International Journal of Environmental Bioremediation & Biodegradation, 2013, Vol. 1, No. 1, 26-36
Available online at http://pubs.sciepub.com/ijebb/1/1/5

DOI:10.12691/ijebb-1-1-5

Microbial Degradation and Decolorization of Methyl Orange Dye by an Application of *Pseudomonas* Spp. ETL-1982

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Received September 27, 2013; Revised October 19, 2013; Accepted October 22, 2013

Abstract The increasing demand for colorfast and non fading textiles leads to continuous growth in the use of reactive dyes, the majority of which are azo dyes. Reactive dyes present in exhausted dye baths and rinsing water are not recyclable and scarcely biodegradable due to their various substituent. Increasing concern about the direct discharge of untreated dye house liquors to water bodies in developing countries and increasingly stringent regulations for textile wastewater in industrial nations has accelerated the need for new treatment schemes. Existing physical and chemical technologies are expensive and often produce large amounts of solid waste. There is a need to find alternative methods of treatment that are effective in removing dyes from large volumes of effluent and are low cost such as biological or combination system. A bacterium identified as *Pseudomonas spp.* ETL-1982 was isolated from dye contaminated soil. This strain rapidly decolorized a methyl orange azo dye solution. Features of the decolorizing process related to biodegradation and biosorption were also studied. The dye was efficiently decolorized in static compared to shaked cultures. The bacterium exhibited a remarkable color removal capability over a wide range of dye concentration (40-120 mg/l), pH (4-10) and temperatures (30-40°C). Dye removal appears to proceed by both enzymatic reductions associated with biosorption process as shown by effect of Chloramphenicol and Penicillin G on process and variation of UV – visible spectra of Azo dye solution after decolorizing cultivation with isolate. The *Pseudomonas spp.* ETL-1982 decolorized the repeated addition of methyl orange dye up to five cycles with variable decolorization rate (9-91%).

Keywords: *azo dyes, biosorption, decolorization, methyl orange*

Cite This Article: Maulin P Shah, Kavita A Patel, and A M Darji, "Microbial Degradation and Decolorization of Methyl Orange Dye by an Application of *Pseudomonas* Spp. ETL-1982." *International Journal of Environmental Bioremediation & Biodegradation* 1, no. 1 (2013): 26-36. doi: 10.12691/ijebb-1-1-5.

1. Introduction

More than 50% azo dyes are used annually, due to simple diazotization reaction mechanism for the production. Around 2000 of them are used in the textile, leather, plastics, paper, cosmetics and foods industries. Azo dyes are characterized by presence of one or more azo groups substituted with aromatic amines. A substituents often found in azo dyes is the sulfonic acid group (-SO3H). The azo dyes containing the substituent are called as sulfonated azo dyes. Sulfonated azo dyes are widely used in the different industries. The fixation rate of these reactive dyes (including azo dyes) in dying is as low as 50%, which results the release of 10-15% water soluble azo dyes into the environment through wastewater discharge, which are usually recalcitrant to conventional wastewater treatment. Physiochemical methods like adsorption and precipitation methods, chemical degradation or photo degradation are financially and often also methodologically damaging, time consuming and mostly not very effective. As a viable alternative biological processes have received increasing interest owing to their cost effectiveness and environmental friendliness. Many microorganisms belonging to different taxonomic groups of bacteria, fungi, actinomycetes and algae have been reported for their ability to decolorize azo dyes [9]. Thus more studies are now focused on their biodegradation. Most of the azo dyes are either inert or non toxic, but they become toxic, mutagenic and carcinogenic upon their biotransformation. The toxic products are usually aromatic amines. Release of azo dyes containing effluents deteriorates water quality and may cause significant impact on human health and the ecosystem. The prerequisite for the complete mineralization of azo dyes is a combination of reductive and oxidative steps. Decolorization of azo dyes normally begins with initial reduction or cleavage of azo bond anaerobically, which results into colorless compounds. This is followed by complete degradation of aromatic amines strictly under aerobic conditions. Therefore anaerobic/ aerobic processes are crucial for complete mineralization of azo dyes [8]. Methyl orange is an azo

dye used in textile and dyestuff industries. Methyl orange has a deep orange color and its presence in effluents posses an environmental threat. We isolated a bacterium with the ability to decolorize high concentration of methyl orange. In addition, characteristics of the decolorization process involving biodegradation and biosorption were examined to establish a feasible color removal process for a wastewater treatment system [7].

2. Materials and Methods

2.1. Screening of Decolorizers

Soil near to textile industrial outlet was used as source for enrichment and isolation of decolorizers. The screening medium (SM medium) contained: peptone, 10g; meat extract, 10g; NaCl, 5g; in 1 liter of distilled water with 0.2 g of Methyl Orange. Methyl Orange dye was sterilized by passing it through a 0.45- µm pore size filter, while other components were sterilized at 121°C for 20 min. Ten grams of soil was then added to a 500-ml Erlenmeyer flask containing 100 ml of SM medium. The cultures were incubated at 30°C on a rotary shaker at 140 rpm. Next, the broth of the decolorized flask was transferred to fresh SM medium to screen the strain having color removing ability. The screening procedure in the liquid culture was conducted repeatedly until a decolorized culture occurred. A small amount of decolorized broth was then poured into an agar plate containing SM medium and it was incubated at 30°C. Colonies surrounded by decolorized zones were selected. Isolates were then tested for their color removal ability in a submerged culture and the best isolate was selected. Finally, identification of the isolate was done by Bergey's Manual of Determinative Bacteriology (2000) [2].

