A Batch Decolorization and Kinetic Study of Reactive Azo Dye RO-13 by Novel Bacterial Strain Alcaligenes faecalis PMS-1

Parin D.Shah, M.S.Rao

Abstract-- An *Alcaligenes faecalis* strain (PMS-1) with high activity for decolorization of Reactive Orange13 (RO-13) was isolated from dye contaminated industrial site. Effects of operation parameters (initial dye concentration, temperature, pH, and salinity) on decolorization of the azo dye by PMS-1 were systematically investigated. In our experiment, an improved decolonization rate of 24.75 mg l^{-1} h^{-1} for 400 mg l^{-1} RO-13 dye was observed which is approximately 38.13 times higher than the reported value. The kinetic characteristics of dye decolorization by the strain PMS-1 were determined quantitatively using the monoazo dye, RO-13. The activation energy (Ea) of decolorization reaction and frequency factor (A_0) were determined using Arrhenius equation. A dye decolorization kinetic study was carried out by three different approaches namely Michaelis – Menten, Lineweaver-Burk and Eadie-Hofstee. The maximum substrate consumption rate (V_{max}) and decolorization rate constant (K_m) were determined.

Index Terms-- *Alcaligenes faecalis* PMS-1, Azo dye, Reactive Orange 13, kinetic study, Decolorization

I. INTRODUCTION

Synthetic dyes are extensively used in textile dyeing, paper printing, color photography, pharmaceutical, food, cosmetic, and leather industries [1], [2]. It is estimated that 280,000 tons of textile dyes are discharged in textile industrial effluent every year worldwide [3].The effluents from textile and dyeing industries have high BOD, COD, color, pH also it contains metal ions [4]. Most synthetic dyes are toxic and highly resistant to degradation due to their complex chemical structures [5]. Such effluents lead to a reduction in sunlight penetration, which in turn decrease photosynthetic activity, dissolved oxygen concentration, and water quality, and have acute toxic effects on aquatic flora and fauna, causing severe environmental problems worldwide [6]. Physicochemical wastewater treatment technologies have proven to be markedly ineffective for handling wastewater of synthetic textile dyes because of the chemical stability of these pollutants [7]. However, microbial or enzymatic decolorization and degradation is an ecofriendly compared to physicochemical treatment methods [8],[9]. Also these methods can lead to complete mineralization of organic pollutants into nontoxic products [7],[10]. Biodegradation is a promising approach for the remediation of synthetic dyes wastewater because of its cost effectiveness, efficiency, and environmentally friendly nature [9],[11]-[13].
    Many researchers had worked for decolorization of various azo dyes using a biological method but less attention

is given to the cyanuric chloride based reactive azo dyes. Keharia et al. (2004) [14] reported that reactive dyes with cyanuric chloride as a reactive group were more resistant to anaerobic reduction in comparison to vinyl sulfone based reactive dyes. In the same report, Keharia et al. (2004) [14] reported average decolorization rate of 0.649 mg l^{-1} h^{-1} for decolorization of RO-13 with an initial concentration of 250 mg l^{-1} by anaerobic sludge bacteria. As per our knowledge, in open literature, no report is available for decolorization of RO-13 using pure bacterial species. Main objectives of the present study are to carry out parametric study for decolorization of RO-13, kinetic study and to determine activation energy and frequency factor for decolorization reaction.

II. MARERIAL AND METHODS

1) Dyes and media

Commercial dye RO-13 or Procion Orange H2R (CAS: 70616-89-6) supplied by Balaji Industries, Vatva, Ahmedabad, India was used as a model dye. Chemical structure and other relevant information for RO-13 dye were mentioned in Fig.1. Nutrient broth and nutrient agar were obtained from HiMedia, Mumbai (India). All other chemicals used were of the highest purity available and analytical grade supplied by Central Drug House (P) Ltd.
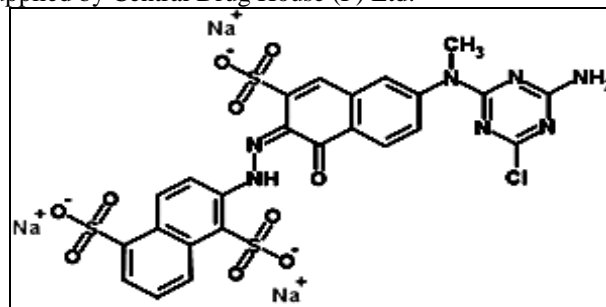

