Kinetic Study on Decolorization of the Dye Acid Orange

Using the Fungus *Phanerochate Chrysosporium*

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Abstract

A dye decolorizing fungal strain, *Phanerochaete chrysosporium* MTC 787 was used in batch experiments for decolorization of the dye, Acid orange10. Effects of initial substrate (dye) and fungal concentrations on the rate and extent of dye decolorization were investigated. The initial pH and oxidation–reduction potential (ORP) was kept at 4.5 and −250 mV, respectively. The rate and extent of dye decolorization increased with increasing dye concentration up to 100 g l^{-1} and then decreased for larger dye concentrations. The rate of enzyme utilization and dye decolorization also increased linearly with increasing initial biomass concentration. A kinetic model describing the rate of enzyme utilization and substrate inhibition as function of the initial substrate and flow rate was developed. The kinetic constants were determined using the experimental data. The initial biomass should be above 3.2 x 10^5cell/ml to obtain high rates and decolorization percentage and to avoid substrate inhibition.

Keywords: *Phanerochaete chrysosporium*, Kinetics, Dye, Model, pH, Fungus, Enzyme, Potential

1. Introduction

Synthetic organic colorants are used for several purposes in the textile, cosmetic, paper making, food and pharmaceutical industries. Textile dyeing processes exhibit highly colored wastewater in large amounts, which is let into the natural water streams. Around 10,000 different types of dyes and pigments are produced and an average of 10-15% of these dyes are let into the wastewater stream (Pearce 2003).Since these dyes exhibit colours in the land, lots of work are carried in the field of decolorizing the dyes before it is let into the land or water resources (O'Neill 1999). Several work involving chemical, physico-chemical, membrane treatment and biological treatment are on its way to purify the wastewater (Francis perineau 1982). In the biological treatment processes, organisms such as *Phanerochaete chrysosporium*, *Coriolus versicolor*, *Pseudomonas* species, *Aspergillus* Sp., are involved in decolorization (Knapp 1995). White-rot fungi are well known for their outstanding ability to produce extra cellular oxidative enzymes, which initiate lignin degradation. The white-rot fungi best involved for treating dyes is *P. chrysosporium* and we have chosen the same for our reactor studies as the enzymes produced by the fungus which causes decolorization are highly non-specific and degrade a variety of dyes (Radha 2005).

While much research has been carried out on the uptake of dye and other pollutants little attention has been given to study the kinetics. Decolorization of dyes using microorganism is affected by several factors (Chulhwan 2006) which include the specific surface properties of the microorganism and the physicochemical parameters of the dye solution such as pH, initial dye concentration and initial biomass concentration (Loukidou 2004). Many other parameters also affect the capacity of microorganism to bind on the immobilizing agents (Wu 2006).The combined effects of two or more component of the microorganism depend on the nature of dye. They compete for the binding sites and in combination of one or more dyes, competence is for the order of the dye addition and it also includes the residence time in the reactor which may initiate the competence level (Aksu 2003).In order for the better utilization of the enzyme and the whole cells they are immobilized onto a solid support which leads to advantageous reactor operation system. The equilibrium established between the dye component and the immobilizing agent and the non-reacted component in solution can be represented through kinetics. The major objective of this study is to investigate the kinetics of dye

solution for a large range of dye concentration and biomass concentrations and to determine the kinetic constants using the experimental data.

2. Materials and Methods

2.1 Experimental set-up

Batch experiments were performed by using sterile Erlenmeyer flasks and a gyratory incubator shaker at 37 °C and 150 rpm. In variable substrate (dye) concentration experiments, the Erlenmeyer flasks (500 ml) were charged with 180 ml of deionized water containing desired concentrations of dye between 10 and 100 g l^{-1} and 200 mg l^{-1}. Na - thioglycolate was the reducing agent which is used to adjust the oxidation–reduction potential (ORP) to −250 mV. The Erlenmeyer flasks were prepared in duplicates, sterilized at 121 °C for 20 min and inoculated with 20 beads calcium alginate   immobilized *P.chrysosporium*. Variable biomass concentration experiments were performed by inoculating the experimental flasks with different amounts of inoculum (10–100 beads) and dye solution (10–100 ml of constant concentration) to obtain a total volume of 200 ml in every flask. The initial pH of the media was adjusted to 4.5. Inoculated flasks were placed on a gyratory shaker at 37 ±2 °C and 150 rpm. Samples were withdrawn aseptically from the experimental flasks periodically for analysis of enzyme activity, biomass and percentage decolorization. A control flask free of fungal immobilized cells containing 100 g l^{-1} dye and 100 mg l^{-1} Na-thioglycolate was used to determine decolorization of any dye in the absence of fungal cells. Duplicate flasks were used to test the reproducibility of the data.