2.2. Dyes

Methyl Orange (MO) was procured from Hi-Media. Dye was checked for its color, solubility in water, ethanol, and absorption maximum. Stock solution of 5000 ppm was prepared by dissolving the dye in distilled water and was filter sterilized and kept at 4°C. Dye at different concentrations (40 ppm, 80 ppm, 120 ppm, 160 ppm and 200 ppm) were used to study their effect on bacterial growth and adsorption after adding to the culture media.

2.3. Decolorization Experiments

All decolorization experiments were performed in three sets. The culture with OD 0.699 at 540 nm at concentration of 4% was inoculated in 250 ml Erlenmeyer flask containing 100ml Screening medium and incubated at 30°C for 24 h. After 24h of incubation, dye was added at concentration of 120 mg/l and 3 ml of the culture media was withdrawn at different time intervals. Aliquot was centrifuged at 5000 rpm for 15 minutes to separate the bacterial cell mass, clear supernatant was used to measure the decolorization at the absorbance maxima of the dye. Abiotic controls (without microorganism) were always included (Parshetti et al., 2006).

The percentage decolorization was calculated as follows

$$\%Decolorization = \frac{Initial\ OD - Observed\ OD}{Initial\ OD} \times 100$$

2.4. Effect of Dye Concentration

The various concentrations of dye (40, 80 120, 160 and 200 mg/l) were added into the culture medium in order to examine the effect of initial dye on the decolorization in static conditions at various time intervals.

2.5. Effect of Temperature

The inoculated SM medium was incubated at various temperatures (10, RT (30), 37 and 55°C) in static conditions for 48hrs. The effect of temperature on dye decolorization was checked spectrophotometrically after 48hrs [9].

2.6. Effect of pH of Culture Medium

The pH of the inoculated screening medium was adjusted to 2, 4, 6, 7, 8 and 10 with 1M HCL or 1M NaOH. The effect of pH on dye decolorization was checked spectrophotometrically after 48 hrs [9].

2.7. Decolorization at Static and Shaking Conditions

Decolorization ability of bacterial isolate was tested in shaking and static conditions at optimum pH (7.0) and temperature (30°C) using screening medium with 120 mg/l of Methyl orange. The supernatant was withdrawn at interval of 24 hrs. for 5 days and was used for analysis of COD and decolorization. Decolorization was monitored by spectrophotometrically and chemical oxygen demand (COD) was determined according to standard method.

2.8. Effect of Glucose and Peptone on Dye Decolorization

To study the effect of carbon and nitrogen sources on decolorization of methyl orange, Mineral medium with trace element addition and varied concentration of glucose / peptone from 1-4% and 120 mg/l of dye was used.

2.9. Effect of Biodegradable Carbon Source on Decolorization Process

Experiments were conducted using whey instead of screening medium with 120 mg/l methyl orange at pH 7 and at room temperature. Decolorization was studied spectrophotometrically at 24 hrs. interval for 5 days.

2.10. Uptake of Dyes in the Presence of Heavy Metals

The experiments were conducted by adding 5mg/l of $K_2Cr_2O_7$, $AgNO_3$, $ZnSO_4$, $CdCl_2$, and $HgCl_2$ into the screening medium containing 120 mg/l of Methyl orange. Control experiment was performed for the dye under similar conditions except that heavy metal was omitted from the growth medium.

2.11. Effect of Biomass Pretreatment on Dye Decolorization

The 24 hrs. old culture of isolate was exposed to different 1% chemical pretreatments viz. NaOH, Acetic acid, EDTA, Methanol and physical pretreatment of autoclaving for 15 min. at 121°C.After saline washing pretreated isolate was added in screening medium with 120 mg/l of methyl orange. The supernatant was withdrawn and checked for dye reduction spectrophotometrically at 24 hrs interval for 5 days.

2.12. Change in Absorption Spectra During Dye Decolorization

The change in peak in absorption spectrum reveals the dye adsorption or biodegradation during decolorization by isolate. Variation of UV- visible spectra of Azo dye solution at concentration of 120 mg/l methyl orange with *Pseudomonas* spp. ETL-1982 was checked at 0, 24 and 48 hrs. intervals spectrophotometrically [2].

2.13. Effect of Chloramphenicol and Penicillin G on Dye Decolorization by Isolate

MIC for the given antibiotics were checked for the isolate and appropriate concentrations of antibiotics were added separately in screening medium with 120 mg/l concentration of methyl orange. Control experiment was carried out by keeping all conditions similar except antibiotic additions [2].

2.14. Assimilation of Dye

An attempt was carried to test the isolate ability to decolorize 120 mg/l methyl orange in mineral medium depleted from carbon or nitrogen or both. The decolorization was read spectrophotometrically after 48 hrs [9].

