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Biodegradation of direct golden yellow, a textile dye by *Pseudomonas putida*

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ABSTRACT

In this study, biodecolorization of Direct golden yellow dye using a bacterial strain *Pseudomonas putida* was investigated. Laboratory scale experiments were performed to investigate the effect of operational parameters such as pH, dye concentration and dissolved oxygen on the decolorization efficiency of the strain. The most efficient decolorization was achieved in the pH range of 7–9 and the biodecolorization process was found to be effective under static and anoxic conditions but aeration strongly inhibited the decolorization. In decolorization, the maximal absorption wavelength in the UV-visible spectra for the dye containing culture shifted from visible light range towards the ultraviolet visible range confirming the structural modifications of the dye molecule during the course of degradation. The strain also exhibited excellent stability during the reported batch experiments. It has been observed that there is an induction of reductase enzyme (azo-reductase), which helps in the anaerobic reduction of azo bonds present in the dye structure. The cells obtained from the late-stationary phase exhibited high azo-reductase activity than the cells from exponential and early-stationary phase. Hence the study reveals the potential of *P. putida* to degrade Direct golden yellow dye effectively under static conditions.

Keywords: Biodecolorization; *Pseudomonas putida*; Direct golden yellow dye; Azo-reductase activity

1. Introduction

Textile processing industries are widespread sectors in developing countries. These industries have shown a significant increase in the use of synthetic complex organic dyes as the coloring material because of their ease, cost effectiveness and its stability in relation to light, temperature, detergent and microbial attack and also due to a variety of colors available compared with the natural dyes [1]. During manufacturing and usage, an estimated 10–15 percent of total dyes are released to the environment [2]. Even at low concentrations water soluble textile dyes can cause waste streams highly colored.

These highly stable reactive dyes, which are not degraded by the conventional wastewater treatment processes, enter into environment in the form of colored wastewater [3]. Over the past decades, biological decolorization has been investigated as a method to transform, degrade or mineralize azo dyes effectively and economically [4]. Various fungal cultures mainly belonging to white rot fungi have been described to oxidize synthetic dyes. Decolorization by fungi is achieved via a completely

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distinct mechanism. However, the long growth cycle and moderate decolorization rate limit the performance of fungal decolorization system. Although decolorization by fungi is normally very time-consuming, most biological decolorization processes reported in the literature applied dye-degrading fungi as the biocatalyst [5].

The recent approach of bioremediation is to improve the natural degradation capacity of bacterial strains. Decolorization of dyes by bacterial strains was typically initiated by enzyme catalyzed anaerobic reduction or cleavage of bonds, followed by aerobic degradation [6]. Although the bacterial degradation and decolorization is an environmental friendly and cost-competitive alternative to the conventional decomposition processes, the development of bacterial color removal processes has not been well documented. So, the need of this work is to meet an urgent demand for the development of treatment concepts of bacterial decolorization which guarantee not only irreversible decolorization but also mineralization of textile dyes.

Dye degradation specially using isolated *Pseudomonas* species as biocatalysts has shown that biological and combination treatment can offer a low cost alternative system that involve the elimination of the reactive dyes [7]. Thus, the main objective of this study was to observe microbial decolorization and biodegradation of Direct golden yellow, a reactive azo dye by *P. putida*.

Release of textile reactive dyes into the environment from the effluents of dye-utilizing industries has become a major concern in wastewater treatment since some textile dyes or their metabolites may be mutagens or carcinogens. These wastewaters are usually treated using conventional physiochemical methods such as flocculation, coagulation and adsorption. Recently, several emerging technologies such as electrochemical destruction, photo-catalysis and sorption (Hu et al., 1992) were identified to have potential for decolorization [8]. However, those approaches often involve complicated procedures or are economically unfeasible.