2.2 Microorganism

P.chrysosporium MTCC-787 obtained from Microbial Tissue culture collection centre, Chandigarh, India in lyophilized form was used in all experiments. The fungal strain was cultivated in laboratory using an incubator shaker under sterile conditions at pH 4.5. Pure cultures grown under respective conditions were used for inoculation of experimental flasks.

2.3 Medium composition

Medium used for cultivation of inoculum culture consisted of glucose (10 g l^{-1}), Malt extract (0.5 g l^{-1}), NH$_4$Cl (0.054g l^{-1}), KH$_2$PO$_4$ (0.136 g l^{-1}), MgSO$_4$ · 7H$_2$O (0.044g l^{-1}), MgCl$_2$.6H$_2$O (0.0775 g l^{-1}), CaCl$_2$.2H$_2$O (0.1g l^{-1}), and 200 mg l^{-1} Na-thioglycolate as the reducing agent at pH 4.5. The initial oxidation–reduction potential (ORP) of the media was nearly about −250 mV indicating the anaerobic conditions. The fungal culture grown on a shaker in the aforementioned media at 37 °C and 150 rpm was used as inoculate for the experimental flasks. The experimental flasks contained desired concentrations of dye and 100 mg l^{-1} Na-thioglycolate (ORP = − 250 mV) in deionized water at pH 4.5. Dye concentration was varied between 10 and 100 g l^{-1} in variable substrate experiments. In the variable inoculum size experiments the initial biomass concentration was varied between 1.2 x 10^{5}cells/ml and 4.5 x 10^{5}cells/ml while the dye concentration was constant at 100 g l^{-1}.The dye Acid orange 10 was obtained from Department of Textile Technology, Anna University, Chennai, India and was kept in sterile state before use.

2.4 Immobilization

The fungus *P.chrysosporium* was inoculated on malt agar and incubated at 35°C, until extensive spore growth occurred. The samples were removed from the flasks periodically and centrifuged at 8000 rpm to remove solids from the liquid media. Analysis was made for the enzyme activity which included lignin peroxidase and manganese peroxidase. Suspensions of 2ml were added to 100 ml of 2% sodium alginate. The mixture was gently stirred at room temperature to produce a uniform suspension and then dropped into 100ml of 20% Calcium chloride solution. The beads so obtained were stored in calcium chloride solution at 4°C for 2 h to complete gel formation (Sheldon 2005). The insoluble and stable immobilized *P.chrysosporium* alginate beads thus obtained were further used for the decolorization studies. Plain beads were prepared without the spore suspension and these were used as the reference beads.

2.5 Analytical methods

Samples were collected at regular intervals and dye concentrations were measured using a spectrophotometer [UV/VIS Shimadzu spectrophotometer (model U 2000)].

Enzyme activities were determined spectrophotometrically using the different enzyme assays individually for lignin peroxidase, manganese peroxidase.

Lignin Peroxidase activity was determined spectrophotometrically (Tien and Kirk 1983) with Veratryl alcohol as substrate. One unit was defined as the amount of enzyme that oxidized 1μmol of Veratryl alcohol in 1 minute at 310nm and the activities are reported as U/ litre.

Manganese Peroxidase activity was assayed at 610nm using Dimethoxyphenol as the substrate as suggested by Field et al., 1993. One unit was defined as the amount of enzyme that oxidized 1μmol of Dimethoxyphenol per minute and the activities are reported as U/ litre.

The samples were analyzed in triplicates and the results were reproducible within 3% deviation. Oxidation reduction potentials (ORP) and pH were measured using a pH meter with either an ORP or a pH probe.