2.15. Fed batch Decolorization of Methyl Orange by Isolate

The fed batch decolorization of methyl orange dye was also studied, in this study 120 mg/l dye was added into the 24 hrs. grown culture of bacterial isolate. After decolorization 120 mg/l dye added into the decolorized broth without supplement of additional nutrient. Dye was added continuously until culture does not lose decolorization ability. The dye concentration was determined by monitoring the absorbance of dye spectrophotometrically. Decolorization of azo dyes in consortium Different Azo dyes viz. Methyl red, Methyl orange, Congo red and tartrazine at the final concentration of 120 mg/l was used in screening medium with isolate and dye reduction was checked spectrophotometrically for 5 days at 24 hrs interval.

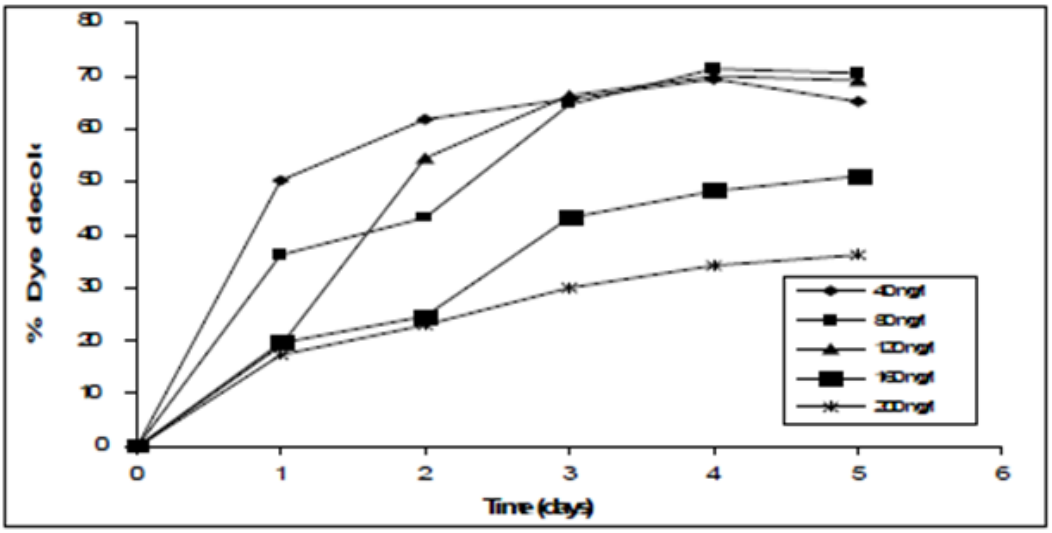

Figure 1. Effect of Methyl Orange dye concentration on decolorization performance of *Pseudomonas* spp. ETL-1982

