-metabolism of DR 28 and degradation kinetic of co-substrate

The co-substrate (glucose) used in this study is defined as the carbon and energy source for microbial growth and maintenance and release of the electrons for cleavage of dyes under reducing environments. The co-metabolism of azo dyes is defined as the removal of non-growth substrates (DR 28) by microorganisms in the presence of a growth (co-substrate) substrate (3000 mg l^{-1} of COD as glucose equivalent). The anaerobic bacteria utilized the energy generated from the glucose degradation for the reductive cleavage of DR 28 dye. In other words, the azo dye was removed by fortuitous metabolism in which the anaerobic bacteria do not derive energy from the dye.

Fig. 4 demonstrates typical residual COD (S_i) levels throughout 300 hours of incubation periods of DR 28 azo dye concentrations varying between 200 and 3200 mg l^{-1}. At lower dye concentrations the COD concentrations dropped almost linearly until degradation was completed while the profile for high dye concentrations exhibited extra phases (indicated by different slopes) during the early stage of degradation. The remaining COD concentration was 500 mg l^{-1} in batch rectors 2 containing 200 mg l^{-1} of DR 28 dye, after 100 hours of the incubation period. Similarly, the residual

COD concentrations were 0 and 954 mg l^{-1} in dye-free and in reactors containing 3200 mg l^{-1} of DR 28 dye, respectively, at the end of 300 hours of the incubation period. This indicates that the COD was biodegraded faster and better in batch reactors containing low concentrations of DR 28 compared to reactors containing higher DR 28 concentrations.

Figures 5 a, b, c, and d show the plots between In $(S_0/S_i)/t_i$ versus $(S_0-S_i)/t_i$, and S_i, ln S_i and $1/S_i$ versus time using equations (2), (5), (6) and (7), respectively in order to determine the kinetic constants through the degradation of co-substrate at DR 28-free samples. The kinetic and correlations coefficients relevant to the Monod, the zero, first and second orders are summarized in Table 2.

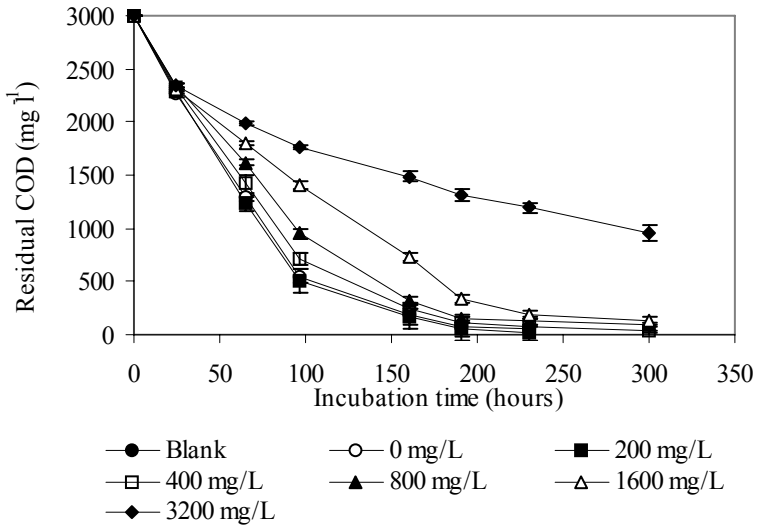

Figure 4. The residual COD (S_i) concentrations throughout 300 hours of incubation period

As shown in this table, the comparison of regression coefficients (R^2) relevant to Monod, zero, first order and second order rate constants (k_0, k_1 and k_2 values) showed that COD was removed according to the first order reaction kinetic. Increases in dye concentrations from 0 to 3200 mg l^{-1} reduced the k_1 values from 1.9 $x10^{-2}$ to 3.5 $x10^{-3}$ h^{-1} in batch studies. Similar batch studies performed by Brás *et al*. (2001) demonstrated that the COD degradation rate constant (first order kinetics) decreased from 4.0 $x10^{-2}$ h^{-1} to 1.6 $x10^{-2}$ h^{-1} while the Acid Orange concentration increased to 300 mg l^{-1}.