3. Results and discussion

3.1 Effect of initial substrate concentration

The Acid orange 10 concentrations were varied between 10 and 100 g l^{-1} in this set of batch experiments while the initial biomass concentration was constant and kept as 25 beads. Variations of dye concentration and enzyme activity with time are depicted in Fig 2a and b, respectively for different initial dye concentrations. Percentage decolorization was almost completed within 72 h when dye concentration was less than 10 g l^{-1}. Complete enzyme utilization took place when dye concentration was larger than 100 g l^{-1} (Fig 2a) due to substrate inhibition at high dye concentrations. Percentage decolorization also reached the maximum level after 72 h of incubation when dye concentration was less than 10 g l^{-1} while complete dye decolorization took longer for higher dye concentrations. An incubation time of 72 h was considered in all further calculations. The pH values dropped from an initial level of 4.5 to 3.0 at the end of 72 h when dye was less than 100 g l^{-1}. The final pH for dye concentrations above 100 g l^{-1} was between 4.7 and 5.2 at the end of 72 h. The ORP decreased from −150 mV to nearly −350 mV in all experiments, except the one with 10 g l^{-1} dye for which the final ORP was −250 mV at the end of 72 h. There was no decolorization taking place in the control flasks. The results of the duplicate experiments were almost the same within 2% deviation indicating the reproducibility of experimental data.   Variations of specific rates of enzyme utilization and decolorization with dye concentration are shown in Fig 3a and b. Specific rates (R_{SX}= (So-S)/ (t.X), g enzyme/g biomass hour) were calculated for the first 72 h. The specific rate of enzyme utilization increased with dye concentrations up to 100 g l^{-1} dye indicating substrate limitations at low dye concentrations. The rate decreased with increasing dye concentrations above 100 g l^{-1} due to substrate inhibition at high dye concentrations (Fig.3a). Similar trends were also observed in the specific rate of dye decolorization (R_{PX}= (P-Po)/ (t.X), g dye/g biomass.h). Dye decolorization rate for the first 72 h period increased at low dye concentrations below 100 g l^{-1} due to substrate limitations, but steadily decreased with increasing dye concentrations for dye larger than 100 g l^{-1} due to substrate inhibition (Fig 3 b). Dye concentration should not exceed 100 g l^{-1} for higher percentage decolorization.

3.2 Effect of initial fungal concentration

Biomass (fungus) concentration is another important parameter affecting the rate and extent of dye decolorization. A series of batch shake flask experiments were performed with varying initial biomass concentrations between 25 and 200 beads containing (approximately 3.2 x 10^5 cells/ml to 8.9 x 10^6 cells/ml) with a constant dye concentration of 100 g l^{-1}. Fig 4 a and b depict variations of enzyme activity and dye concentrations with time for different initial biomass concentrations. Enzyme utilization was completed within 24 and 30 h when biomass concentrations were above 100 and 150 beads respectively. However, enzyme utilization was rather slow for biomass concentrations below 150 beads since the rate is directly proportional with the biomass concentration. Enzyme utilization was completed after 72 h when biomass concentration was less. (Fig.4a). Decolorization percentage also reached the maximum level after 72 h of incubation when biomass concentration was above 50 beads. Nearly 120 h were required for maximum decolorization when biomass concentrations were lower than 50 beads as shown in Fig.4b. pH values in experimental flasks decreased from an initial pH of 4.5 to pH 3.6–3.8 depending on the initial biomass concentrations. Therefore, pH variations were not significant to require pH control. The final oxidation reduction potentials (ORP) at the end of 72 h were between −250 and −275 mV with an initial ORP of −250 mV for all experimental flasks. Biomass concentrations increased with time to give a yield coefficient of 1.1 g X g S^{-1} on the average. There was no enzyme utilization and decolorization in the control flask free of biomass. The results of the duplicate experiments were almost the same within 2% deviation.