2 Materials and methods

2.1. Experimental set up for the initial studies

2.1.1. Dye stuff and chemicals

The water-soluble Direct golden yellow ME (mild exhaust) dyes were taken for biodecolorization studies. It is a mixed bi-functional reactive dye where vinyl sulphone group is linked to chromophore through a mono-chloro tri-azine group as a bridge link. The dye was used at a quality identical to that being used in the textile industry. The dye was of industrial grade and was a generous gift from local textile industry located in Tirupur, India. Direct golden yellow dye was used as a model dye for all the decolorization experiments. All other chemicals used were of an analytical grade.

2.1.2. Microorganism culture conditions and acclimatization

P. putida was used as an indicator strain to study the biodecolorization performance to decolorize Direct golden yellow dye. Pure culture isolated from soil sample was maintained on nutrient agar slants at 4ºC. Subcultures were made routinely. The culture of *P. putida* was grown in 250 ml Erlenmeyer flask containing nutrient broth (NB) medium of following composition (g/l): 10 g peptone, 10 g sodium chloride and 5 g yeast extract at 30 ± 2ºC for 24 h. The pH of the medium was adjusted to be in the range of 6.8–7.0. Growth of the microorganisms was monitored spectrophotometrically. The cell pellet obtained upon centrifugation at 1957 x g for 15 min was re-suspended in distilled water and its absorbance was studied at 600 nm. The relationship between the cell concentration and absorbance (Abs) of cell culture at 600 nm is as follows:

$$\text{Dry cell weight}\left(\text{g/l}\right) = (\text{Absorbance of cell culture at 600 nm})/0.32 \qquad (1)$$

The strain was acclimatized by transferring aliquots of *P. putida* culture from the fresh nutrient medium to medium containing various concentrations of dye. The dyes containing nutrient agar plates were inoculated with suspension from flasks containing acclimatized organisms. The isolated colonies were transferred to dye containing nutrient broth and used for biodecolorization studies.

2.1.3. Batch decolorization experiments

The biodecolorization performance of the strain *P. putida* was studied as given in the following experiments. To obtain synchrony in the division of *P. putida* culture for study, a loopful of acclimatized *P. putida* seed taken from an isolated colony on a nutrient agar plate was pre-cultured in 50 ml NB medium kept under shaking incubation. Pre-cultured broth (1% by volume) broth was then inoculated into fresh medium for obtaining required concentration of culture containing *P. putida*. Once the cells had grown to late exponential or early stationary phase (i.e., 12–15 h), the culture was taken and used for decolorization experiments. All experiments were performed in duplicate to guarantee reproducibility. The growth was followed by measuring the optical density of the crude samples at 600 nm.

The concentration of the dye was primarily determined by measuring the optical density (OD) of the

supernatant sample after centrifugation at 1957 x g for 15 min. The degradation of the dye was followed by analyzing UV-visible spectrum of the samples drawn at various intervals. A sterile cell free medium was chosen as a control.

2.1.4. Biodecolorization studies at static and shaking conditions

In order to study the effect of Dissolved Oxygen on biodecolorization efficiency of *P. putida*, the studies were carried out in shaking as well as static incubation conditions. The strain was grown for 24 h at 30ºC in 100 ml NB to study the effect of static and shaking incubation on biodecolorization performance of microbial culture. After 24 h, 100 mg/l dye was added in each and incubated at static and as well as shaking conditions on orbital shaker. An aliquot (5 ml) of the culture media was withdrawn at different time intervals, and analyzed for growth and decolorization.

2.2. Study of physico-chemical parameters

It is well known that the some of the physico-chemical parameters affect both the enzyme activity and microbial growth that consequently interferes with microbial biodecolorization efficiency. Hence, decolorization was studied by varying pH (5, 6, 7, 8, 9, 10 and 11) and dye concentrations (100–2000 mg/l).

2.3. Analysis of the effect of growth phase on decolorization

P. putida cells were collected from various growth phases to compare the specific biodecolorization efficiency and the cell age. The cells were harvested periodically from the culture stating from mid-log phase (8 h) to late-stationary phase (25 h). These cells were inoculated into a fresh nutrient broth containing dye. The samples were withdrawn periodically and analyzed for residual dye concentration.