Fig. 1. Chemical structure of RO-13, C.I. No.: 18270,   Dye type: Monoazo, λ_{max}: 488 nm, Molecular Wt. 762.03 (g mol^{-1})

2) Isolation, preservation and maintenance of RO-13 decolorizing bacteria

    The isolation of bacterial species was carried out from soil samples contaminated with dyes. Samples were collected from dye manufacturing industry Balaji Industries, Vatva Ahmedabad, India. The nutrient broth along with RO-13 (50 mg l^{-1}) was inoculated with 1% (w/v) of soil. The flask was incubated at 30 ± 2 ℃ temperature under the static condition. After 48 h of incubation, 1.0 ml of the culture was serially diluted, and 0.1 ml samples were withdrawn from

10^{-5} dilution. The samples were inoculated on the nutrient agar plates by spread plate method containing 100 mg l^{-1} dye. After 2 days of incubation, the bacterial growth colonies were screened for their ability to form a clear zone on the plate. The bacteria showing higher zones of decolorization were isolated and used for further study. Pure culture was maintained on nutrient agar slants and stored in test tubes at 4 °C and sub-cultured monthly.

3) Identification of RO-13 decolorizing microorganism by 16S rDNA sequencing method

The identification of RO-13 dye decolorizing microorganisms was carried out as reported by Kalyani et al. (2008) [15]. Out of all isolates, one which demonstrated faster decolorization was considered for identification. A preliminary identification of the culture was done based upon biochemical tests. 16S rDNA sequencing of isolated bacteria was carried out at Ocimum Biosolutions, Hyderabad, India. The nucleotide sequence analysis of the sequence was done at BlastN site at NCBI server (http://www.ncbi.nlm.nih.gov/BLAST) and corresponding sequences were downloaded. The sequence was refined manually after cross checking with the raw data to remove ambiguities and submitted to the NCBI. Isolated pure bacterial species are designated as *Alcaligenes faecalis* PMS-1 with a NCBI accession number GenBank ID: JF297973.

4) Experimental investigation for parameter optimization

Decolorization experiments were also performed with different initial dye concentration (50-2000 mg l^{-1}), temperature (25-45 °C), pH (5-10) and common salt (NaCl) concentration (0-10 w/v %). If otherwise mentioned, experiments were performed in 100 ml Erlenmeyer flasks containing nutrient broth growth medium (13 g l^{-1}) supplemented with RO-13 (400 mg l^{-1}) dye and 10 % inoculum (v/v) containing 10^7 cells ml^{-1} at 30 ± 2 °C temperature under static condition. pH in the experiments were adjusted by adding 0.1 N NaOH or 0.1 N HCl. All decolorization experiments were performed in triplicates.

5) Quantitative analysis of dye decolorization using UV–Vis spectrophotometer

The extent of decolorization was determined using a UV-Vis spectrophotometer (Shimadzu UV-1800) at 488 nm, which is the wavelength of maximum absorbance of the RO-13 dye solution. Decolorization has been evaluated using following equation % Decolorization= [(Initial absorbance - Observed absorbance) / Initial Absorbance] x 100. The samples were centrifuged at 3500 RPM for 20 min by using a centrifuge (REMI R-4C). This was done for all samples for separation of biomass prior to measurement of absorbance.

6) Kinetic Studies
6.1 Determination of activation energy of RO-13 decolorization reaction

The activation energy (Ea) of RO-13 decolorization was determined by the Arrhenius equation (equation (1)).

$$k = A_0 \exp(-Ea/RT) \qquad (1)$$

where k is the rate constant depending on reaction order, A_0 is the frequency factor with the same unit as k, Ea is the activation energy (J mol^{-1}), R is the gas constant (8.314 J K^{-1} mol^{-1}) and T is the temperature (K).