Fig 5 a and b depict variations of volumetric rates of enzyme utilization and decolorization with the initial fungus concentration. The time periods considered for calculating the rates were until complete utilization of enzyme (24, 31 and 48 h for different biomass concentrations and 120h for decolorization), since decolorization continued a little while after complete enzyme utilization. The volumetric rate of enzyme utilization increased with biomass concentration almost linearly yielding nearly 200 mg l^{-1} h^{-1} enzyme utilization rate at 25 beads biomass concentration (Fig.5a). Percentage decolorization rate also increased with biomass concentration as shown in Fig.5 b. The maximum dye decolorization rate of 0.105 ml l^{-1} h^{-1} was obtained with 100 beads containing 5.2 x 10^5 cells/ml of initial biomass concentration.

3.3 Kinetic modeling and estimation of the kinetic constants

The following kinetic model was used to describe the initial rate of substrate utilization [12].

$$v = \frac{kX_o S_o}{k_s + S_o}\left(\frac{k_{SI}}{k_{SI} + S_o}\right)\tag{1}$$

Where V is the initial rate of dye utilization (g S l^{-1} h^{-1}); Xo and So are the initial biomass and the substrate (dye) concentrations (g S l^{-1}); K is the rate constant (g S g X^{-1} h^{-1}); K_s is the saturation constant (g l^{-1}); K$_{SI}$ is the substrate inhibition constant (g l^{-1})

The first term in the right hand side of Eq. (1) represents enzyme utilization rate at low dye concentrations according to Monod equation and the second term represents substrate (dye) inhibition at high concentrations. According to the data presented in Fig 3 a, enzyme utilization rate increased with dye concentration up to 75 g l^{-1} and then decreased for greater dye concentration due to substrate inhibition. For dye concentrations less than 75 g l^{-1}, the inhibition term in Eq. (1) can be neglected and the Eq. (1) takes the following form:

$$v = \frac{kX_o S_o}{k_s + S_o} = \frac{R_m S_o}{k_S + S_o} \tag{2}$$

Where $R_m = K X_o$ is the maximum rate of substrate (dye) utilization in g S l^{-1} h^{-1}.

In the double reciprocal form Eq. (2) takes the following form:

$$\frac{1}{v} = \frac{1}{Rm} + \left(\frac{k_S}{R_m}\right)\left(\frac{1}{S_o}\right) \tag{3}$$

When the experimental data (Table 1) for dye concentrations below 75 g l^{-1} was plotted in form of $1/V$ versus $1/S_o$ the following constants were found for R_m and K_s. Therefore, Eq. (2) takes the following form for $S_o < 75$ g l^{-1}:

$$v = \frac{kX_o S_o}{k_s + S_o} = \frac{17X_o S_o}{570 + S_o}$$

(4)

Extremely high value of K_s indicated that the kinetics can be approximated to first order. Since S_o is much lower than K_s (i.e., $S_o/K_s < 0.13$) for $S_o < 75$ g l^{-1}, then S_0 in the denominator may be neglected to yield

$$v = \left(\frac{k}{k_s}\right)X_o S_o = 0.0298 \, X_o S_o \tag{5}$$

For dye concentrations above 75 g l^{-1}, substrate inhibition was observed as presented in Fig. 4 a. Therefore, at high substrate concentrations ($S_o > 75$ g l^{-1}) only the inhibition term was considered and the Eq. (1) was approximated to the following expression:

$$v = R_{sm}\left(\frac{k_{SI}}{k_{SI} + S_o}\right) = k'X_o\left(\frac{k_{SI}}{k_{SI} + S_o}\right) \tag{6}$$

In double reciprocal form, Eq.(3) takes the following form:

$$\frac{1}{v} = \frac{1}{R_{sm}} + \frac{S_o}{R_{sm} k_{SI}} \tag{7}$$

When the experimental data for $S_o > 75$ gl^{-1} (Table 1) was plotted in form of $1/V$ versus S_0 the following constants were obtained from the slope and intercept of the line:

$R_{sm} = 1.62$ g S l^{-1}h^{-1}, K$_{SI} = 93$ g l^{-1}, K' = 3.24 g X^{-1}h^{-1} since Xo was 0.5 g l^{-1}. Then, Eq. (7) takes the following form:

$$v = k'X_o\left(\frac{k_{SI}}{k_{SI} + S_o}\right) = 3.24X_o\left(\frac{93}{93 + S_o}\right)$$

(8)

V values for $S_o < 75$ gl^{-1} and $S_o > 75$ gl^{-1} were estimated using equations (6) and (8), respectively. Table 1 summarizes the experimental and the predicted values of V for all dye concentrations tested. Good agreement between the predicted and the experimental values of V indicated accuracy of the kinetic constants and the validity of the rate expressions for the experimental conditions used.