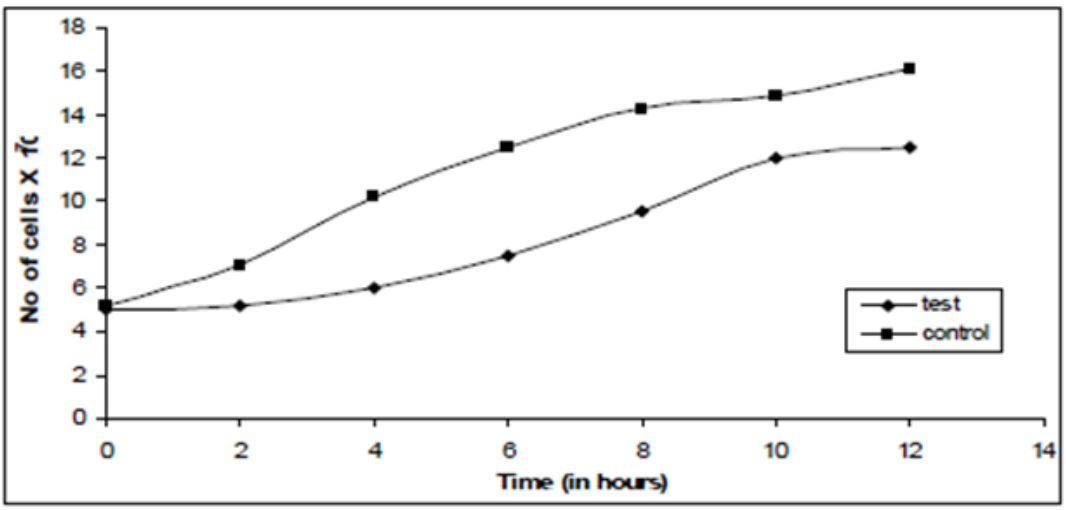

Figure 2. Effect of Methyl Orange dye on growth of *Pseudomonas* spp. ETL-1982

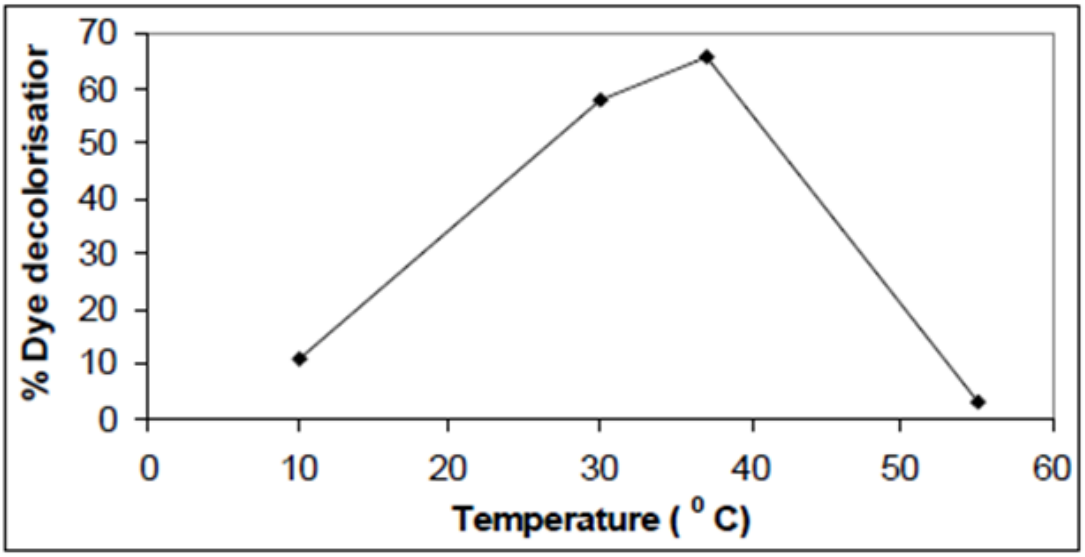

Figure 3. Effect of temperature on dye decolorization by *Pseudomonas* spp. ETL-1982

3. Results and Discussion

The textile industry is one of the industries that generate a high volume of waste water. Strong color of the textile waste water is the most serious problem of the textile waste effluent. The disposal of these wastes into receiving waters causes damage to the environment. Dyes may significantly affect photosynthetic activity in aquatic life because of reduced light penetration and may also be toxic to some aquatic life due to the presence of aromatics, metals, chlorides, etc. Synthetic dyes are extensively used in the textile and printing industries. Azo dyes are the most important group of synthetic colorants. These are the largest class of dyes, and more than half of the annually produced dyes. Dye waste water from textile or dye stuff industry is one of the most difficult to treat because dyes have various synthetic origin and they contain complex aromatic molecular structures, which make them more stable and more difficult to be degraded. The removal of dyes from the textile waste effluent has been carried out by physical & chemical methods, such as flocculation, membrane filtration, electrochemical techniques, ozonation, coagulation and adsorption. In recent years, a number of studies have focused on some microorganisms which are able to biodegrade and biosorb dyes in waste waters. A wide variety of microorganisms capable of decolorizing a wide range of dyes include some bacteria, fungi and algae. The use of microorganisms for the removal of synthetic dyes from industrial effluents offers considerable advantages. The process is relatively inexpensive, it is simple method and the running costs are low and the end products of complete mineralization are not toxic. The present study was designed to test decolorization of azo dye by bacteria isolated from textile effluent drainage site. For this purpose soil near to textile effluent outlet was collected & enrichment and isolation for azo dye decolorizing bacteria was carried out in screening medium containing azo dye. Microorganism showing maximum decolorization in less time was selected & using Bergy`s

Manual of Determinative Bacteriology (2000) was identified as *Pseudomonas* spp. ETL-1982. The physical properties (data not shown) and time course of methyl orange decolorization was studied at different initial concentrations (40-200 mg /L) in static cultures. Data in Figure 1 depict that at the lowest dye concentration (40-120 mg/L) the dye was decolorized more than 70 % after 4 days incubation. Similar data were reported by Yusef et al., 2008. 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, 34% decolorization was attained upon using 200 mg /L of the dye after 4 days. Bacterial growth in presence of methyl orange was also studied using control without dye, which showed the dye has inhibitory effect on growth of bacteria as number of bacteria were decreased in presence of methyl orange as compare with control without dye (Figure 2). The rate of chemical reaction is the direct function of temperature. Bacteria require optimum temperature for growth. Since dye decolorization is metabolic process hence shift in temperature from optimum results into decrease in dye decolorization as high temperature causes thermal inactivation of proteins & possibly of such cell structures such as membrane. The operating temperature of the incubation process varied between 10°C, 30°C, 37°C & 55°C, to study the effect of temperature on the decolorization process (Figure 3). At temperature below 37°C, due to slow growth of the bacteria, it took more days for decolorization and at temperatures above 37°C, the activity of *Pseudomonas* spp. ETL-1982 and hence percentage of decolorization decreases. The variation in pH of the growth medium results in change in activity of bacteria & hence the bacterial growth rate as well as decolorization. Bacteria are active over certain range of pH. The optimum pH for the growth is the same for the dye decolorizing activity as it is mainly the metabolic process. In contrast with other decolorizing microbes like fungi with narrow pH range, *Pseudomonas* spp. ETL-1982 cells proved to be of desirable characteristic, removing methyl orange color over a wide range of pH (6-10) with optimum at pH 7 (71.7% dye decolorization). Large decrease in decolorization occurs at high acidic pH (2-4)