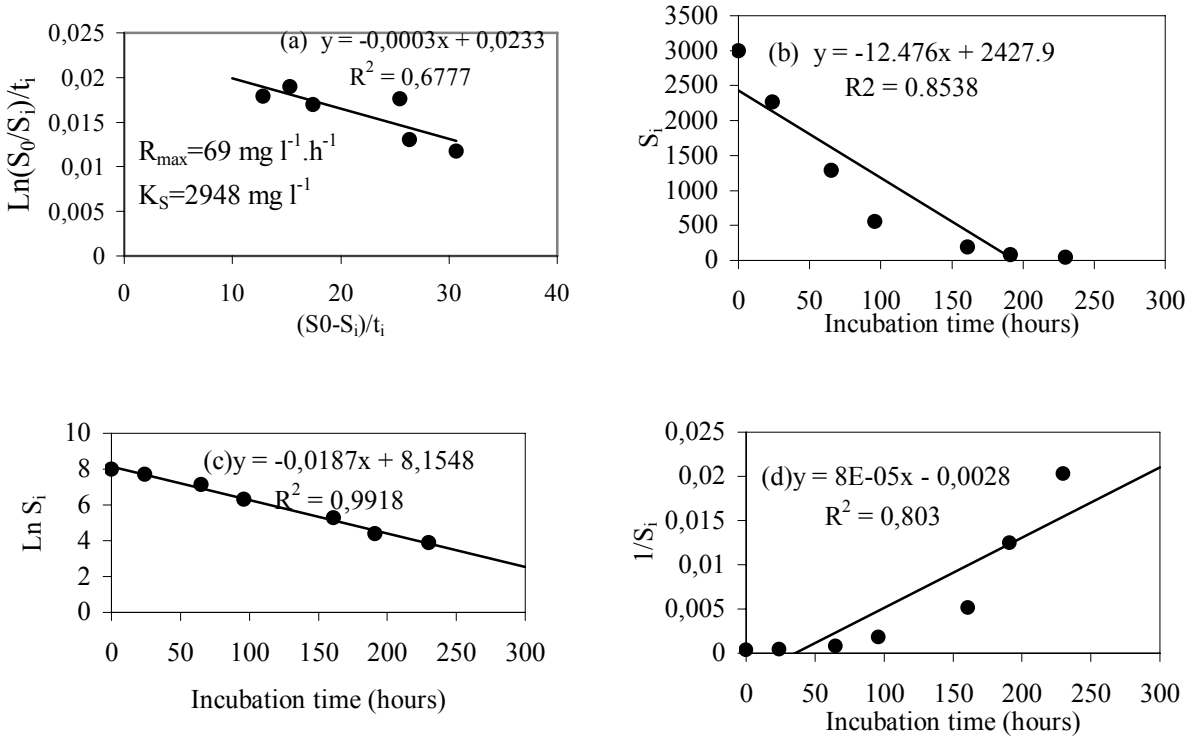

Figure 5. Monod (a), zero (b), first (c), and second (d) order reaction kinetic for 0 mg/L of DR 28 azo dye

Table 2. Monod, zero, first and second order kinetic constants obtained in anaerobic batch tests during COD degradation

Kinetics	Constants	0 mg l^{-1}	200 mg l^{-1}	400 mg l^{-1}	800 mg l^{-1}	1600 mg l^{-1}	3200 mg l^{-1}
Monod	R_{max} (mg $l^{-1}.h^{-1}$)	69	57	74	72	375	-
	K_S (mg l^{-1})	2948	2064	3799	4832	36400	-
	R^2	0.678	0.760	0.570	0.332	0.010	
0. order	k_0 (mg $l^{-1}.h^{-1}$)	12.5	12.6	12.6	12.6	11.9	6.9
	R^2	0.854	0.847	0.800	0.837	0.911	0.894
1. order	k_1 (h^{-1})	1.9 x10^{-2}	2.1 x10^{-2}	1.6 x10^{-2}	1.3 x10^{-2}	1.1 x10^{-2}	3.5 x10^{-3}
	R^2	0.992	0.987	0.990	0.963	0.974	0.973
2. order	k_2 (l $mg^{-1}.h^{-1}$)	7.9 x10^{-5}	1.4 x10^{-4}	5.1 x10^{-5}	3.4 x10^{-5}	1.8 x10^{-5}	2.1 x10^{-6}
	R^2	0.803	0.697	0.781	0.917	0.848	0.989

4.2. Decolorization kinetics of DR 28

Azoreductase enzyme systems help bacteria to decolorize high concentrations of azo dye with a co-substrate under anaerobic conditions (Carliell et al., 1995; Wuhrmann et al., 1980;Yoo, 2002). Taking these characteristics into consideration, the effect of increasing DR 28 azo dye concentrations on decolorization of dye was determined.

To determine the decolorization kinetic of DR 28 dye, experiments with constant initial substrate (3000 mg l^{-1} of glucose COD) and different initial dye concentrations varying between 200-3200 mg l^{-1} were performed in order to detect the residual dye concentrations and percentages throughout 54 hours of the incubation period. The residual dye percentages for different initial dye concentrations are depicted in Fig. 6.

