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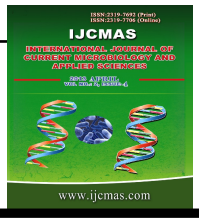

Original Research Article

Decolorization and degradation of Azo dye - Remazol Black B by newly isolated *Pseudomonas putida*

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Keywords

Decolorization; Glucose; *Pseudomonas putida;* Remazol Black B; Textile effluent.

A B S T R A C T

Disposal of dyes into the environment causes serious damage and also they may be toxic to some aquatic organisms due to their breakdown products. The chemical and physical methods have many disadvantages which can overcome by biological method because it is cost saving and environmentally benign. Among different decolorizing microorganisms bacteria can degrade and even completely mineralize many dyes under certain conditions. In the present study an attempt was made to examine the potential of newly isolated *Pseudomonas putida* for decolorization of azo dye- Remazol Black B in batch reactor. The influence of different concentration of glucose, pH and temperature on decolorization was studied to find the optimum conditions required for maximum decolorization and degradation. pH 7.0 and 35^0C were considered to be the optimum decolorizing conditions because in these conditions only the maximum decolorize was found. 5g/L glucose present media showed the maximum decolorization. This new isolate grew well in a high concentration of dye (300mg L^{-1}) and 97.12% decolorized within 48 h and also tolerated upto 1000mg/L of dye. Colorless cells of *P. putida* and UV–Visible spectroscopic analyses suggested that the decolorizing activity only through biodegradation not by inactive surface adsorption. The above results show the potential of this bacterial strain to be used in the biological treatment of textile effluent under optimum condition.

Introduction

Nowadays, water pollution has become a matter of great concern in our society. Most of the water pollutions are related to the industrial effluents. Textile industry is the one of the most important industry in all over world and this industry uses large volumes of water in wet processing operations and thereby, generates substantial quantities of wastewater containing large amounts of dissolved dyestuffs and other products. Textiles are

made of a variety of materials and may contain a large number of chemicals that are employed during the production of fibers as preservative, finishing, and coloring agents.

More than 10,000 dyes are used in the textile industry and <280,000 tonnes of textile dyes are discharged every year worldwide as untreated effluents in the form of wastewater into public drains that eventually empty into rivers (Hsueh *et al*., 2005). Most of them are recalcitrant in nature, especially azo dyes. Azo dyes (N = N group) form the largest class of synthetic dyes with a variety of colour and structure (Minussi *et al.*, 2001; Gharbani *et al*., 2008). These dyes account for approximately 60-70% of all dyes used in food and textile manufacture. Worldwide at the time of production and application about 2- 50% of these dyes are lost as waste effluents (Olukanni *et al*., 2009).

Discharge of these dyes may significantly affect photosynthetic activity in aquatic life by reducing light penetration and phytoplanktons form abnormal colouration (Duran and Esposito 2000; Mester and Tien 2000; Wu, *et al*., 2011). This also alters the pH, increases the biochemical oxygen demand (BOD) and chemical oxygen demand (COD), and gives the rivers intense colourations and public is greatly concerned about water quality. The presence of unnatural colours is aesthetically unpleasant and tends to be associated with contamination. Without adequate treatment these dyes will remain in the environment for an extended period of time (Olukanni *et al*., 2006).

Furthermore, the dye bearing effluents are considered to be a very complex and inconsistent mixture of many pollution substances ranging from organic chlorine based pesticides to heavy metals and is considered to be recalcitrant and non biodegradable. So the removal of dyes from water body has draw great attention within environmental research. Dye wastewater is usually treated by physical or chemical treatment processes. Although they can remove dyes partially, various limitations prevent them to be economical and thus cannot be used widely and economically (Chen *et al*., 2003).

Currently, biological methods are using often to remove dyes in wastewater because of its excellent decolorization ability, cheaper and environment friendly. Microbial decolorization is an Ecofriendly and cost competitive alternative to chemical decomposition process (Moosvi *et al*., 2005). A number of microorganisms have been found to be able to decolourize textile dyes including bacteria, fungi, and yeasts (Olukanni *et al*., 2006). They have developed enzyme systems for the decolourization and mineralization of azo dyes under certain environmental conditions (Pandey *et al*., 2007). In the case of enzymatic remediation of azo dyes, azo reductases and laccases seem to be the most promising enzymes. Laccases have been shown to decolourize a wide range of industrial dyes (Reyes *et al*., 1999; Rodriguez *et al*., 1999).