2.4. Decolorization assay

The residual concentration of the dye was primarily determined by measuring the OD of the supernatant after centrifugation at 1957 x g for 15 min. The un-inoculated dye free medium was used as a blank. All assays were performed in duplicate and compared with controls. Biodecolorization was determined by measuring the absorbance of decolorization medium at 420 nm, which is the maximal absorption wavelength specific to the dye and the percentage biodecolorization was calculated as follows:

$$\text{Percentage decolorization} = \frac{\text{Initial absorbance} - \text{Observed absorbance}}{\text{Initial absorbance}} \times 100 \quad (2)$$

2.5. Determination of azo-reductase activity

In order to study the effect of enzyme on biodecolorization, the cell free extract from the cultures was prepared as follows. *P. putida* was grown in NB medium at 30ºC for 24 h and the culture sample was centrifuged and the cell pellets were suspended in the sodium phosphate buffer (0.1 M, pH 7.0) and sonicated (60–75 W; 5 min) with an ultrasonic processor. The temperature was maintained below 4ºC. This homogenate was centrifuged and supernatant was used as a source of enzyme.

Activity of azo-reductase was assayed spectrophotometrically in cell free extract and also in culture supernatant. The residual dye concentration in the reaction mixture was measured as a function of time, and the initial rate of dye disappearance was determined from the concentration profiles to determine the enzyme activity. Azo-reductase activity was determined based on the procedures described by Zimmermann and Kulla [6]. In general, 1 ml of cell-free extract (typically at a concentration of 2–3 mg protein/ml) was added to 0.1 M NaP buffer (pH 7.0) containing 24 μM of dye and 0.35 mM of NADH; the total volume of the reaction mixture was 2 ml. All enzyme assays were carried out at room temperature. Reference blanks contained all components except the enzyme.

2.6. Estimation of the kinetic parameter

To evidently reveal the decolorization capability of *P. putida* to direct yellow dye, time courses of extra cellular dye concentration in batch cultures with various initial dye concentrations were used for determination of kinetic parameters. Considering the whole cells as enzymes (or biocatalysts), we might apply the Monod kinetic model to depict this decolorization. It is assumed that the cell solution has a fixed number of bio-available catalysts to which dye substrate can bind [9]. At high substrate concentrations, all these cell catalysts may be occupied by dyes or the biocatalysts are saturated. The scheme of Monod kinetics involves a reversible step for "*cell-substrate complex*" formation and a dissociation step of the complex. The decolorization rate, V is expressed in Eq. (3):

$$V = \frac{-\mathrm{d}S}{\mathrm{d}t} = \frac{V_{\max} S}{K_S + S} \quad (3)$$

where, S and t are dye concentration and time. Parameters V_{max} and K_S are the maximal decolorization rate and saturation constant or half velocity constant. K_S indicates the affinity of the cell to the dye substrate. Using these parameters, time courses of residual dye concentrations were then predicted.

2.7. TOC analysis

The total organic carbon (TOC) of the initial dye sample and the samples withdrawn from culture during the course of decolorization was determined using TOC analyzer.

3. Results and discussions

The synthetically prepared Direct golden yellow dye was subjected to biodecolorization studies by using bacterial strain called *P. putida*. The biodecolorization performance of the strain was investigated by varying the operational parameters such as pH, initial dye concentration and dissolved oxygen. All experiments were conducted in a batch mode at room temperature.

3.1. Characterization of P. putida

The characteristics of the *P. putida* strain were found to be Gram negative and rod shaped under microscope. It has very simple nutritional requirements and can grow well at a temperature of 37°C and at a pH of 7. The colony morphology appears to be circular with low convex.

3.2. Growth curve of P. putida

Since the growth phase of the organism determines the rate of microbial growth, death rate and the extent of secondary metabolic activity in a specific medium, the growth curve is found to be an important characteristic of the strain used for biodecolorization studies. The plot was made by inoculating a single colony of *P. putida* cells in a fresh NB medium at pH 7.0 and analyzing the growth by observing the cell density for every 45 min at 600 nm.