6.2. Determination of maximum substrate consumption rate (V_{max}) and decolorization rate constant (K_m)

Batch experiments of RO-13 dye decolorization were performed in optimum operating conditions with different initial dye concentrations. The dye decolorization rate was also determined. A dye decolorization kinetic study was carried out by three different approaches namely Michaelis Menten, Lineweaver-Burk and Eadie-Hofstee and the maximum substrate consumption rate (V_{max}) and decolorization rate constant (K_m) were determined.

III.   RESULTS AND DICUSSION

1) Effect of initial dye concentration
Jadhav et al. (2007) [16] reported that dye-house effluent typically contains 600-800 mg l^{-1} dye. Varying amounts of these dyes are lost in wastewater streams. The influence of initial dye concentration on the decolorization ability of PMS-1 was investigated. Experiments were performed with initial dye concentrations of 50, 100, 400, 800, 1600 and 2000 mg l^{-1}.
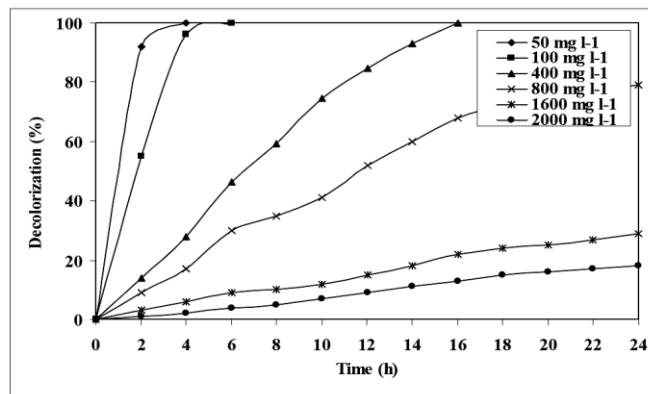

Fig. 2. Effect of initial dye concentration on decolorization of RO-13 by PMS-1

At initial concentrations of 50–400 mg l^{-1} almost 100% decolorization was achieved whereas only 79, 29 and 18% decolorization was observed at dye concentrations of 800, 1600 and 2000 mg l^{-1} respectively (Fig. 2). The time required for complete decolorization of 50 mg l^{-1} dye was 4 h, whereas 16 h required for 400 mg l^{-1} dye. In our experiment, an improved decolonization rate of 24.75 mg l^{-1} h^{-1} for 400 mg l^{-1} RO-13 dye was observed which is approximately 38.13 times higher than the reported value. Decline in decolorization rate may be due to the poisonous effect of the dye on bacteria, obstruction of active sites of azoreductase enzyme by complex dye structure and/or insufficient production of biomass for decolorization of higher concentrations of dye. Increase in initial dye concentration decreased the decolorization rate and at high concentrations

inhibition was observed. Similar observations were also made by Gopinath et al. (2009) [13].

2) Effect of temperature

With an increase in temperature from 25 to 37 °C, the decolorization rate was increased from 14.75 to 25.0 mg l^{-1} h^{-1}. With further increase in temperature from 37 to 45 °C, decolorization rate gradually decreased to 18.0 mg l^{-1} h^{-1}. Similar observations were also made by Saratale et al. (2009) [17]. From the experimental results, the optimum temperature for decolorization was found to be 37 °C (Fig. 3).
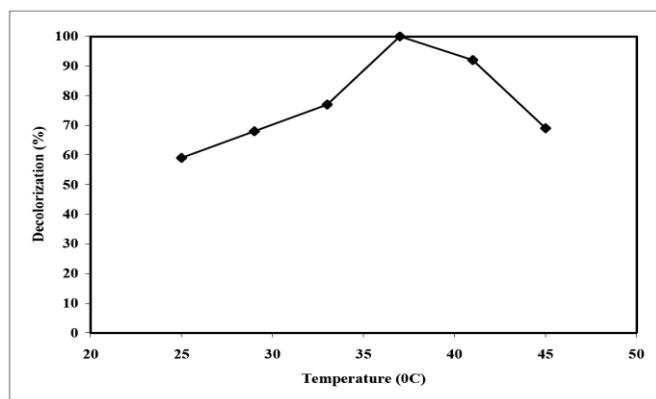