(Figure 4). This is an advantage of this bacterium for developing a practical bioprocess in treating dyeing mill effluents. In case of fungi increase in pH greater than 5.5 resulted in the fragmentation of mycelia pellets & below 5.5 there is no appreciable growth of fungi hence percentage of decolorization decreases. Hence the bacteria are preferred over fungi for dye decolorization. The pattern of methyl orange decolorization in static as well as in shaked cultures was elucidated in medium. Figure 5, shows that lower decolorization percentages were exhibited in shaked cultures compared to static ones. Maximal efficiency of MO decolorization (84.47%) was achieved in 4 days incubated statically. These observations suggest that the decolorization performance of *Pseudomonas* spp. ETL-1982 was better in the presence of low oxygen content. The reason could be due to competition of abundant oxygen and the azo compounds for the reduced electron carriers under aerobic condition. Yusef et al., 2008, also reported that to achieve an effective color removal, agitation and vigorous aeration should be avoided. The cell growth in shaking condition was higher than static condition but there was less decolorization (68.61%) with more COD removal (40 %) under shaking condition, while 84.47 % decolorization with less COD removal (32.85%) under static condition within 4 days (Figure 6). These findings are consisted with result shown by Guven Ozdemir et al., who suggested COD removal is more under shaker condition. Addition of a carbon source such as glucose at different concentrations has an effect on the percentage of decolorization (Figure 7). The concentration of glucose was varied from 1% to 4 % and it was found that the percentage of decolorization increases with the increase in concentration of glucose due to decrease in lag period. The percentage decolorization decreases with the increase in concentration of peptone up to maximum peptone concentration of 1% (71.5% dye decolorization) and after which there is decrease in percentage of decolorization. The decrease decolorization results from nitrate or nitrite, a reducing equivalent that cells generated from peptone consumption. These metabolites of nitrate/nitrite may compete with the azo dye and result in less decolorization (Figure 8). The reduction of azo dyes depends on the presence and availability of a co-substrate because it acts as an electron donor for the azo dye reduction. The rate of azoreduction process also depends on the type of cosubstrate used and chemical structure of the azo dyes. Many different co-substrates were found to suite as electron donor, like glucose yeast extract & whey. Decolorization was found to be more in whey (85% in 4 days) when it was compared with the screening media supplemented with yeast extract peptone & NaCl (70%) (Figure 9). Hence one can use whey water as cheap growth medium for dye decolorization. Similar data was reported by Padmavathy et al., 2003**.** In addition to a complex mixture of dyes, the textile mill effluents often contain heavy metals which generally affect the uptake and metabolism of azo dyes. Results obtained in the presence of different heavy metals are shown in Figure 10. Data indicates that the process of color removal is significantly inhibited by the presence of Mercuric chloride (11%) & Potassium dichromate (12%) especially during the initial period (1-2 days) of the incubation. Marginal inhibition in color uptake is noticed in the presence of Silver Nitrate, Zinc Sulphate & Cadmium Chloride. Hence the bacteria are able to tolerate the toxic effect of Silver Nitrate, Zinc Sulphate & Cadmium Chloride to achieve decolorization. Slow rates of color uptake in the presence of Chromium & Mercury may be related to heavy metal inhibition of enzymes and metabolic pathways. Similar data were in presence of chelating agent 1% EDTA, drastic decrease in decolorization (Figure 11). It might be because of complete inhibition of laccase activity which is involved in decolorization. Hence the presence of EDTA in dye waste water greatly affects its removal. Also decolorization by pretreated *Pseudomonas* spp. ETL-1982 with 1% acetic acid, 1% NaOH and 1% Methanol was studied (Figure 11). 1% NaOH & 1% Methanol had little effect on decolorization where as 1% acetic acid decreases the decolorization at larger extent (25.6%). Dye decolorization was studied by the autoclaved cells of *Pseudomonas* spp. ETL-1982 and compared with that of live cells. Autoclaved cells decolorized 120 mg/L of methyl orange only by 47.5 % (Figure 11). The strain does not exhibit extra cellular decolorizing activity. Only intact cells were responsible for decolorization of the dyes. Decolorization by autoclaved cells indicates that dye decolorization is primarily due to adsorption. However nearly 25 % of the dye decolorization can be assumed to be due to degradation by the bacterial system which was confirmed by studying the growth kinetics of the bacterial cultures in minimal media containing no Nitrogen or Carbon source except for the dye. These results indicate that the bacteria could utilize the dye as the carbon and nitrogen source for its survival and multiplication (Figure 12). Growth & decolorization in presence of antibiotics were studied by adding Penicillin G & Chloramphenicol in 1.25 U/ml & 5 mg/L with respect to their MIC for the isolate (Figure 13). Penicillin G inhibits cell wall synthesis and Chloramphenicol inhibits protein synthesis. Cell growth impaired greatly in Chloramphenicol and cells grew in presence of Penicillin G. Figure 144 reveals that about 53.8% of dye removal was observed in the decolorization culture in the presence of Chloramphenicol & 76.69% in presence of Penicillin G. Thus, biodegradation in the decolorization process may result from the action of azo reductase inside the resting cell. Decolorization of the dye solution by bacteria could be due to adsorption to microbial cells or to biodegradation. In adsorption, examination of the absorption spectrum would reveal that all peaks decreased approximately in proportion to each other. If dye removal is attributed to biodegradation, either the major visible light absorbance peak would completely disappear or a new peak would appear. Dye adsorption would result in cell mats which are deeply colored because of adsorbed dyes, whereas those retaining their original colors are accompanied by the occurrence of biodegradation. Figure 15 displays the change of UV-visible spectra of Methyl orange, using the supernatant fluid of the culture at 0, 24, and 48 hrs decolorizing cultivation with *Pseudomonas* spp. ETL-1982. The absorbance peak at 440nm disappears after cultivation. The fed batch decolorization study was carried out to check the ability of isolate for the decolorization of repeated added dye. The *Pseudomonas* spp. ETL-1982 decolorized the repeated addition of methyl orange dye up to 5 cycles (each 24 h) with variable decolorization rate