Abiotic tests performed with autoclaved anaerobic partially granulated sludge showed that the microbial decolourization was preceded primarily by biological degradation. As shown in Fig. 6, the colour remained almost completely in abiotic sample, and only 2 % of the colour was removed in this way in test carried out with the dye. In other words, compared to more than 98% color removal under biotic conditions incubation under abiotic conditions led to only 2 % colour removal**,** which was probably due to physical adsorption of dye molecules by the cells dying or by auto-oxidation. This fact also implies that the physical adsorption of dye molecules on cell mass was a negligible mechanism in colour removal.

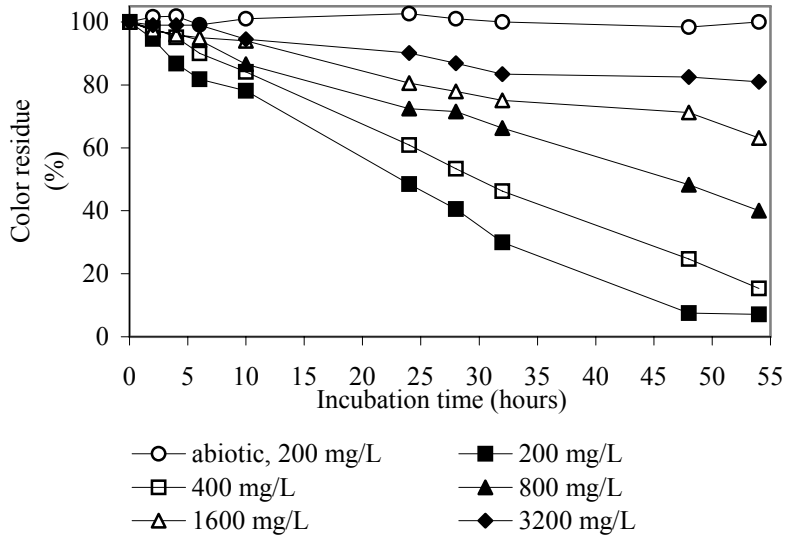

Figure 6. Percentages of residual colour at different DR 28 concentrations throughout the 54 hours of the incubation period.

The average residual color (%) was between 78 % and 95 % in batch reactors containing 200, 400, 800, 1600 and 3200 mg l^{-1} DR 28**,** in the first 10 hours. Fig. 6 shows the residual color percentages for DR 28 dye during the incubation period. After 54 hours of the incubation period the residual color percentages were almost 10**,** indicating the color was not completely removed for 200 mg l^{-1} of DR 28.

As explained above, the reactions proceeded without a lag phase. The color removal efficiency of the dye was lower, resulting in relatively high residual dye concentration. Samples

containing higher DR 28 concentrations had a slow rate of decolorization**,** which was not complete even after 54 hours of the incubation period (See Fig. 6).

Experimental data obtained from the batch tests were plotted in form C versus time, ln C versus time, and ln C/Co versus time using equations (8), (9) and (10), respectively. In the following step, the color removal rate constants were found for zero, first and second order reaction kinetic from the slopes of the best fit lines, respectively (See Table 3). Among the data obtained, the rate constants with the highest regression coefficients and relevant reaction kinetic were accepted as the most suitable kinetic and kinetic coefficients. A high degree linear relationship (R^2 >0.95) between the dye concentration (C) and time showed that the color was removed according to zero order kinetic in all batch decolorization tests.

The zero, first, and second order rate constants (K_0, K_1 and K_2), through color removals, resulting from the fitting equation (8), (9) and (10) and the whole test results are listed in Table 3.

Table 3. The rate constants from decolorization kinetic test

Constants	**200 mg l^{-1}**	**400 mg l^{-1}**	**800 mg l^{-1}**	**1600 mg l^{-1}**	**3200 mg l^{-1}**
K_0 (mg $l^{-1}.h^{-1}$)	3.6	6.4	8.7	10.4	11.8
R^2	0.981	0.998	0.996	0.973	0.967
K_1 (h^{-1})	5.0 x10^{-2}	3.2 x10^{-2}	1.6 x10^{-2}	8.0 x10^{-3}	4.1 x10^{-3}
R^2	0.949	0.957	0.976	0.975	0.974
K_2 (l $mg^{-1}.h^{-1}$)	1.2 x10^{-3}	2.1 x10^{-4}	3.1 x10^{-5}	6.2 x10^{-6}	1.4 x10^{-6}
R^2	0.789	0.806	0.924	0.970	0.980

As seen in Table 3, the decolorization rate constants of samples containing higher concentration of DR 28 dye are significantly higher than the rate constants of samples containing lower concentration of DR 28 dye. The decolorization constants were found to be between 3.6-11.8 mg $l^{-1}.h^{-1}$ at DR 28 concentrations of 200 mg l^{-1} and 3200 mg l^{-1}, respectively**.** This result contradicts the study performed by Weber (1991) who reported that the degradation of benzidine based azo dye in an anaerobic pond sediment followed pseudo first order kinetic with half lives ranging from 2 to 6 days.