Fig. 3. Determination of optimum temperature (°C) for decolorization of RO-13

3) Effect of pH

At pH 5.0, extent of decolorization and decolorization rate were observed 21% and 3.5 mg l^{-1} h^{-1}respectively. As pH increased decolorization also increased up to pH 7. Highest decolorization of 98.2% and the highest average decolorization rate of 24.5 mg l^{-1} h^{-1} were observed at about pH 7. With further increase in pH, extent of decolorization was decreased gradually. At a pH of 10, extent of decolorization and decolorization rate were observed 17% and 2.83 mg l^{-1} h^{-1} only. The results suggest that pH variation had a significant effect on the decolorization of RO-13 by PMS-1. The optimum pH range for decolorization was observed between 7 and 8 (Fig.4).

4) Effect of salt

Decolorization experiments were performed in the presence of different initial concentrations of commercial grade NaCl salt. Experimental results were as shown in Fig. 5.

For salt concentration up to 5%, the decolorization activity of PMS-1 maintained approximately 81%. As the salt concentration increased, percentage decolorization decreased drastically. At 10% salt concentration, only 12% decolorization was achieved. From the experimental study, it was found that the maximum tolerable salt concentration was 5%, to achieve reasonable decolorization.
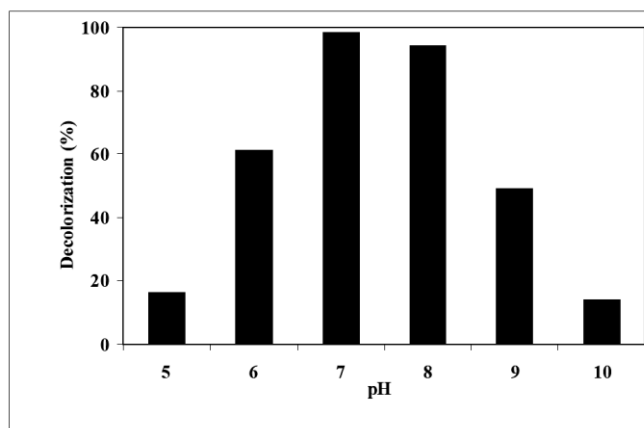

Fig. 4. Determination of optimum pH condition for decolorization of RO-13
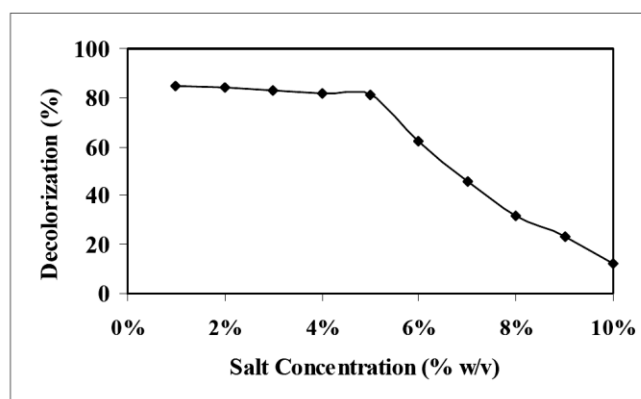

Fig. 5. Effect of salt concentration

5) Kinetic Study of RO-13 decolorization
5.1. Determination of reaction order

Decolorization data from the batch tests were used to determine the order of the decolorization experiment. The kinetic equations used for 0th, 1st and 2nd order kinetic study are given in equations (2) – (4). Dye concentration (C) versus time, ln C versus time and (1/C) versus time graphs were plotted respectively for equations (2), (3) and (4).

$$C_t = C_0 - k_0 t \qquad (2)$$
$$C_t = C_0 \exp. (-k_1 t) \qquad (3)$$
$$(1/C_t) = (1/C_0) + k_2 t \qquad (4)$$

The rate constants of decolorization experiments and coefficients of least square method analysis are tabulated in Table 1. The correlation coefficients (R^2) were in the range of 0.93-0.99, which approximates the first order reaction kinetics. The rate of decolorization is inversely proportional to the initial dye concentration. As the dye concentration increases decolorization rate decreases. The first order kinetics with respect to dye concentration has been reported by Karatas et al. (2009) and Isik and Sponza (2004)[18],[19].