(9-91%). In first cycle 91.85% decolorization occurred, 82.85% decolorization in second cycles & the percent decolorization goes on decreasing (up to 9% at 5th cycle) as the number of cycle increases (Figure 16). Our isolate also has the ability to decolorize following azo dyes viz. Methyl orange, Methyl red, Congo red & Tartrazine in consortium. Figure 17 & Figure 18, depict that it can decolorize these mixed dye up to 64 % in 5 days.

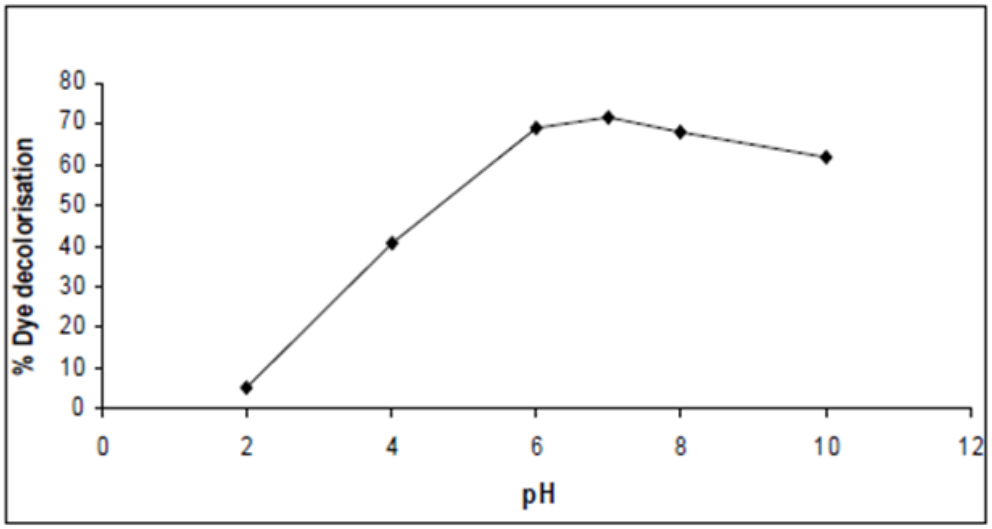

Figure 4. Effect of pH on dye decolorization by *Pseudomonas* spp. ETL-1982

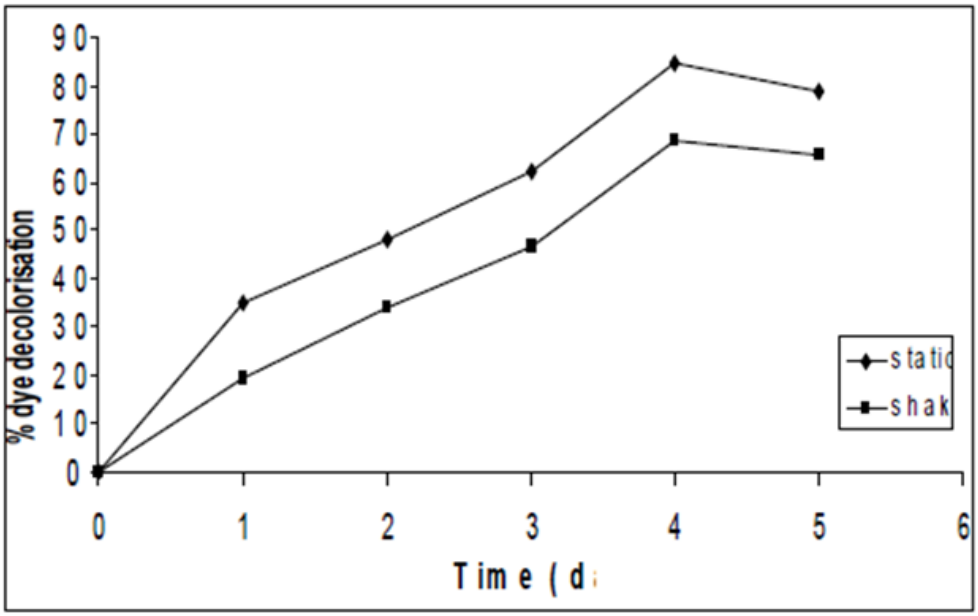

Figure 5. Dye decolorization by *Pseudomonas* spp. ETL-1982 under shaking and static condition