The values of time and absorbance over a period of growth monitored and growth curve was plotted and given in Fig. 1. From the results, the acclimatization period of the strain to the medium was found be between 1 and 4 h from the time of inoculation. It has been inferred that *P. putida* reached the exponential growth phase in the time period of 4–6 h, in which the cell concentration tends to increase logarithmically. After that a stationary phase was observed, in which secondary metabolites such as enzymes and other products were expected to be produced and subsequently lead to the microbial degradation process. This stationary period of growth was considered to be significant for dye biodecolorization studies [10].

3.3. Biodecolorization performance under static and shaking conditions

The major mechanism of colour removal by bacterial species is reported to be by means of adsorption onto cell walls and the remaining by means of secondary metabolism of the species (Hu et al., 1992). In order to study the biodecolorization mechanism of Direct golden yellow by *P. putida*, the effect of shaking and static incubation condition on growth rate and colour removal rate was found to be necessary.

To study the growth and decolorization pathway of bacterial culture under static and shaking condition, the dry cell weight and colour removal was plotted against time period under both the incubation conditions. The results are given in Fig. 2. From the results, it is observed that agitated cultures grew well compared to static condition. As the agitation was continued during the stationary phase, no decolorization was seen. However, when agitation was terminated for static condition, a significant decrease in dye concentration was observed.

It was also found that the cell concentration remained nearly unchanged during the static incubation period, probably due to the depletion of Dissolved Oxygen in accordance to an earlier study, which had already proven that bacterial biodecolorization was induced only when the Dissolved Oxygen concentration dropped to zero, because of the inhibitory effect of oxygen on decolorizing enzymes [11]. In contrast to color removal under

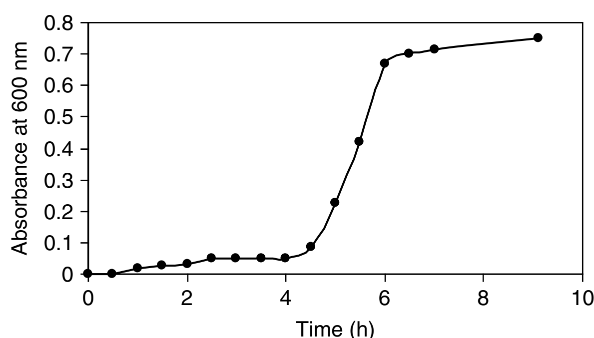

Fig. 1. Growth curve of *Pseudomonas putida*.

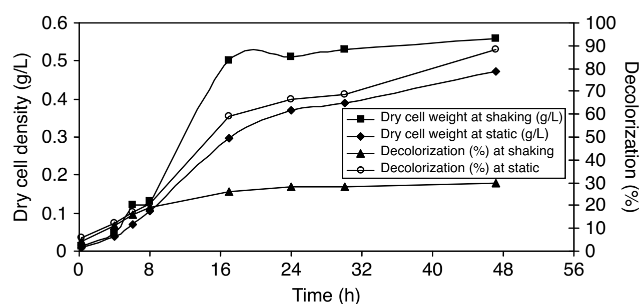

Fig. 2. Effect of static and shaking condition on decolorization.

stationary incubation condition, cell growth rate was slow compared to that under agitated incubation condition.

As it is seen in the Fig. 2, the bacterial culture exhibited a maximum of biodecolorization of 92% within 48 h of static incubation condition whereas the culture showed only 30% of biodecolorization under shaking condition. The results clearly indicated that decolorization was not dependent on biomass concentration but was significantly correlated with Dissolved Oxygen. However, the maintenance of cellular viability is of importance, as a basic metabolism to express enzyme activity which must be sustained for the decolorization to proceed.

3.4. Effect of dissolved oxygen (Dissolved Oxygen) on biodecolorization

Although the oxygen effect on biodecolorization has been recognized, quantitative analysis of the correlation between (Dissolved Oxygen) and the biodecolorization rate should be reported. The Dissolved Oxygen levels were periodically monitored using Dissolved Oxygen meter and rate of biodecolorization was analyzed for finding the influence of oxygen on biodecolorization rate and microbial growth.