TABLE 1
RATE CONSTANTS OF DECOLORIZATION EXPERIMENTS WITH
RESPECT TO DYE CONCENTRATION

Constants	Concentration (mg l⁻¹)					
	50	100	400	800	1600	2000
k_0 (mg l⁻¹h⁻¹)	12.5	17.05	25.958	27.538	19.56	16.319
R^2	0.8096	0.8957	0.985	0.655	0.9918	0.9913
K_1 (h⁻¹)	0.978	0.8118	0.2945	0.0699	0.0145	0.009
R^2	0.9725	0.9678	0.9371	0.9892	0.9927	0.9908
k_2 (l mg⁻¹ h⁻¹)	0.115	0.06	0.0019	0.00002	0.000015	0.000001
R^2	0.99	0.7881	0.6831	0.9507	0.9899	0.97158

5.2. Activation energy of decolorization reaction

   The effect of temperature on the decolorization of RO-13 by PMS-1 was shown in Fig. 3. In the temperature range of 25-37 ℃, the decolorization increased with temperature and depended on the activation energy (Ea) of the reaction as given by Arrhenius equation (Eq. (1)).Since the decolorization of RO-13 by the strain PMS-1 followed a first-order kinetic with respect to dye concentration, the first-order constants k_1 (h⁻¹) were obtained in each temperature test (Table 2). The value of ln (k_1) was plotted against the reciprocal of temperature (Fig.6). The high-degree linearity (R^2=0.9275) gives reliable estimations of the activation energy (Ea) and frequency factor (A_0). The obtained values were Ea = 73.5 J mol⁻¹ (17.56 cal mol⁻¹) and A_0= 3.98 x 10¹¹ h⁻¹. It is reported that the general activation energy range of enzymatic-catalyzed reactions is usually within 4-20 cal mol⁻¹ [20].

TABLE 2
RATE CONSTANTS OF DECOLORIZATION EXPERIMENTS WITH
RESPECT TO TEMPERATURE

Constants	Temperature (° C)					
	25	29	33	37	41	45
k_0 (mg l⁻¹ h⁻¹)	12.38	12.967	15.736	25.958	20.909	13.736
R^2	0.949	0.9054	0.9389	0.985	0.9524	0.9189
k_1 (h⁻¹)	0.058	0.0669	0.1001	0.1825	0.1774	0.736
R^2	0.9931	0.973	0.9899	0.9341	0.9557	0.9859
k_2 (mg⁻¹h⁻¹)	0.0003	0.0004	0.0011	0.0007	0.0012	0.0005
R^2	0.9619	0.9492	0.7076	0.8574	0.7932	0.9659

5.2 Determination of maximum substrate consumption rate (V_{max}) and decolorization rate constant (K_m)

   Experimental results for RO-13 decolorization were as shown in Table 3. A dye decolorization kinetic study was carried out by three different approaches namely Michaelis – Menten, Lineweaver-Burk and Eadie-Hofstee. The maximum substrate consumption rate (V_{max}) and decolorization rate constant (K_m) were determined.
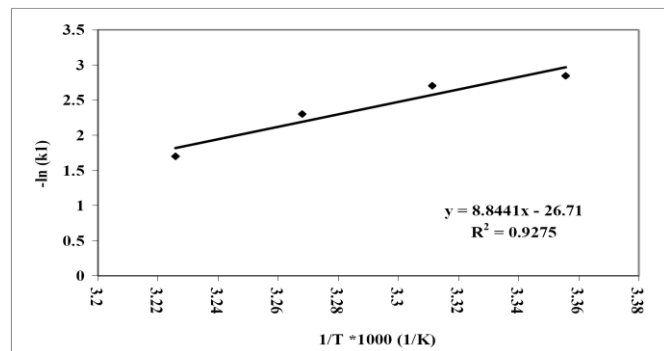