The general approach of bioremediation is to improve the natural degradation capacity of the native organisms. There are many variables or factors affecting enzyme production and decolorization that are expressed by different taxa and culture conditions. The ability of microorganisms to carry out dye decolorization has received much attention and several bacteria capable of dye decolorization, either individually or in consortia (Verma and Madamwar, 2003; Mossvi *et al*.,

2007). The present study focused on isolation and screening of a new potent strain for decolorizing Remazol Black B (Azo dye) and optimize its culture parameters to maximizing the decolorization.

Materials and Methods

Isolation and screening the decolorizing strain

The soil samples were collected from three different sites of dye industry in sterile bags and brought to laboratory within 24hrs to isolate potent dye decolorizing bacteria. The isolation of bacterial strains were carried out by serially diluting the soil samples in saline water and subsequently plating on Nutrient Agar medium using pour plate method. Seven different colonies were obtained through serial dilution method and then serial streaking on nutrient agar. Each strain was then inoculated into nutrient broth and incubated 24 h at 35^{o}C. Each colony was named as MS1, MS2, MS3, MS4, MS5, MS6 and MS7. These test cultures were grown in mineral salt medium amended with Remazol Black B dye (1000 mgL^{-1}) and screened for dye decolorization. Strain that showed high decolorizing potential was chosen for further optimization study.

Identification and characterization of the strain

Morphological, physiological and biochemical characteristics of the potent dye decolorizing strain, MS7 was determined by the method described in "Bergey's Manual of Determinative Bacteriology" (Holt *et al*., 1993).

Growth medium

Mineral salt medium (MSM) was prepared with the following composition and was used for all the studies (g l^{-1}): Na_2HPO_4 (2.0), $(NH_4)_2SO_4$ (3.0), $MgSO_4.7H_2O$ (0.25), $CaCl_2.7\ H_2O$ (0.25), 0.5% (w/v) yeast extract, glucose (10) and 1 mlL^{-1} of mineral solution (1 g/l $MnSO_4 \cdot H_2O$, 0.5g/l $CoCl_2 \cdot 6H_2O$, 0.2g/l $CuCl_2 \cdot 2H_2O$, 0.1 /l $ZnCl_2$ and 0.4 g/l $Na_2MoO_4 \cdot 2H_2O$). The pH of the medium was adjusted to 7.0 by addition of either 1N NaOH or 1N HCl.

Decolorization by Shake flask culture

Optimization of dye decolorization was carried in 250 ml Erlenmeyer flasks with 100 ml of medium and incubated at 150 rpm for 24 h at 35^{o}C. This batch experiment was run under various growth conditions varying one at a time while keeping others constant. All experiments were performed in triplicate.

Effect of pH

Media pH was adjusted into 3, 4, 5, 6, 7, and 8 by addition of either 1N NaOH or 1N HCl in order to study its effect on decolorization of Remazol Black B by newly isolated MS7. At the same time other parameters kept constant.

Effect of temperature

In order to study the effect of temperature on decolorization of Remazol Black B by MS7 was carried out at different incubation temperatures ranges 25, 30, 35 40 and 45^{o}C while kept other parameters constant.

Effect of different concentration

To determine the effect of different initial concentrations of Remazol Black B on dye decolorization by MS7, media was amended different initial concentrations such as 100, 150, 200, 250, 300, 350, 400, 450 and 500mg L^{-1} and incubated for 24 hrs. This experiment was studied at a constant condition.

Effect of different initial concentration of glucose

To find the optimum concentration of glucose for dye decolorization by MS7 glucose free media was supplemented with various initial concentration of glucose (1, 2.5, 5, 7.5 and 10 gl^{-1}) and cultures were incubated at an optimum condition for 24hrs.

