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Exploring the Strength of *Pseudomonas Putida* ETL-7 in Microbial Degradation and Decolorization of Remazol Black-B

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Abstract Azo dyes represent a major group of dyes causing environmental concern because of their colour, biorecalcitrance nature and potential toxicity to living beings. In the present study an attempt was made to examine the potential of 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 350 C were considered to be the optimum decolorizing conditions because in these conditions only the maximum decolorize was found. 5 g/L glucose present media showed the maximum decolorization. The bacterium exhibited a remarkable color removal capability over a wide range of dye concentration (50-200 mg/l), 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.

Keywords: *Pseudomonas, Decolorization, pH, temperature, Remazol Black-B*

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1. 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. 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 present investigation focused on isolation and screening of a new potent strain for decolorizing Remazol Black B and optimize its culture parameters to maximizing the decolorization.

2. Materials and Methods

2.1. Isolation and Screening

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°C. Each colony was named as ETL-1, ETL-2, ETL-3, ETL-4, ETL-5, ETL-6 and ETL-7. 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.

2.2. Identification and Characterization of the Strain by Bergey's Mannual

Morphological, physiological and biochemical characteristics of the potent dye decolorizing strain, ETL-7 was determined by the method described in Bergey s Manual of Determinative Bacteriology (Holt *et al*., 1993).

2.3. Identification of Bacteria using 16S rRNA

The total genomic DNA of ETL-7 was isolated using Promega WIZARDR Genomic DNA Purification kit. The forward primers, FD1 (5'-AGAGTT TGATCC TGGCTCAG-3') and the reverse primer, RD1 (5'-AAGGAGGTGATCCAGCC-3') were used to amplify the 16S rRNA sequence of ETL-7. PCR conditions included an initial denaturation for 5 min at 95°C followed by 30 cycles of 1 min at 95°C, 1min at 50°C and 2 min at 72°C. The amplicons were purified and sequenced. The 16S rRNA sequence of ETL-7 was analyzed using Basic Local Alignment Search Tool (www.ncbi.nlm.nih.gov/BLAST).

2.4. Phylogenetic Analyses of MPS-2 16S rRNA Gene Sequences

Genomic DNA was isolated from the pure culture pellet using consensus primers and partial 16s rRNA genes were amplified by PCR using forward primer (5' GAGCGGATAACAATTTCACACAGG-3'), reverse primer (5'-CGCCAGGGTTTTCCCAGTCACGAC-3') and internal primer (5'- CAGCAGCCGCGGTAATAC-3'). The amplified 16s rRNA gene was sequenced. The obtained sequence data was aligned and analyzed for identification and finding the closest homology for the isolate. The next closet homo- logy was found with *Pseudomonas* and it was designated as *Pseudomonas spp.* ETL-7. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 4 (Wuhermann, 1980). The evolutionary history was inferred using the Neighbor- Joining method (Pagga, 1986). The bootstrap consensus tree inferred from 500 replicates is taken to represent the evolutionary history of the taxonomy analyzed (Pearce, 2003). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxonomy clustered together in the bootstrap test (500 replicates) is shown next to the branches (Pearce, 2003). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Rafii, 1990) and are in the units of the number of base substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 1420 positions in the final dataset. This tree was rooted with gram negative bacteria *Eschericia coli* strain K12 MG1655.

2.5. 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 $MnSO_4{\cdot}H_2O$, 0.5 g/l $CoCl_2{\cdot}6H_2O$, 0.2 g/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.

2.6. Decolorization Study

Optimization of dye decolorization was carried in 250 ml Erlenmeyer flasks with 100 ml of medium and incubated at 140 rpm for 24 h at 35°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.

2.7. 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 ETL-7. At the same time other parameters kept constant.

2.8. Effect of Temperature

In order to study the effect of temperature on decolorization of Remazol Black B by ETL-7 was carried out at different incubation temperatures ranges 25, 30, 35 40 and 45°C while kept other parameters constant.