During the aerobic process (Dissolved Oxygen = 4.6 mg/l), the culture was agitated in nutrient broth medium containing 100 mg/l of dye. The residual dye concentration dropped, despite a rapid increase in the cell concentration during the shaking incubation condition. The Dissolved Oxygen level only decreased to 1.5 mg/l and remained the same for longer time. As a consequence, no significant colour removal was observed. The correlation between Dissolved Oxygen level and the biodecolorization percentage is shown in Fig. 3. As the agitation continued, no decolorization was observed.

But after 6 h of static incubation, the Dissolved Oxygen quickly dropped to nearly 0.5 mg/l, with a significant decrease in dye concentration. This indicates the decolorization of Direct golden yellow dye by *P. putida* which is initiated only when the Dissolved Oxygen level becomes very low. The results could also be compared with the findings about the indirect effect of oxygen on induction of enzymes which are necessary for secondary metabolic biodecolorization [12].

The specific reason for decreased biodecolorization during agitation could be competition between oxygen and dye compounds for the reduced electron carriers such as NADH under anaerobic condition. The presence of oxygen normally inhibits the activity of some enzyme since aerobic respiration may dominate the utilization of NADH; this impedes the electron transfer from NADH to dye compounds [4].

3.5. Effect of pH on biodecolorization

The variation of initial pH on the decolorization of dye during bacterial degradation is presented in Fig. 4. It shows that an increase in pH from 5.0 to 7.0 led to a three-fold increase in the specific decolorization rate, which remained essentially same for pH 7.0 to 9.0. This seems to indicate that neutral and basic pH values would be more favourable for decolorization of the dye. The trend of pH dependence on decolorization is similar to that obtained in *P. putida* [13].

As can be seen from Fig. 5, the maximum color removal efficiency of 92 % was achieved at 50 h of biodecolorization process carried out at a pH of 7. Other pH values brought only lesser removal percentage within the same period of time. That again confirms that the best suitable pH is 7 for bacterial decolorization studies. Since the pH has a direct effect on both the enzyme activity and cell growth rate, this was considered to be a significant operational parameter for the batch decolorization as confirmed by the present study.

It is well known that the pH affects both the enzyme activity and microbial growth. It is evident from the

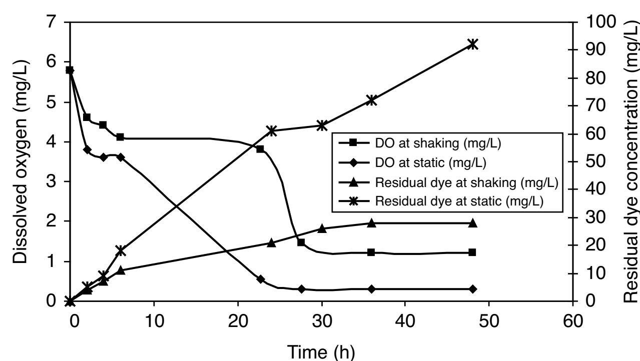

Fig. 3. Effect of dissolved oxygen on decolorization of direct golden yellow dye.

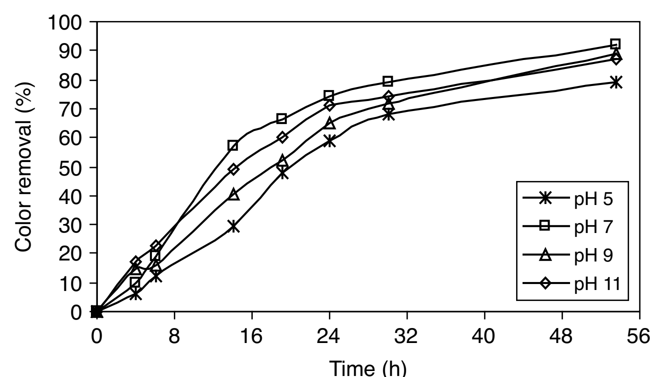

Fig. 4. Biodecolorization performance of *P. putida* at various pH.