4. Conclusions

Dye decolorization using Acid orange 10 was investigated as functions of the substrate (dye) and biomass concentrations using batch experiments. The rate and extent of Percentage decolorization of the dye or dye utilization

increased with increasing dye or dye concentration up to 100 g l^{-1} indicating substrate limitation at low dye or dye concentrations. Further increase in dye concentration above 100 g l^{-1} resulted in gradual decrease in the rate and extent of percentage decolorization indicating substrate inhibition at high dye concentrations. Dye concentrations should be kept below 100 g l^{-1} in batch experiments to avoid substrate inhibition possibly due to high osmotic pressure. Fed-batch experiments may also be used to overcome substrate inhibition at high dye concentrations. Increasing biomass concentrations resulted in improved enzyme utilization and percentage decolorization. Both the rate and the extent of percentage decolorization increased almost linearly with the biomass concentrations between 100 and 200 beads containing approximately 8.9 x 10^6 cells ml^{-1}. Maximum dye decolorization of 99.65% (vv^{-1}) was obtained with 200 beads where there was high biomass concentration. Optimum biomass concentrations above 50 beads were generally advantageous resulting in shorter time period and higher percentage decolorization. Substrate utilization kinetics was found to be first order for low dye concentrations of less than 75 g l^{-1} above which substrate inhibition was observed. The developed kinetic model used was found to be suitable for representation of the experimental data.

Acknowledgement

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Legends for figures:

Figure 1. Enzyme production by the fungus *P.chrysosporium*.

(▲) LiP production (⬘)MnP production

Figure 2a. Variation of dye concentration with time.

(b) Variation of enzyme activity with time.

Dye concentration (g l^{-1}): (⬘) 10, (▲) 50, (□) 100, (■) 150, (○) 200 and (●) 225.

Figure 3a. Variation of specific rate of enzyme utilization with dye concentration.

(b) Variation of specific rate of percentage decolorization with dye concentration.

Figure 4a. Variation of enzyme utilization with time.

(b) Variation of percent dye concentration with time.

Biomass concentration (number of beads):

(△) 25, (▲) 50, (□) 100, (■) 150, (○) 200 and (●) 250.

Figure 5a. Variation of dye utilization rate with initial biomass concentration.

(b) Variation of decolorization rate with initial biomass concentration.

Legends for Table:

Table 1. Experimental and the predicted rate data used for kinetic modeling

Where X_0 = 0.5 g l^{-1}, R_m = 8.5 g S l^{-1}h^{-1}, K_{SI} = 570 g l^{-1} and K = 17 g S g X $^{-1}$

Table 1. Experimental and the predicted rate data used for kinetic modeling

S_o (g l^{-1})	$1/S_o$	$V_{theor.}$ (gl^{-1}h^{-1})	$1/V$	$V_{predicted}$ (gl^{-1}h^{-1})
25	0.04	0.358	2.79	0.357
50	0.02	0.673	1.49	0.685
75	0.013	1.006	1.00	0.988
100	0.01	0.792	1.26	0.781
125	0.008	0.685	1.46	0.690
150	0.0067	0.628	1.59	0.620