2.9. Effect of Dye Concentration

The various concentrations of dye (100 200, 300, 400 & 500 mg/l) were added into the culture medium in order to examine the effect of initial dye on the decolorization at various time intervals.

2.10. Effect of Glucose Concentration

To find the optimum concentration of glucose for dye decolorization by ETL-7 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.

2.11. Decolorization Assay

Samples were withdrawn and centrifuged at 9,000 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:

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

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

2.12. Statistical Analysis

Correlations analysis was performed to find the degree of relationship between the variables. This was done by Software – MINITAM Release 12.2.

3. 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 ETL-1, ETL-2, ETL-3, ETL-4, ETL-5, ETL-6 and ETL-7. All the strains were cultivated in MSM amended with 1000 mgL^{-1} of Remazol Black B for 24hrs at 35°C. Among the isolated colonies, colony ETL-7 showed the maximum decolorization then other strains. ETL-7 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).

3.1. Characterization and Identification

Table 1 shows the results of the morphological, physiological and biochemical characteristics of strain ETL-7. Morphologically ETL-7 showed a pinpoint, slimy surface, smooth margin, raised, transferent, with an entire margin on nutrient agar plates. It was a gram negative rod shape.

It could hydrolyze urea and utilized citrate. Strain ETL-7 was showed positive and negative responses for various biochemical tests (Table 1). Based on the results from morphological, physiological and biochemical characteristics, strain ETL-7 was identified as *Pseudomonas putida*.

Table 1. Morphological, physiological and biochemical characteristics of strain ETL-7

Morphological Characteristics	
Colony morphology	Slimy surface, pinpoint, entire margin
Cell morphology	Rod shape
Physiological Characterisitics	
Growth under aerobic condition	Positive
Growth under anerobic condition	Negative
Biochemical Characteristics	
Catalase	Positive
Oxidase	Positive
Indole production	Negative
Methyl-red	Negative
Voges-Praoskauer	Negative
Gelatin hydrolysis	Negative
Nitrate reduction	Negative
Citrate utilization	Positive
Urea hydrolysis	Positive
Glucose, Fructose	Positive
Sucrose, Mannitol, Starch degradation	Negative

3.2. Sequence Analyses of Gene Encoding for the 16S rRNA from Bacterium ETL-7

The 16s rRNA gene sequences were compared by using BLAST similarity searches and the closely related sequences were obtained from GenBank. On the basis of morphological and biochemical analysis in combination with phylogenetic analysis, the strain ETL-7 was identified as *Pseudomonas*. The phylogenetic tree (Figure 1) constructed by the MEGA4 (Shah MP, 2013) displays ETL-7's position in relation to other members of the

Pseudomonas spp., and 98% most closely related to *Pseudomonas otitidis* strain 81f and *Pseudomonas* spp. GD6.