Zero order kinetics with respect to dye concentration have also been reported by several researchers (Dubin and Wright, 1975; Brown, 1981), whereas others found first order kinetics (Carliell et al., 1995; Wuhrmann et al., 1980; Weber, 1991; van der Zee et al., 2001; Weber, Wolfe, 1987).

4.3. The effect of increasing dye concentrations on methane production during simultaneous biodegradation and decolorization

Fig. 7 shows the cumulative methane gas productions during the incubation period in batch reactors containing 3000 mg l^{-1} glucose COD, and DR 28 dye separately at concentrations varying between 200 and 3200 mg l^{-1}. The quantities of cumulative methane gas showed that a quick initial methane production occurred due to hydrolysis and degradation of glucose together with azo dye. Afterwards, the methane production became slower because of the accumulation of aromatic amine and volatile fatty acids, which would make the methanogenic stage progress less readily (Beydilli et al., 1998; Donlon et al., 1995). In all assays**,** the cumulative methane decreased as the azo dye concentrations increased. This could have occurred during the solubilization of azo dye degradation products or intermetabolites and these products could inhibit the activity of anaerobic bacteria. 3000 mg l^{-1} of glucose–COD was completely converted to methane gas in dye-free batch reactors. As shown in Fig. 7, the cumulative gas measured at DR 28 concentrations varying between 800 and 3200 mg l^{-1} are about 97 % and 70% of samples containing the dye-free and 200 mg l^{-1} of dye, respectively, after 300 hours of incubation. It is clear that high concentrations of DR 28 dye caused inhibition of anaerobic degradation of glucose**,** resulting in lowering of methane gas during incubation. 30 % lower methane gas production was achieved in 3200 mg l^{-1} of dye-amended cultures at the end of 300 hours of the incubation periods.

Carliell et al. (1995), Razo-Flores et al. (1997), and Chinwetkitvanich et al. (2000) reported that the decolorization took place in the methane production stage. The bacteria could be using the azo bonds in the chromophores of the dye as an electron acceptor together with electrons coming from a H-donor co-substrate**,** resulting in decolorization. As a result, the level of methane formation should reflect the degree of the color removal. In our studies, the methane gas production corresponds with the residual dye concentrations measured in the batch reactors seeded with partially granulated mixed (methanogenic+acidogenic bacteria) sludge. This finding is also in agreement with those of Carliell et al. (1995) and Razo-Flores et al. (1997) but contradicts Panswad et al. (2000) who found that decolorisation with some facultative versus anaerobes. The study performed by Işık and Sponza (2003) also showed that non methanogenic bacteria are responsible for the color reduction in these processes.

4.4. The variations of total aromatic amine (TAA), volatile fatty acids (VFA), Bicarbonate alkalinity (B. Alk.) and pH variations in different concentrations of DR 28 azo dye

The high residual COD concentrations could be attributed to the presence of residual dye and dye metabolites that were not completely mineralized. The dye contribution to the fed COD was

746 mg l^{-1} in 1000 mg l^{-1} of DR 28 solution**,** so the measured levels of residual COD (data not shown) and TAA in supernatants indicate that the metabolites seem to introduce a significant level of COD in the batch reactors. This result suggests that degradation of intermediates did not take place, contrary to the data obtained by Brás *et al*. (2001).

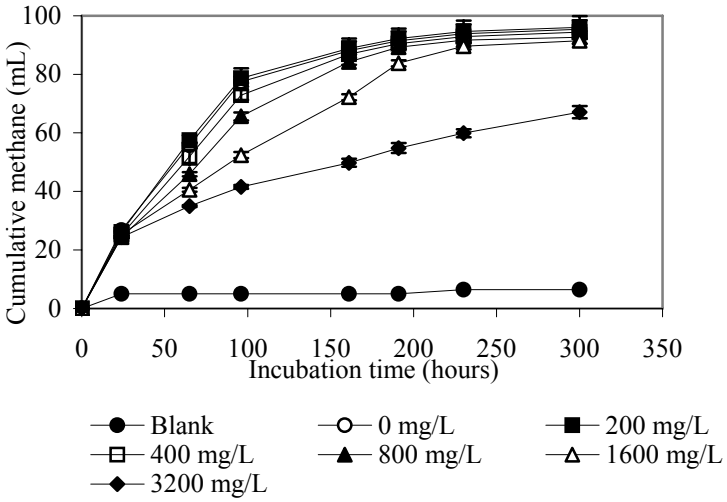

Figure 7. The effect of different concentrations of DR 28 on cumulative methane production.