X_0 = 0.5 g l^{-1}, R_m = 8.5 g S l^{-1}h^{-1}, K_S = 570 g l^{-1} and K = 17 g S g X $^{-}$
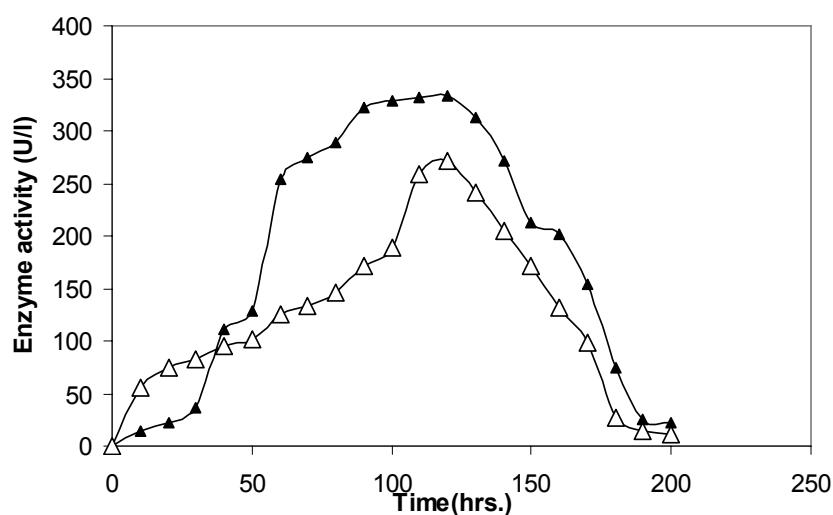
Figure 1. Enzyme production by the fungus *P.chrysosporium*
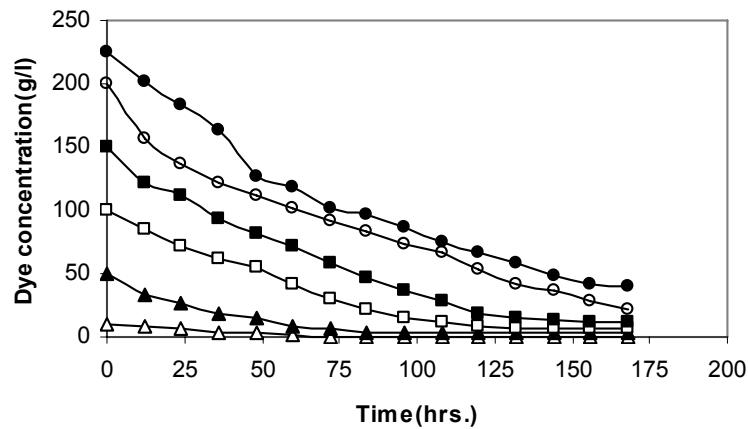
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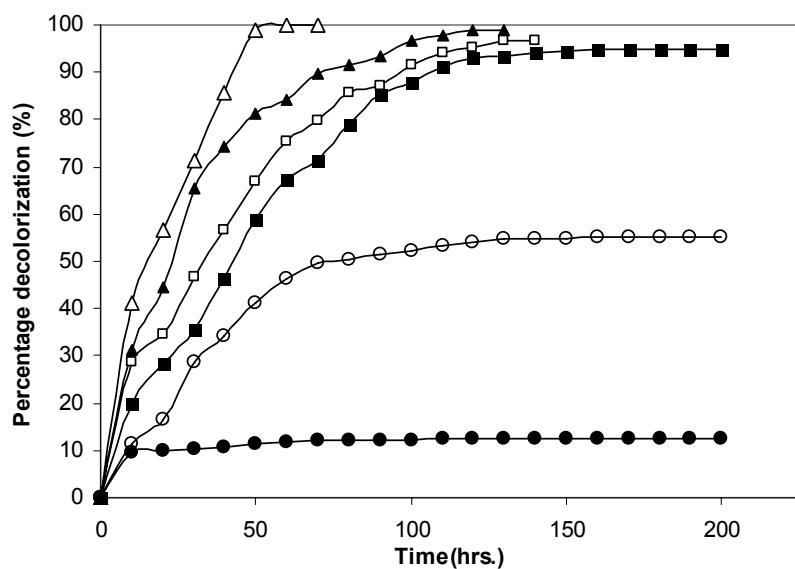