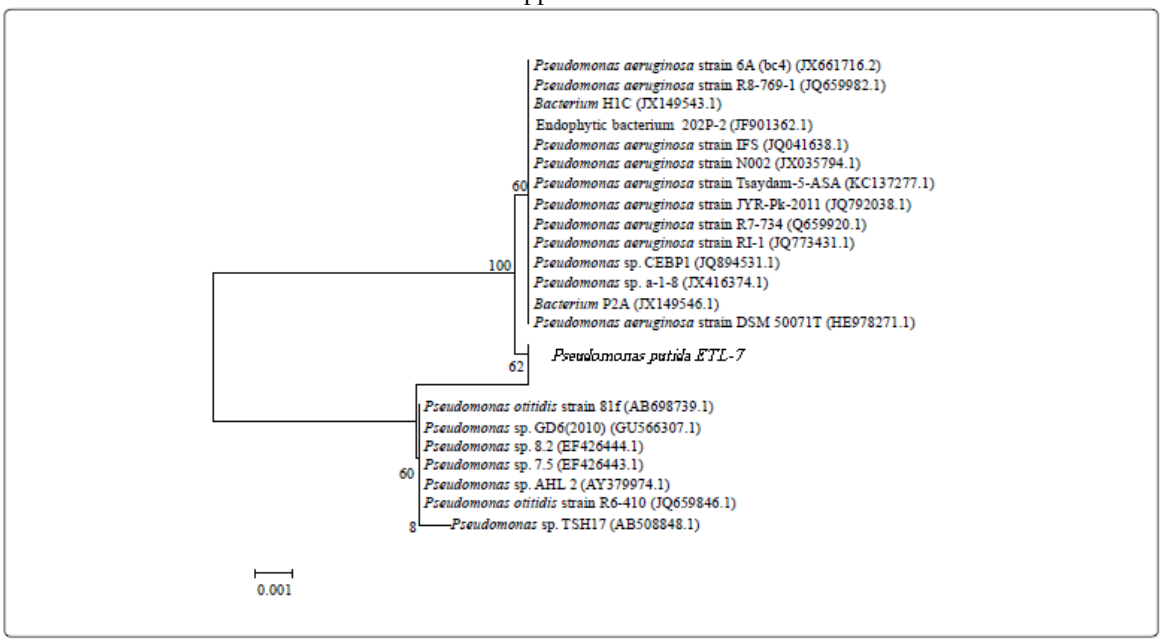

Figure 1. Phylogram (neighbor-joining method) showing genetic relationship between strain ETL-7 and other related reference microorganisms based on the 16S rRNA gene sequence analysis

3.3. Effect of pH

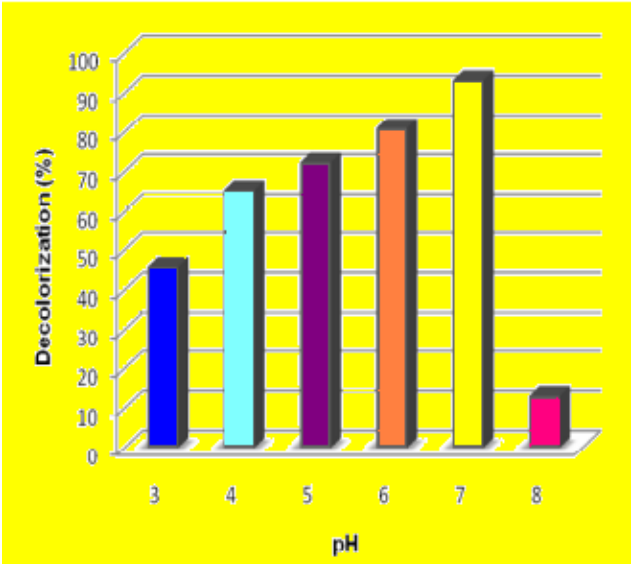

Figure 2. Effect of pH

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 (Figure 2). 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.

3.4. Effect of Temperature

The best decolorization was achieved at temperature 35°C and 40°C with 94.25% and 83.65% decolorization respectively in 48 h (Figure 3). 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).