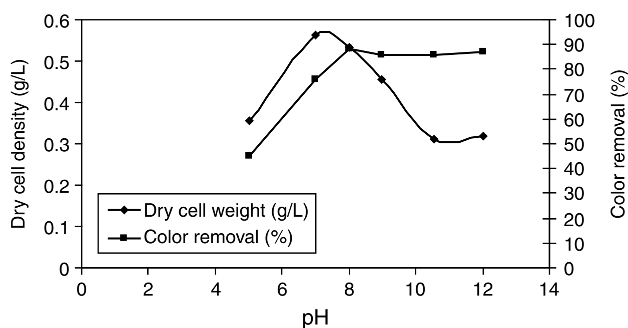

Fig. 5. Effect of pH on microbial growth and decolorization efficiency.

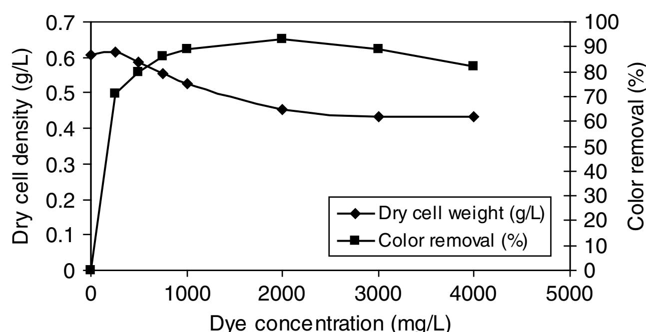

Fig. 6. Biodecolorization profile of *P. putida* at various dye concentrations.

results that decolorization efficiency has not much decreased with an increase in pH (Fig. 5) and remained almost same for higher pH. It was also observed that the increasing pH values have brought a significant decrease in the microbial growth (Fig. 4). This indicates that bacterial decolorization was a non-growth associated enzymatic process (Hu et al., 1992).

3.6. Effect of dye concentration on biodecolorization

The biodecolorization activity of *P. putida* was studied at various initial dye concentrations ranging 250 to 4000 mg/l. The results are presented in Table 1. It is seen from the results that as the concentration of dye increased, the cell growth also increased gradually. That confirms the stability of *P. putida* even at high concentration of dye.

It has been found that *P. putida* can decolorize up to 2000 mg/l of dye after that it seemed to be toxic for cell growth. The dye decolorization is strongly inhibited beyond 2000 mg/l dye in the medium. It is well known that the substrate concentration affects both the enzyme activity and microbial growth. It is evident from the results (Table 1) that biodecolorization efficiency increased with an increase in dye concentration.

Table 1
Dye degradation efficiency of *P. putida* under static incubation

Dye concentration (mg/l)	Dry cell density (g/l)	Absorbance of culture supernatant at 420 nm	Percentage color removal
0	0.605	0.834	0
250	0.616	0.323	71
500	0.585	0.242	80
750	0.555	0.215	86
1000	0.525	0.204	89
2000	0.453	0.167	93
3000	0.432	0.204	89
4000	0.431	0.245	82

Fig. 6 depicts that a substrate inhibition effect may occur at dye concentrations higher than 2000 mg/l. This may be explained to be due to the inhibition of enzyme activity with increasing concentration of dye. It was also observed that biodecolorization occurred essential after 24 h of incubation (late stationary phase) during which there was no microbial growth. This indicates that it was a non-growth associated enzymatic reduction in the dye chromophore. Since the maximal rate took place at a relatively high dye concentration, the strain seems to be suitable for biodecolorization of an environment with a high dye loading.