TAA, VFA, B. Alkalinity levels and pH values were measured in all batch reactors containing different concentrations of DR 28 azo dye at the end of the incubation period. The concentrations of total aromatic amines released from the azo dye increased as the DR 28 concentrations were increased. Fig. 8 shows the variations of TAA (a) and VFA, B.Alk. concentrations, and pH values (b) for increasing concentrations of DR 28 dye. At higher DR 28 concentrations, while the pH and the B. Alkalinity levels decreased, the VFA concentrations increased. This could be due to the inhibition of methanogenesis and accumulation of inter-metabolites through simultaneous degradation and decolorization of dye (Beydilli *et al*., 1998; Brás *et al*., 2001; Donlon *et al*., 1995). The amount of recovered TAA accounted for 100 % of expected TAA which is calculated from equations on graphs in Fig. 3a and b. Fig. 8a clearly shows the accumulation of aromatic amines (as benzidine) during the degradation of increasing concentrations of DR 28 azo dye. Higher DR 28 concentrations cause lower methane production because of the accumulation of aromatic amines and VFA, which are known to be toxic to methanogenic bacteria.

As a result, increases in TAA, VFA concentrations and decreases in pH were observed with increases in DR 28 concentrations while the B. Alkalinity decreased as it was consumed to neutralize the medium through fatty acid production.

The accumulation of some intermediates, which could be explained by structural modifications of the azo dye molecule due to the azo bond reduction, producing metabolites such as

aromatic amines, was reported by many researchers (Wuhrmann *et a*l., 1980; Brás *et al*., 2001; Knapp & Newby , 1995).

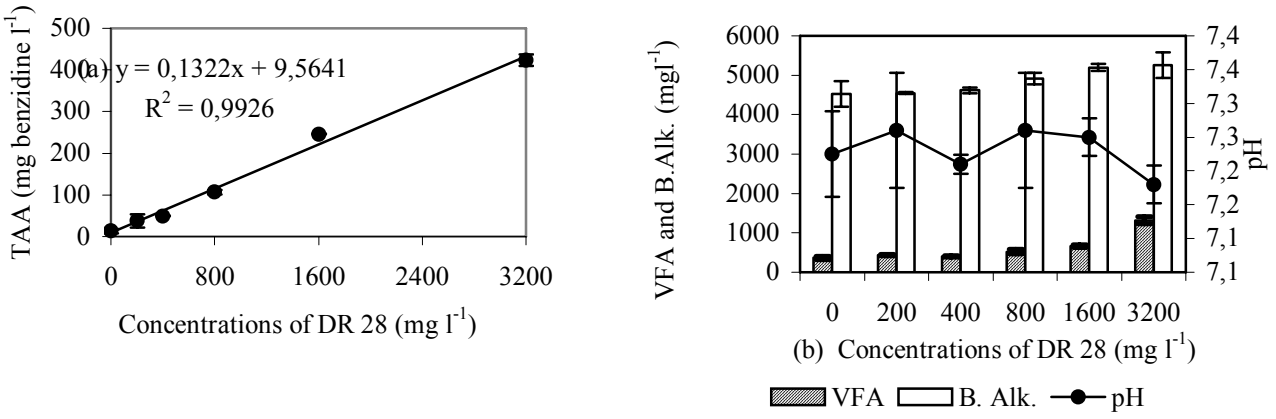

Figure 8. Variations in TAA (a), and VFA (mg CH_3COOH/L), B.Alk. (mg $CaCO_3$/L) concentrations and pH values (b) after reduction of DR 28 dye at increasing concentration.

4.5. Determination of inhibition kinetics of DR 28 azo dye

The substrate removal rates (R_i) and the the half velocity constants (K_S) were calculated using equations (19) and (20), respectively, in order to determine the type of inhibition. R_{max} value given in equation (20) is the maximum substrate removal rate and was calculated from the initial glucose-COD concentration of 3000 mg l^{-1} to the first substrate removal interval in which the first remaining COD was measured in the batch course. Fig. 9 depicts the calculated substrate removal rates (R_i) plotted versus substrate concentration (S_i) for batch test performed in dye free sample. As shown in this Fig. R_{max} was calculated to be 30.7 mg $l^{-1}.h^{-1}$.

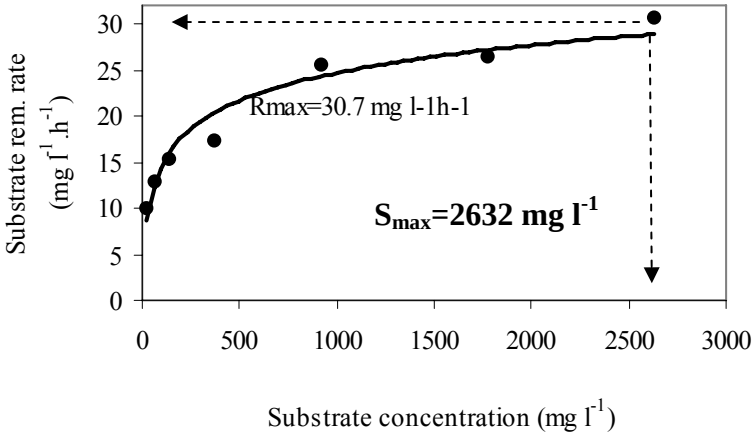

Figure 9. Substrate removal rate (R_i) versus substrate concentration (S_i) for dye free sample in batch reactor.