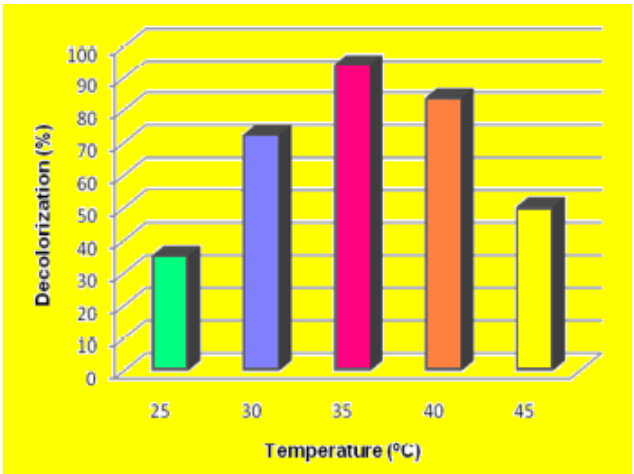

Figure 3. Effect of Temperature

3.5. Effect of Remazol Black B Concentration

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. Data in Figure 4 depict that at the lowest dye concentration (50-200 mg/L) the dye was decolorized more than 84% after four days incubation. Similar data were reported by Yan et al., 2004. As the dye concentration increased in the culture medium, a decline in color removal was attained. This might be attributed to the toxicity of dye to bacterial cells through the inhibition of metabolic activity, saturation of the cells with dye products, inactivation of transport system of the dye or the blockage of active sites of azoreductase enzymes by the dye molecules. Under given experimental conditions, 60% decolorization was attained upon using 500 mg /L of the dye after four days. 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.

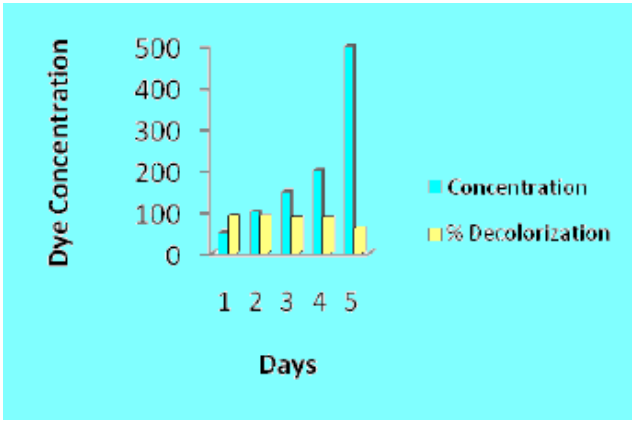

Figure 4. Effect of Dye concentration

3.6. Effect of Glucose Concentration

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 5 g/l glucose present media with 93.24% (Figure 5). 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. In this study, *Pseudomonas putida* ETL-7 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* ETL-7 is 300 mgL^{-1}. In the lab scale study it showed the maximum decoloriaztion at pH 7.0, temperature 37°C 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.

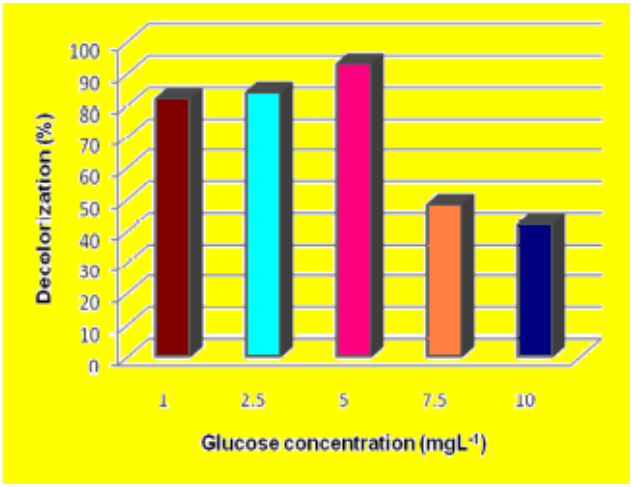

Figure 5. Effect of Glucose concentration

4. Conclusion

A novel strain having a high capacity for rapid decolorization of Remazol Black-B as a azo dye was isolated from a site near to textile industrial outlet. The isolate was identified as Pseudomonas putida ETL-7. The effects of pH, Temperatures, Glucose concentration and dye concentration on the decolorization of remazol black-B were investigated. Examination of the mechanism of the Decolorization process indicated that it proceeded primarily by biological degradation associated with a minor portion of the dye adsorbing onto the cell surface. Identification and toxicity study of the products from the degradation of Remazol Black-B dye by Pseudomonas putida ETL-7 is now in progress.

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