3.7. Effect of growth phase on biodecolorization

P. putida cells were collected from various growth phases to compare the specific biodecolorization percentage and the cellular age. It can be assumed that the mechanism of biodecolorization in microorganisms is mostly because of biotransformation enzymes. The role of these enzymes in biodecolorization may be different for each organism. This study compares the specific decolorization rates of *P. putida* cells collected from various growth phases. The data in Table 2 shows that the decolorization activity of *P. putida* cells increased slightly from mid-log phase (3.8 mg dye g/cell/ h) to late-stationary phase (4.5 mg dye g/cell/h). The variation in growth phase

Table 2
Effect of growth phase on decolorization

Growth phase	Time (h)	Decolorization activity of *P. putida* (mg dye g/cell/h)
Mid-log phase	8	3.8
	10	4.0
Early-stationary phase	12	4.15
	18	4.3
Late-stationary phase	24	4.42
	30	4.5

Table 3
Azoreductase activity of *P. putida* by cell-free extract from various growth phases

Growth phase		Initial dye concentration (μM)	Residual dye concentration (μM)	Azoreductase activity (nM Mg/protein/min)
Phase	Time (h)			
Mid-log phase	8	24	18	1.5
Early-stationary phase	12	24	12	2.1
Late-stationary phase	15	24	10	3.8

seems to cause only minor effects on the intact cell decolorization activity, since the deviation is very less.

3.8. Enzyme activities during the batch biodecolorization process

As a comparison, the Direct golden yellow dye reduction activity was assayed for cell-free extracts obtained from cultures at different growth phases. As indicated in the Table 3, the cell-free extract originated from the late-stationary phase exhibited the best azo-reductase activity, while the lowest activity belonged to cell-free extract from mid-log phase. These observations seem to indicate that azo-reductase is probably not an essential enzyme for cell growth, since higher enzyme activity occurs at stationary phase, during which the cell growth is not the dominant physiological activity.

3.9. Kinetic analysis

A numerical solution to the Monod model gave an optimal value of 12.94 mg dye/g cell/h for maximum rate (V_{max}) and 8.6 mg/l for the saturation constant (K_s). The kinetic parameters K_s and V_{max} can be evaluated from the intercept and slope of Hanes–Woolf plot [14]. It is already been concluded by Chang et al. that dye with smaller K_s generally tends to have a stronger affinity between enzyme and substrate [11]. From the K_s value obtained for the biodecolorization studies the affinity between azo-reductase and Direct golden yellow dye was confirmed. The rate of the biodecolorization process was also observed to be moderate for this batch process.

3.10. Degradation product analysis

UV-visible analysis (400–800 nm) of supernatants of different time intervals showed decolorization and decrease in dye concentration from batch culture. The visible color removal was measured at 420 nm. The concentration of dye during the biodecolorization was determined based on the calibration curve given in Fig. 8.

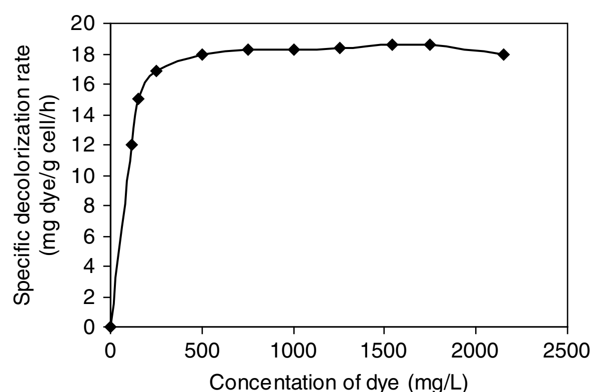

Fig. 7. The profile of dye concentration on the specific decolorization rate of direct golden yellow dye.

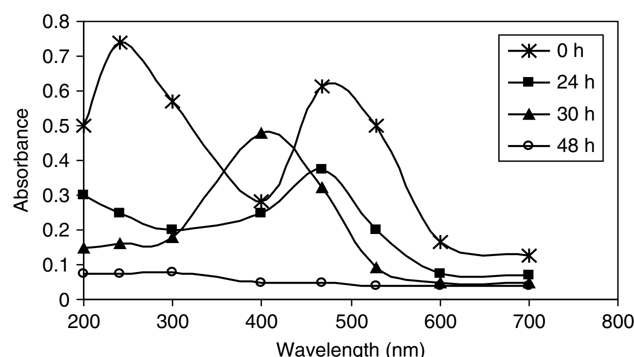

Fig. 8. UV-visible spectra during decolorization of direct golden yellow dye.