Figure 2b. Variation of enzyme activity with time
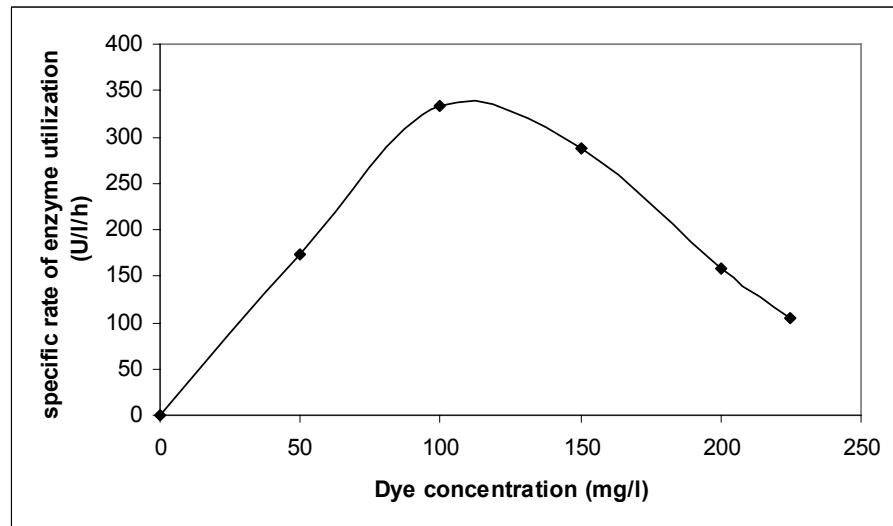
Figure 3a. Variation of specific rate of enzyme utilization with dye concentration
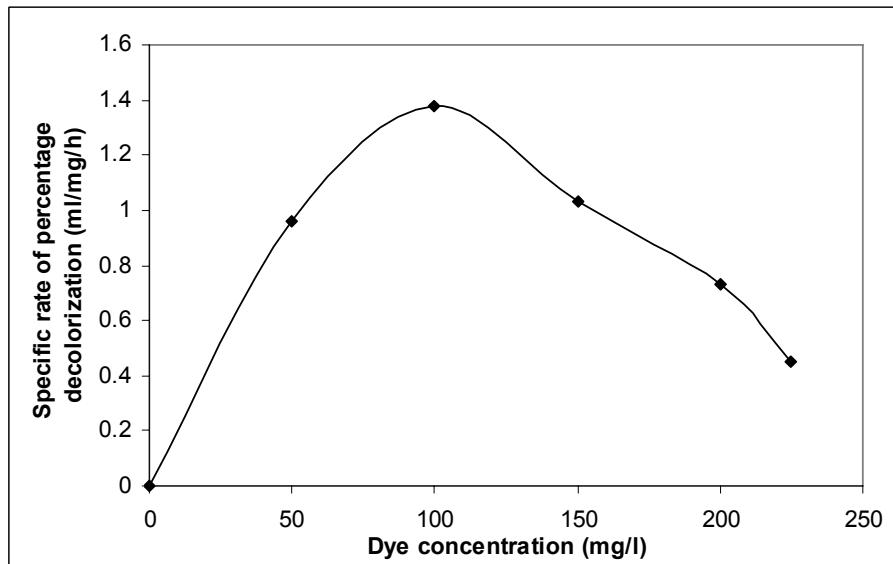
Figure 3b. Variation of specific rate percentage decolorization with dye concentration
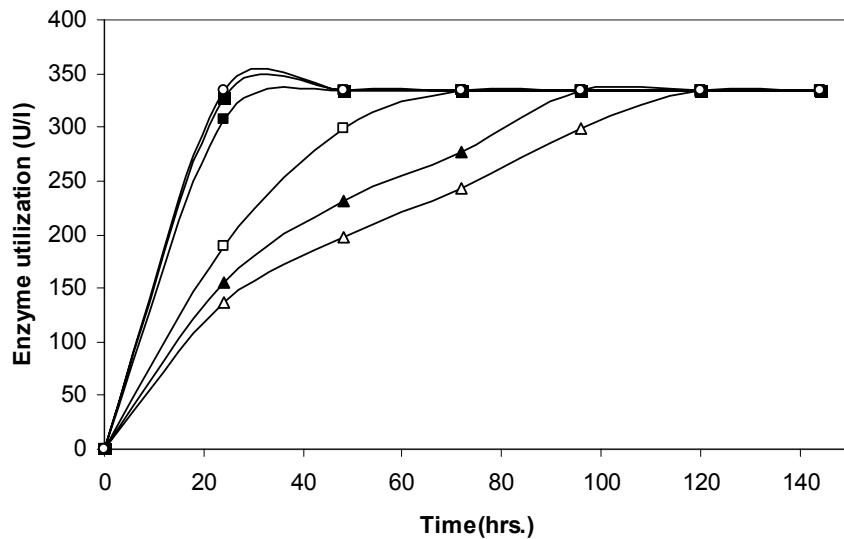