The batch studies performed clearly showed that the inhibition of increasing dye concentrations on methane gas productions was accomplished with the inhibition of glucose degradation. The reciprocal fits (Lineweaver-Burk plots) of inhibition given in equations (11), (12) and (13) ($1/R_i$ versus $1/S_i$) showed that DR 28 azo dye caused competitive inhibitions on glucose-COD removals. The slopes and the intercepts indicating the type of inhibition were found from the Lineweaver-Burk plots at increasing dye concentrations. Fig. 10 shows the plots between 1/R and 1/S for dye concentration varying between 0 and 3200 mg l^{-1}. The K_S and R_{max} values were calculated from the equations (19) and (20) in dye-free samples and were substituted in reciprocal fits of the competitive inhibition in order to determine the inhibition constants (K_{ID}). The slope of the dye free plot is (K_S/R_{max}) whereas the slope of increasing dye containing plots is (K_S/R_{max})*(1+I_D/K_{ID}). In other words, increases in the intercepts and slopes on the 1/S axis of plots containing dye indicate that the slopes (K_S/R_{max}) in inhibited fits increased by a factor 1+(I_D /K_{ID}). As shown in Fig. 10, in the competitive inhibition, the maximum substrate rates (R_{max}) did not change while the K_S values increased.

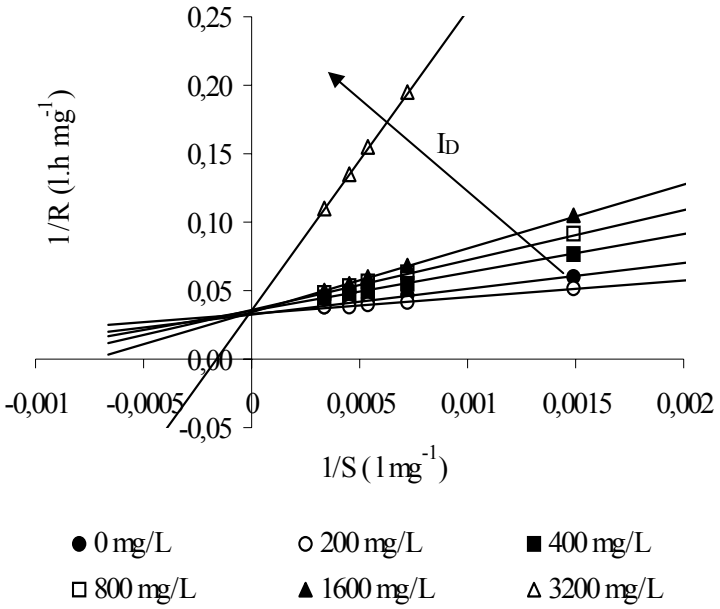

Figure 10. Lineweaver-Burk plots

The kinetic data calculated from the aforementioned plots were documented in Table 4. As shown in this table the apparent K_S for dye and glucose degrading/decolorizing samples are greater than the K_S measured in samples treating only glucose. The K_S values increased from 580 to 5977 mg l^{-1} as the dye concentrations increased from 200 to 3200 mg l^{-1} while the R_{max} in dye-free samples was 30.7 mg $l^{-1}.h^{-1}$.

Table 4. Maximum substrate utilization rate, half saturation concentration, and inhibition constant values

Constants	0 mg l^{-1}	200 mg l^{-1}	400 mg l^{-1}	800 mg l^{-1}	1600 mg l^{-1}	3200 mg l^{-1}	Average
R_{max}(mg $l^{-1}.h^{-1}$)	30.7	29.6	28.3	27.3	28.6	27.3	28.6
K_S (mg l^{-1})	580	354	789	994	1330	5977	1671
K_{ID} (mg l^{-1})	-	No inhibition	842	862	1095	302	511

The mean R_{max} value was 28.6±1 mg $l^{-1}h^{-1}$ at increasing DR 28 concentrations. Therefore, it can be assumed that the increasing dye concentrations were not affected the maximum reaction rates. However, it is important to note that the K_S is a much more important kinetic parameter than R_{max} governing the reaction and, therefore, the inhibition, since the reaction rate is the function of R_{max}, K_S, I_D concentrations and K_{ID} values.