Two absorbance peaks were observed during the scanning of Direct golden yellow at a pH of 6.5. The peak observed in the UV region at 226 nm was due to the internal structure of the dye molecule. The visible peak at 420 nm was due to the yellow color of the dye chromophore. Peak observed at 420 nm (0 h) decreased without any shift in the λ_{max} up to complete decolorization of medium. According to Brown and Hamburger 1987, biodecolorization of dyes by bacteria could be due to the adsorption by microbial cells, or to biodegradation. During the course of degradation, a new peak

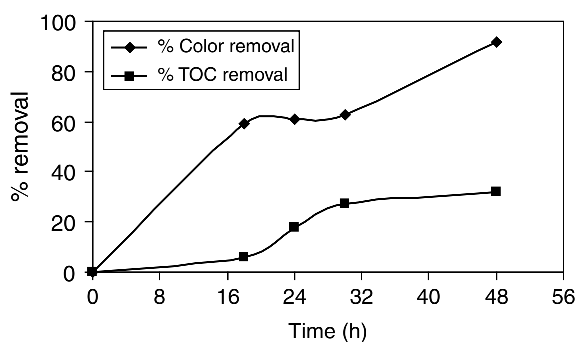

Fig. 9. TOC removal during the biodegradation of direct golden yellow dye.

formed in the 24 h sample, which confirms the production of new metabolite. In the case of adsorption, UV-visible adsorption peaks decrease approximately in proportion to each other, whereas in biodegradation, either the major visible light peak disappears completely, or a new peak appears.

3.11. TOC analysis

Fig. 9 presents the TOC removal pattern during the degradation of Direct golden yellow dye. The complete removal of colour was achieved within an incubation period of 48 h. At the same time TOC removal was only 26% as shown in Fig. 9. It is well known that the removal of color is due to the cleavage of chromophore bond in the dye molecule. But the oxidation of aromatic ring compound takes a long time and hence the removal of TOC is very less [15].

4. Summary and conclusions

In this investigation water soluble Direct golden yellow dye was taken for biodecolorization studies. The experiments were conducted in batch mode to study the influence of operational parameters such as pH, dissolved oxygen and dye concentration on biodecolorization performance of *P. putida*. Results obtained from this work show that *P. putida* possesses decolorization efficiency to a maximum of 92%within a 48 h of static incubation. The biodecolorization rate accelerated in high dye concentration and the maximum removal percentage occurred at 2000 mg/l of dye. Hence, the strain is able to decolorize high concentration of Direct golden yellow effectively.

It is also observed that decolorization is not growth dependent, but is metabolic activity dependent. To ensure an effective dye concentration with the *Pseudomonas* strain, it requires a strict control of the Dissolved Oxygen concentration in the system. In general, operations at a Dissolved Oxygen level higher than 0.3 mg/l should be avoided. Kinetic characteristics of dye decolorization by *P. putida* were determined quantitatively using Direct golden yellow dye as the model substrate. Monod kinetic model provided better predictions to dye decolorization at initial time period due to significant intermediate accumulation at longer period of time. The results showed that the dependence of specific decolorization rate on dye concentration could be described by conventional substrate-inhibition model. Ability of this organism to decolorize the textile dye indicates its commercial availability.

From the described biodecolorization studies, it is observed that the culture exhibited good decolorization ability at a pH of 7.0 and quantitative relation between Dissolved Oxygen, microbial growth and decolorization percentage were established. A significant increase in the azo-reductase activity in cells obtained after decolorization indicates involvement of this enzyme in the decolorization process. Its expression, during the studies, was found to be higher during the stationary phase. However, further experiments with purified enzymes should be made to elucidate which enzyme is mainly responsible for decolorization and degradation of this dye. Ability of this organism to decolorize various textile dyes indicates its commercial availability. In order to develop an efficient bacterial decolorization process, knowledge regarding the kinetics of decolorization and the environmental factors affecting the rate of decolorization need to be well identified quantitatively. In addition, the stability and reusability of the bacterial strain during repetitive decolorization operations has to be examined in order to check the industrial applicability of the strain.

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