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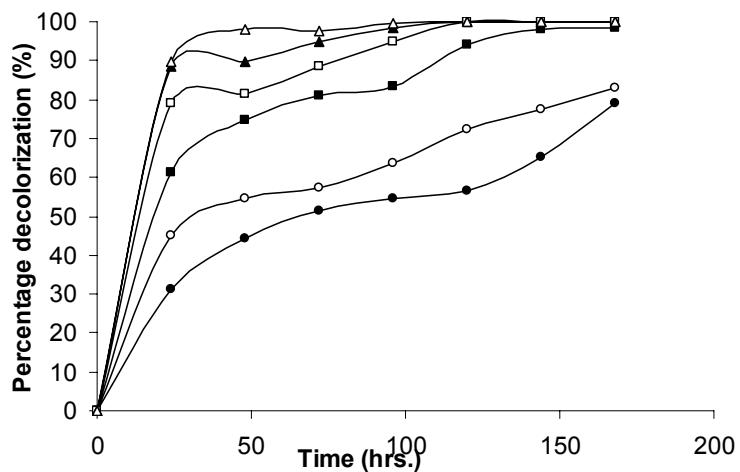
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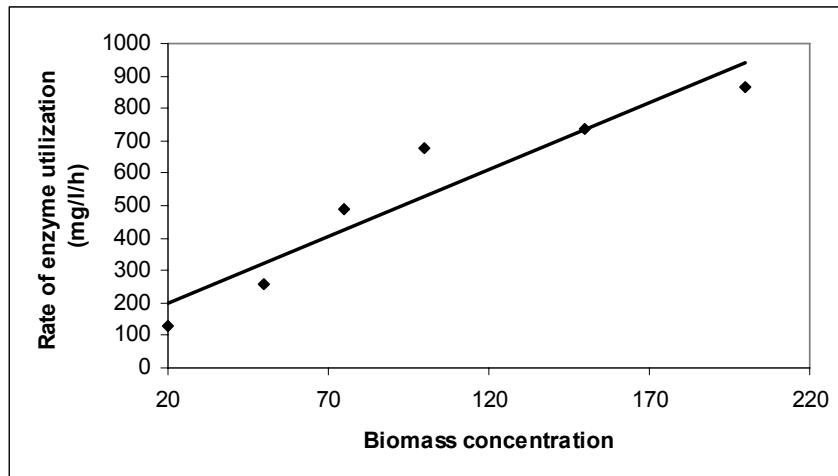
Figure 5a. Variation of enzyme utilization rate with initial biomass concentration
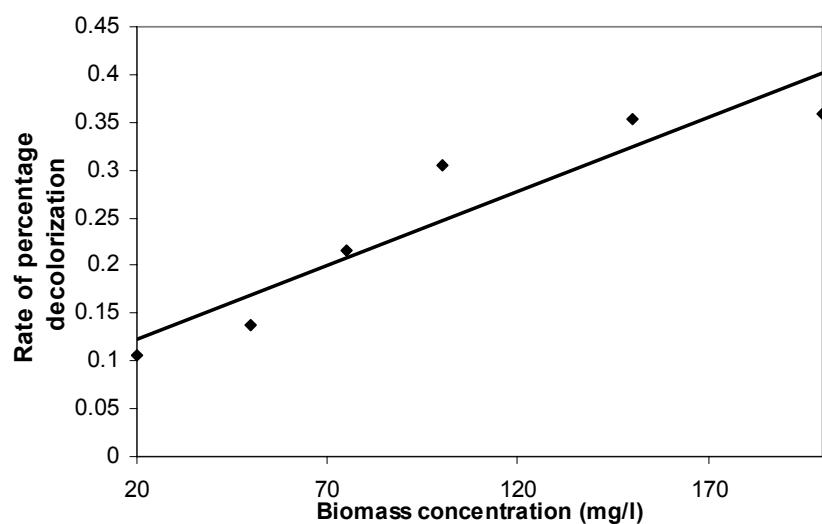
Figure 5b. Variation of decolorization rate with initial biomass concentration