Decolorization assay

Samples were withdrawn and centrifuged at 10,000 rpm for 5 min and collected supernatant to estimate the % of decolorization. The supernatant was read at 595nm by UV- Visible spectrophotometer. 1mM same dye was used as blank. The percentage of decolorization was calculated by the following formula

$$\text{Decolorization (\%)} = \frac{I_i - I_f}{I_i \text{ X } 100}$$

Where, I_i and I_f are initial and final absorbance of the dye solution. Each decolorization value is a mean of two parallel experiments.

Statistical Analysis

Correlations analysis (Karl Pearson) was performed to find the degree of relationship between the variables. This was done by Software - MINITAM Release 12.2.

Results and Discussion

Isolating and developing new strain will be beneficial in textile wastewater treatment. The isolation of efficient dye decolourisation bacteria from the samples collected from dye contaminated soil and wastewater indicates the natural adaptation of these microorganisms to survive in the presence of the toxic dyes (Khadijah *et al.*, 2009). In the present study soil samples collected from the dye industry when subjected to serial dilution and subsequently plated on a solid enrichment media yielded nearly 7 distinct bacterial colonies. Each colony was named as MS1, MS2, MS3, MS4, MS5, MS6 and MS7. All the strains were cultivated in MSM amended with 1000 mgL^{-1} of Remazol Black B for 24hrs at 35^0C.

Among the isolated colonies, colony MS7 showed the maximum decolorization then other strains. MS7 was a potent dye decolorizing strain and hence it was selected for further studies. Several authors have been reported about the isolation and screening of microorganisms capable of decolourising various azo dyes from sludge samples collected from wastewater treatment sites contaminated with dyes. (Chen *et al.,* 2003; Senan and Abraham, 2004; Khadijah, *et al.*, 2009).

Characterization and identification of decolorizing strain

Table 1 shows the results of the morphological, physiological and biochemical characteristics of strain MS7. Morphologically MS7 showed a pinpoint,

slimy surface, smooth margin, raised, transferent, with an entire margin on nutrient agar plates. It was a gram-negative rod shape.

Table. 1 Morphological, physiological and biochemical characteristics of strain MS7.

Test Characteristics	Response
Morphological characteristics	
Colony morphology	Pinpoint, slimy surface, smooth margin, Raised, transferent, entire margin
Cell morphology	Rod shape
Gram reaction	-
Motility	-
Physiological characteristics	
Growth under aerobic condition	+
Growth under anaerobic condition	-
Growth in liquid medium	Turbid
Biochemical characteristics	
Catalase	+
Oxidase	+
Indole production	-
Methyl red	-
Voges-Proskauer	-
Gelatin hydrolysis	-
Casein hydrolysis	-
Nitrate reduction	-
Citrate utilization	+
Urea hydrolysis	+
Lysine decarboxylase	+
Glucose	+
Sucrose	-
Fructose	+
Mannitol	-
Arabinose	-
Maltose	-
Starch degradation	-

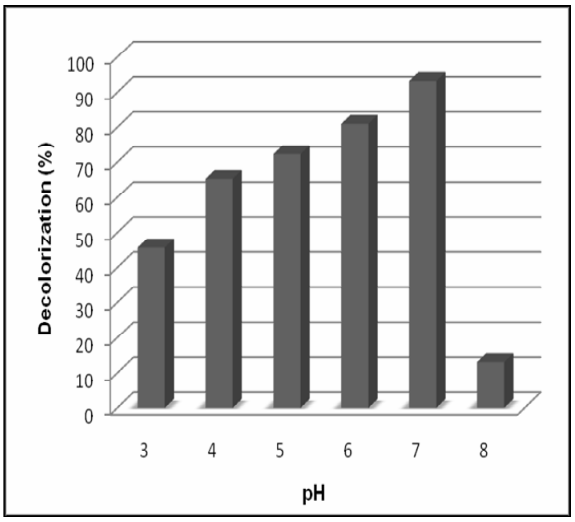
It could hydrolyze urea and utilized citrate. Strain MS7 was showed positive and negative responses for various biochemical tests (Table1). Based on the results from morphological, physiological and biochemical characteristics, strain MS7 was identified as *Pseudomonas putida*.