As seen in Table 2 the first order kinetic constants (k_1) decreased from 0.0187 to 0.0035 h^{-1} as the accumulation of glucose-COD, dye and corresponding intermetabolites such as volatile fatty acids and aromatic amines took place contributing to high K_S values. The inhibition constants (K_{ID}) were between 842 and 302 mg l^{-1} for batch reactors containing 400 and 3200 mg l^{-1} of DR 28 dyes. No inhibition was observed in the samples containing 200 mg l^{-1} of DR 28. This finding is also in agreement with the study performed by Kalyuzhnyi and Sklyar (2000). They reported that low concentrations of azo dyes have a stimulatory effect on degradation of substrate.

Since the reaction rate is a function of the maximum substrate removal rate, half velocity constant, substrate concentration, inhibitor (dye) concentration and inhibition constant, the following rate equations can be proposed for COD removal in wastewater containing 800 mg l^{-1} of DR 28 with 1000 mg l^{-1} of glucose-COD resulting in a competitive inhibition:

$$R_{800} = \frac{27.3 * 1000}{994 * (1 + \frac{800}{862}) + 1000} = \frac{27300}{2817 + 1000} = 9.35 \text{ mg } l^{-1}.h^{-1} \qquad (22)$$

The K_S values increased up to 5977 from 580 mg l^{-1} indicating the low affinity of glucose-COD and dye to anaerobic microorganisms in batch reactor containing 3200 mg l^{-1} of DR 28 dye. Low K_{ID} values in samples containing higher DR 28 dye concentration indicate the accumulation of this dye resulting in a severe inhibition. For S_i=1000 mg glucose-COD l^{-1}, 82 % decrease in the reaction rates were observed in reactors containing 3200 mg l^{-1} of DR 28 dye, compared to dye-free reactors.

5. Discussion

In this study glucose COD is the electron donor and is consumed to provide the reducing equivalents throughout azo dye cleavage by azoreductase catalyzed reduction as reported by Zimmermann *et al*. (1982). The azo bonds were cleaved under anaerobic conditions to form corresponding intermediate aromatic amines while glucose-COD was converted to VFA, which was accomplished with methane conversion as reported by Carliell et al. (1995). Similarly, the experimental data found in this study showed that the glucose-COD transformed to VFA resulting in methane production while the azo bonds of DR 28 dye cleaved and transformed to aromatic amines. At high dye concentrations, when TAA and VFA were accumulated, dyes are competing for the active sites of azoreductase enzyme together with substrate (glucose COD). Dye and the intermediate substances may block the active sites of this enzyme, which inhibits cleavage of the azo bond. In other words, at higher DR 28 concentrations, the accumulation both in VFA and TAA resulting in low methane productions was observed, corresponding with lower rate of azo dye co-metabolism.

Many metabolic enzymes and cofactors are induced by utilization of growth substrate through decolorization of azo dye. A non-growth substrate (dye) is biotransformed by the specific enzymes, but it can not be utilized by microorganisms to support their growth during color removal period. As the growth substrate, glucose is first converted to volatile fatty acid by acidogen bacteria while the color of the dye is removed by the cleavage of azo bond with electrons transferring from glucose under reductive environments. The kinetics of decolorization were shown to be first order with respect to dye concentration and this confirms the findings of Carliell *et al*. (1995), Wuhrmann *et al*. (1980), and Weber and Wolfe (1987), whereas other researchers found zero order kinetics Dubin & Wright, 1975; Brown, 1981; Harmer & Bishop, 1992). A probable explanation of these contradictory observations is that the rate limiting step in the reduction of azo dye may differ between the different co-substrate and dye with different reactive groups studied.

The results of the kinetic studies indicated that the inhibition of azo dye is typified by competitive inhibition. In other words, a competitive inhibition coefficient model described the fate of glucose-COD exposed to high concentrations of DR 28 in batch reactor systems. In this type of inhibition the adverse effect of azo dyes results in the cessation or decreasing of enzymatic reduction (azoreductase enzyme) of azo bonds. Partial reduction of azo bonds (-N=N-) to form a single bond (-NH-NH) result results from incomplete breakdown of the azo bond. The inhibitor (dye) ceased the complete breakdown of azo bond to form aromatic amines as well as partial reduction of azo bond. As the inhibition increased the activity of microorganism or the azoreductase enzyme was severely inhibited (Chung & Stevens, 1993). Since the key enzymes of azo reduction

are in cytoplasm, the inhibition of the dye molecules from transferring into the cells for decomposition results in the cessation of color removal (Pasti-Grigsby *et al*., 1992). In the competitive inhibition the inhibitor (dye) combined with the free enzyme in such a way that it competed with the normal substrate (glucose) for binding at the active site of enzyme azoreductase and other related enzymes. Dye bounded to active site on the enzyme and deformed the enzyme, so that it did not form glucose-substrate complex at its normal rate and, once formed, the complex did not decompose at the normal rate to yield product, methane. In other words, the high azo dye concentration probably blocks the active sites of the aforementioned enzymes, which prevents the biodegradation and**,** partially, the decolorisation.