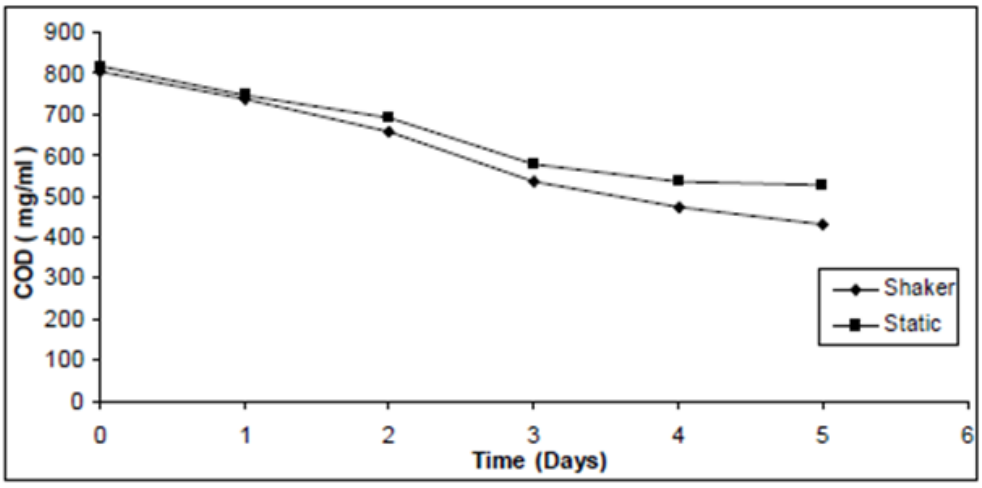

Figure 6. COD removal during dye decolorization by *Pseudomonas* spp. ETL-1982 under static and shaking condition

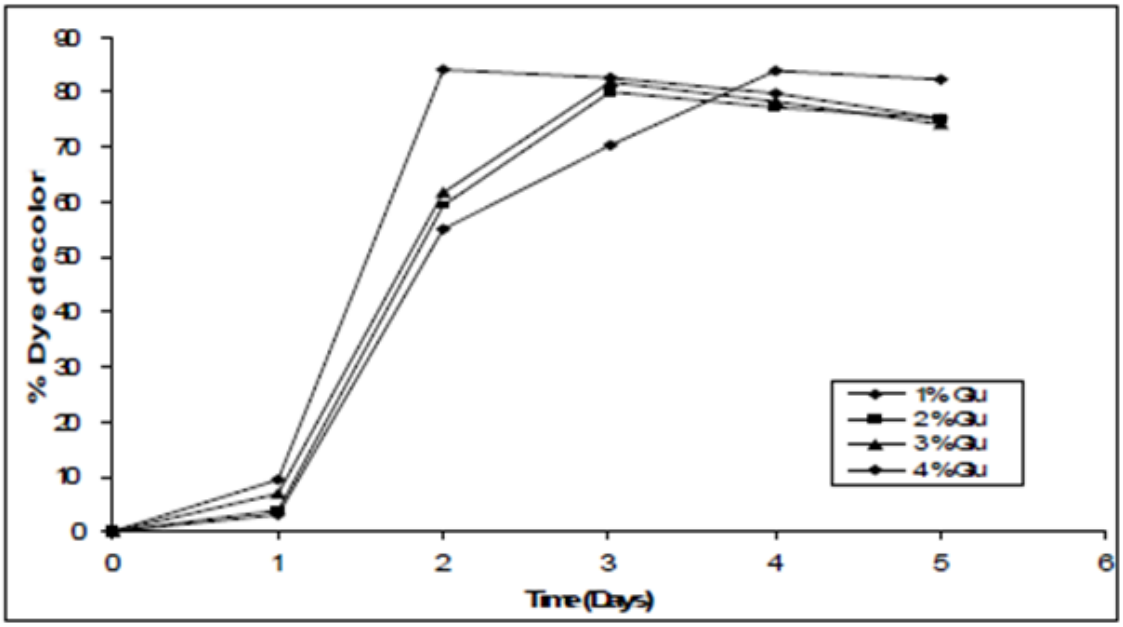

Figure 7. Effect of Glucose concentration of % dye decolorization by *Pseudomonas* spp. ETL-1982