Effect of pH on decolorization of Remazol Black B

The pH tolerance of decolorizing bacteria is quite important because reactive azo dyes bind to cotton fibers by addition or substitution mechanisms under alkaline conditions and at high temperatures (Aksu, 2003). pH has a major effect on the efficiency of dye decolorization, and the optimal pH for color removal is often between 6.0 and 10.0 (Chen *et al.,* 2003; Guo *et al.,* 2007; Kilic *et al.,* 2007).

In the present study the maximum decolorization of Remazol Black B was achieved at pH 7.0 with 93.23% in 48 hrs (Fig.1). The optimum pH of the growth of *Pseudomonas putida* was neutral. This result accordance with Bhatt Nikhil *et al*., (2012) they found that the consortium SpNb1 exhibited optimum decolorizing activity at pH 7.5 with maximum dye decolorization 94.95 ± 0.09 % and 29.98 mgL^{-1} h^{-1} dye removal rates within 9.30 hrs at 300 ppm dye concentration. Further increase in pH, dye decolorizing activity of the culture was decreased. This may be related to the transport of dye molecules across the membrane, which is considered a rate limiting step.

Fig. 1 Effect of pH on decolorization of Remazol Black B by *Pseudomonas putida*.



Effect of temperature on decolorization of Remazol Black B

The best decolorization was achieved at temperature 35°C and 40°C with 94.25% and 83.65% decolorization respectively in 48 h (Fig.2). This could be owing to a greater production of enzymes and maximal growth conditions of the bacterial culture for its dye decolonization ability. Saratale *et al*., (2010) and Bhatt Nikhil *et al*., (2012) reported that 37°C temperature gave maximum decolorization by bacterial consortium. Decolorizing activity was significantly suppressed at 42°C, which might be due to the loss of cell viability or deactivation of the enzymes responsible for decolorization at 42°C (Cetin and Donmez, 2006; Panswad and Luangdilok, 2000).

Effect of initial concentration of Remazol Black B

The biodegradation abilities of microorganisms can be enhanced by gradually exposing them to higher concentrations of synthetic organic chemicals. Adaptation of a microbial community toward toxic or recalcitrant compounds is found to be very useful in improving the rate of decolorization process (Dafale *et al.,* 2008). Generally the microorganisms are performing their metabolic processes at the optimum substrate concentration.

Different experimental parameters are affecting the enzyme kinetics. Enzyme kinetics follows the principles of general chemical reaction kinetics. At lower substrate concentration, the initial reaction velocity is proportional to substrate concentration (1st order reaction). Further increase in substrate concentration does not affect the reaction rate and the latter became constant (zero order reaction). Above the optimum level of substrate concentration may be stopped the process or reduced reaction rate. In the present investigation the maximum decolorization was obtained with 97.12% at 300mg L^{-1} (Fig.3).

Fig.2 Effect of temperature on decolorization of Remazol Black B by *Pseudomonas putida*.

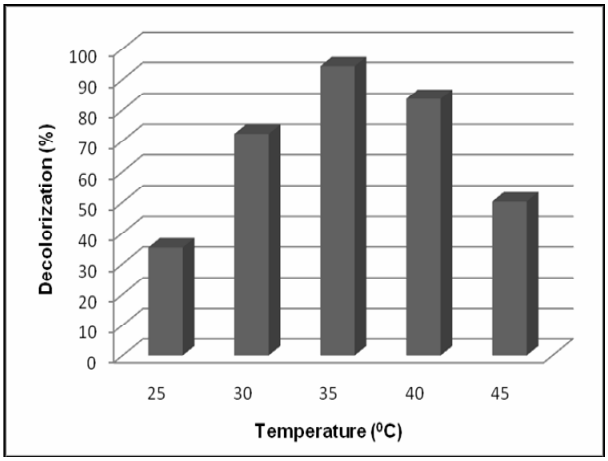
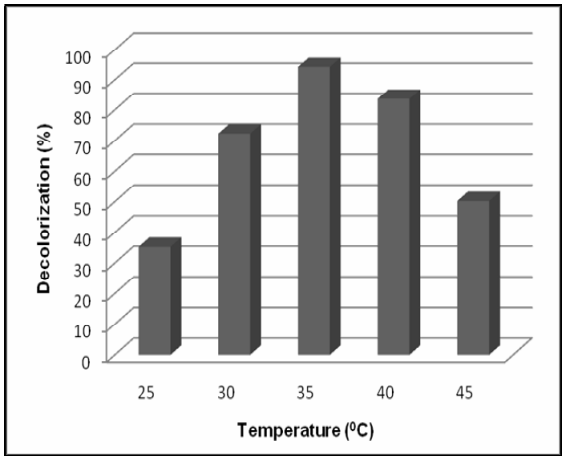