Fig. 6. Estimation activation energy of decolorization of RO-13 using Arrhenius equation

TABLE 3
EXPERIMENTAL RESULTS FOR RO-13 DECOLORIZATION

Initial Dye Concentration S (mg l⁻¹)	Final Dye Concentration (mg l⁻¹)	Time (h)	V (mg l⁻¹ h⁻¹)
100	2.8	6	16.2
200	62.6	6	22.9
250	110.2	6	23.3
300	154.2	6	24.3
350	194	6	26
400	237.4	6	27.1

5.2.1 Michaelis-Menten kinetics

   As the biodegradation of RO-13 is due to decolorizing enzyme, the Michaelis-Menten equation could be used to describe the reaction kinetics. Michaelis-Menten equation is given as follows: V= V_{max} x S/ (K_m + S). Where V_{max} is the maximum substrate (i.e. RO-13) consumption rate in mg l⁻¹ h⁻¹; V is the substrate consumption rate in mg l⁻¹ h⁻¹; S is the substrate concentration in mg l⁻¹; K_m is the Michaelis-Menten constant in mg l⁻¹. K_m is equal to the concentration of the substrate when the reaction rate is half of the maximum velocity.

   Fig.7 shows that experimental data can be fitted quite well with Michaelis-Menten equation. By calculating the sum of the squared derivations and let it be minimized, the equation coefficients can be obtained. The results are ½V_{max} = 14.0 mg l⁻¹h⁻¹, K_m = 105 mg l⁻¹, V_{max} = 27.1 mg l⁻¹h⁻¹.

5.2.2. Lineweaver-Burk Plot

   Michaelis- Menten equation is transformed by a double reciprocal approach to Lineweaver- Burk equation as follows: 1/V = K_m/ (V_{max} S) + 1/ V_{max}. A plot of 1/ V versus 1/S gives 1/V_{max} as intercept on the ordinate when 1/S approaches zero, and an intercept of (-1/K_m) on the abscissa for V approaches zero. The Lineweaver–Burk plot is shown in Fig.8.

   By calculating the sum of the squared deviations, and let it be minimized, another set of k_m and V_{max} can be

obtained. These were $K_m = 109.62$ mg l^{-1}, $V_{max} = 34.12$ mg l^{-1} h^{-1}.
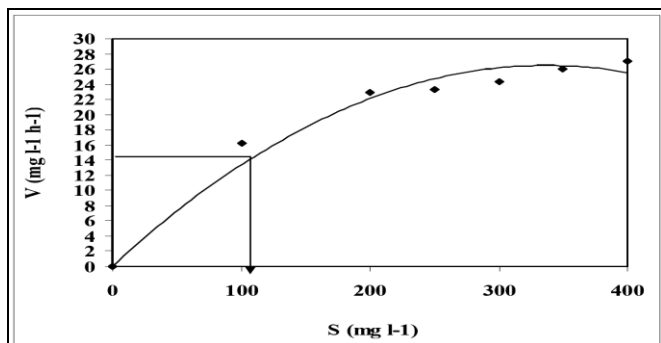

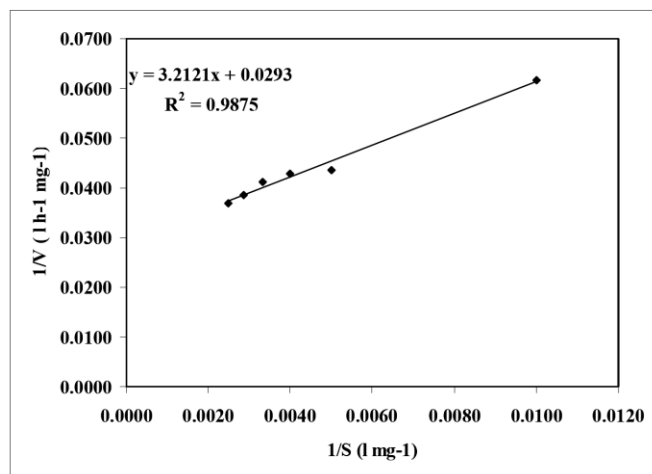Fig.7. Michaelis-Menten decolorization rate curve of RO-13