Larger K_S values seem to indicate a low affinity between the primary substrate/dye and the anaerobic microorganisms in batch reactor resulting in accumulation of substrate/dye and low color/glucose-COD removal. The dye/substrate accumulation shows that the dye was not utilized and the azo bond in the dye was not cleaved, since the electrons released from glucose and NADH utilization stopped as reported by Kudlich et al. (1997). The azo dyes causing competitive inhibition do not affect the intercept on the 1/R axis. $1/R_{max}$ plots crossed the Y axis on the same point, indicating that they do not interfere with the maximum rate of breakdown of the enzyme-substrate complex.

Small values of K_{ID} in DR 28 indicate the high inhibition potential because K_{ID} is in the denominator in the competitive inhibition. Lower K_{ID} in samples containing high concentrations of DR 28 dye, represents the degree to which the microorganisms are significantly inhibited by increasing dye concentrations compared to low concentrations of the dye. The high concentration of dye had a severe inhibitory effect on azo bond reduction and cleavage and the electrons did not reach azo bond cromotophore since most of the active sites of azoreductase enzyme were bonded with dye. Furthermore, the concentrations of aromatic amines released from the decolorization of DR 28 at higher concentration might be more toxic than DR 28 at lower concentrations. In other words, higher K_S and lower K_{ID} values, in the competitive inhibition, can be characterized as a system with low affinity to substrate and with more inhibition.

Of relatively few kinetic studies performed relevant to inhibition of dye only four study results could be compared with our data:

Hu (2001) found that *P. luteola* showed lower K_M values (113-135 μM) for the mono azo dyes (Reactive Red 22, Reactive Violet 2 and Direct Blue 15) and higher K_M values (222-397 μM) for the triazo (Direct Black 22, Direct Violet 9 and Direct Blue 15) dyes. The Lineweaver-Burk plot of azoreductase enzyme produced by *Pseudomanas luteola* to different dye concentrations indicated the existence of a competitive inhibition model. The maximum specific removal rate (V_{max}) values

varied between 4.08 and 5.24 n mole mg^{-1} protein.min^{-1} for all the aforementioned dyes. A substrate inhibition model was applied to describe the dependence of specific decolorization rate on Reactive Red 22 (RR 22) azo dye concentration. The maximum decolorization rate (V_{max}) and the kinetic constants (K_1 and K_2) were found as 3160 mg RR 22 g^{-1} cell. h^{-1}, 48.9 mg l^{-1} and 160.8 mg l^{-1}, respectively, at 2000 mg l^{-1} bacteria and a RR 22 concentration varying between 0-5000 mg l^{-1} (Chang & Kuo, 2000). In a study performed by Yongjie and Bishop (1994), 15 mg l^{-1} of acid orange exhibited non-competitive and mixed inhibition resulting in increases in reaction rate while slight increases in K_S values were observed.

The K_S and R and K_{ID} values obtained in our study are comparatively lower than the data obtained by Chang *et al*. (2000) and Hu (2001). This could be due to differences in the dyes, co-substrates, microorganisms used and anaerobic operation conditions applied in the studies. On the other hand, the rate of azo reduction was affected by the structure of, and the substituents on the aromatic ring of dyes such as OH^-, $-NH_2$, and $-SO_3H$ resulting in inhibition (Hu, 2001; Donlon et al., 1995). As reported by Urushigawa and Yonezawa (1977) and Zimmermann *et al*. (1982) azo group with hydroxy and amino group are more likely to be reliably degraded than those with a $-CH_3$, $-NO_2$, $-SO_3H$, $-OCH_3$ group. The benzidine released as anaerobic intermetabolite caused increases in toxicity of DR 28 dye due to carcinogenic properties of this organic substance as reported by Chung and Steven (1993). They observed that the toxicity of parental dye on methanogens increased with time as aromatic amines (benzidine) were produced.

The anaerobic conditions clearly provide an unexplored area of potential advantage for dye decolorization even if the dye concentrations are as high as 1600 and 3200 mg l^{-1}. In practice, the observed inhibition may not be important because real textile wastewater contains only 100-500 mg l^{-1} of these azo dyes. Thus, it may not inhibit the decolorization of textile wastewaters containing the azo dye but it is important in degradation of anaerobic substrates since continuous exposure of anaerobic biomass to potentially toxic and variable loadings of the dye could have adverse effects on the anaerobic COD removals.

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