Fig. 3 Effect of initial concentration of Remazol Black B on decolorization by *Pseudomonas putida*.



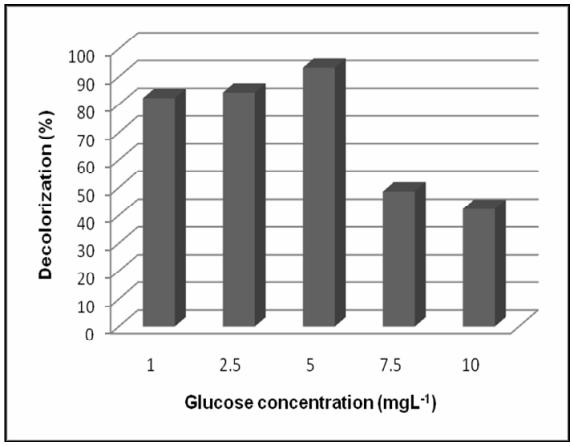
When increase in initial dye concentration decrease in decolorization due to toxicity of the dyes to the growing microbial cells at higher dye concentrations. Gopinath *et al*,. (2009), studied that the biodegradation of Congo Red by a strain of *Bacillus sp* obtained from tannery industry effluent, the increase in initial dye concentration decreased the decolorization rate, and at high concentrations (1500 and 2000 mg L^{-1}), inhibition was observed.

Effect of different initial concentration of glucose

There are only very few bacteria that are able to grow on azo compounds as the sole carbon source; these bacteria cleave –N=N– bonds reductively and utilize amines as the source of carbon and energy for their growth, but such organisms are specific towards their substrate (Pandey *et al.*, 2007). In the present study the maximum decolorization of Remazol Black B was achieved 5g/l glucose present media with 93.24% (Fig.4).

Above this concentration not supported the decolorization. Wang *et al.*, (2009) reported that the lack of glucose inhibited the Reactive Red 180 decolorizing activity of *Citrobacter* sp. CK3 since only 26.72% color removal was observed after 120 h incubation. When glucose supplemented, *Citrobacter* sp. CK3 exhibited strong decolorizing activity with about 90% decolorization extent in 48 h, except that when the glucose concentration was 0.5 gl^{-1} or 12 gl^{-1}, the decolorization efficiencies (64.19% and 67.23% in 120 h, respectively) were much lower. The reason low glucose (0.5 gl^{-1}) concentration could not meet the growth requirements of the bacteria. When the glucose concentration was much higher, such as 12 gl^{-1}, the bacteria could utilize glucose preferentially, thus resulting in lower decolorization extent.

Fig.4 Effect of different initial concentration of glucose on decolorization of Remazol Black B by *Pseudomonas putida*.



In this study, *Pseudomonas putida* MS7 was isolated from industrial effluents. This bacterial strain, showed decolorizing activity through a degradation mechanism rather than adsorption. The maximum azo dye- Remazol Black B tolerant capacity of *P. putida* MS7 is 300 mgL^{-1}. In the lab

scale study it showed the maximum decoloriaztion at pH 7.0, temperature 37^0C and glucose 5 mgL^{-1}. This newly isolated strain has potential in the decolorization of various dye effluents. Statistical analysis - correlation indicated that the above physical and chemical parameter and % decolorization are interdependent since they showed high degree of correlation.

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