Fig. 8. Lineweaver–Burk plot of decolorization of RO-13 by PMS-1

5.2.3. Eadie-Hofstee Plot

Another way to calculate k_m and V_{max} was the use of Eadie-Hofstee Plot. The rearranged Michaelis-Menten equation $V = - V (K_m/S) + V_{max}$ was used to plot V against V/S gives a straight line with y intercept = V_{max} and slope = - K_m.

By calculating the sum of the squared error, and let it be minimized, another set of k_m and V_{max} can be obtained. These are $K_m = 106.76$ mg l^{-1}, $V_{max} = 33.84$ mg l^{-1} h^{-1} (Fig.9). A comparison of K_m and V_{max} values obtained from above three approaches is shown in Table 4. It shows that these results are comparable. This suggests that, decolorization of RO-13 by PMS-1 is a first order irreversible process.
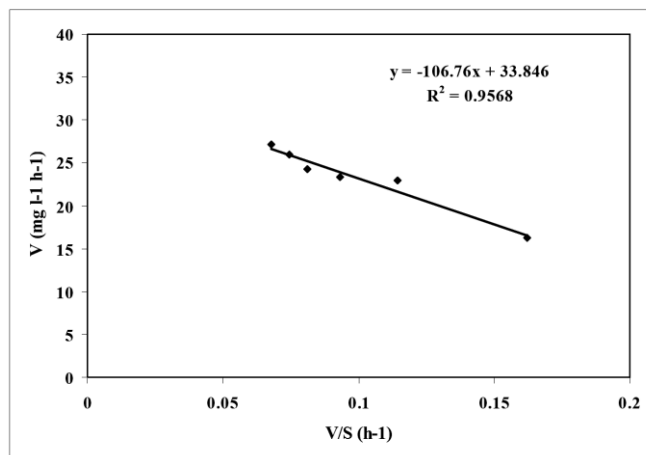

Fig. 9. Eadie-Hofstee plot of decolorization of RO-13 by PMS-1

TABLE 4
RESULTS OF KINETIC STUDY AND ANALYSIS

Plot type	V_{max} (mg l^{-1} h^{-1})	K_m in (mg l^{-1})
Michaelis Menten	27.1	105.0
Lineweaver–Burk	34.12	109.62
Eadie-Hofstee	33.84	106.76

IV   CONCLUSIONS

Isolated pure bacterial species are designated as *Alcaligenes faecalis* PMS-1 with a NCBI accession number GenBank ID: JF297973. In this work, decolorization of cyanuric chloride based azo dye RO-13 was carried out systematically using bacterial strain PMS-1. The dye decolorization rate depends on various operating parameters such as initial dye concentration, temperature, pH, and salt concentration. The ability of PMS-1 to decolorize the reactive azo dye under a broad range of pH suggested that isolated strain could be useful in biological treatment of industrial wastewater. Kinetic study of decolorization experiments approximate first order reaction with respect to temperature and dye concentration. The calculated activation energy value and frequency factor were found to be 73.5 J mol^{-1} and 3.98 x 10^{11} h^{-1}, respectively. The V_{max} and K_m were found to be 27.1 mg l^{-1} h^{-1} mg l^{-1} h^{-1} and 105 mg l^{-1}, respectively by using Michaelis-Menten kinetics. In our experiment, an improved decolonization rate of 24.75 mg l^{-1} h^{-1} for 400 mg l^{-1} RO-13 dye was observed which is approximately 38.13 times higher than the reported value. Enhanced RO-13 decolorization ability could be due to the selection of efficient strain and optimization of the parameters.

V.   ACKNOWLEDGEMENTS

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