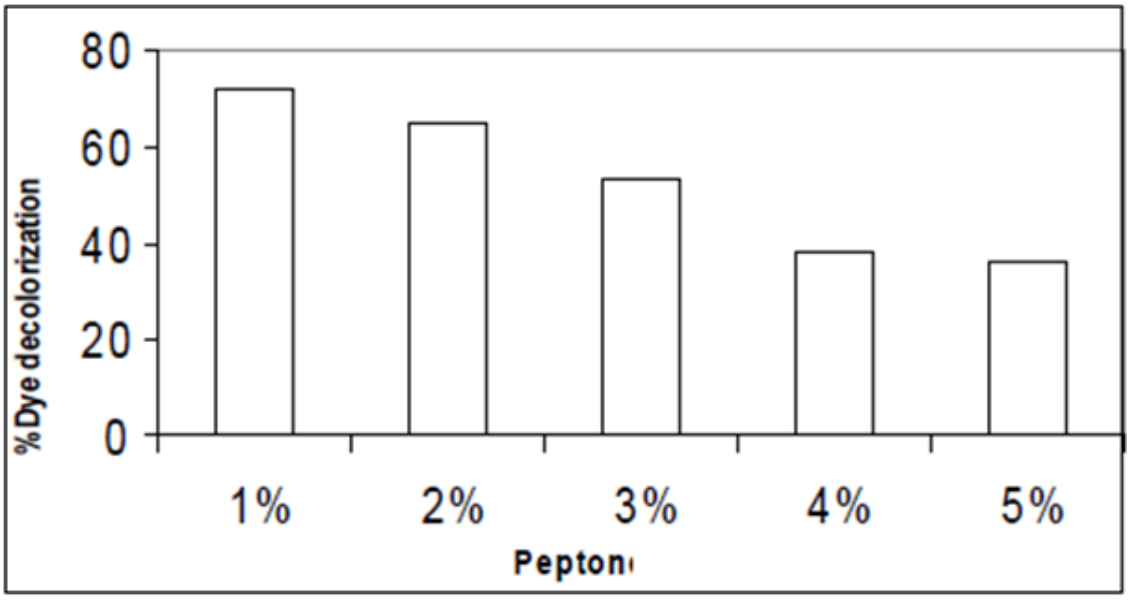

Figure 8. Effect of Peptone concentration of % dye decolorization by *Pseudomonas* spp. ETL-1982

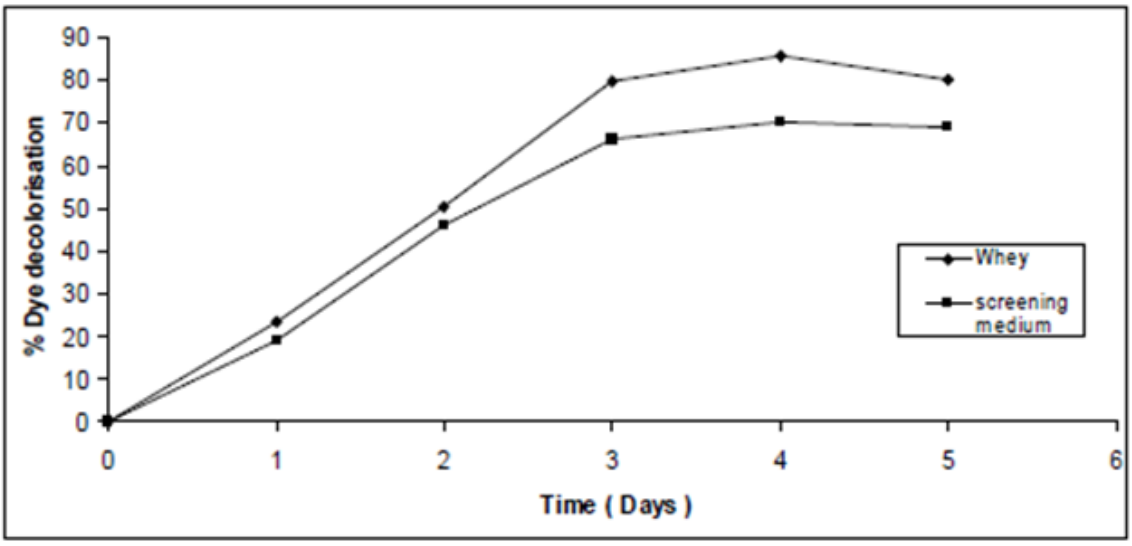

Figure 9. Dye decolorization by *Pseudomonas* spp. ETL-1982 in presence of whey as co-sustrate

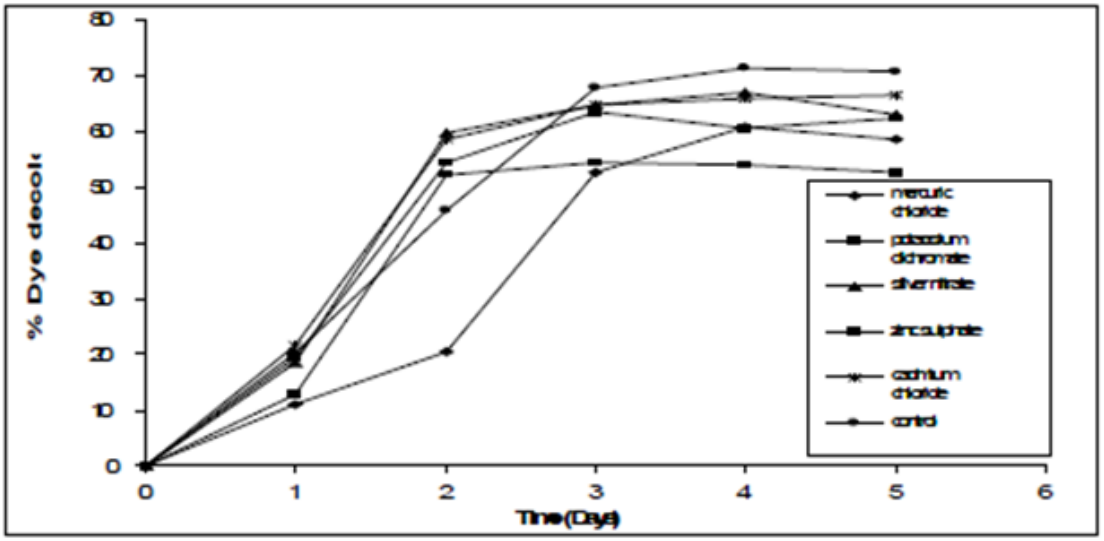

Figure 10. Influence of heavy metals on rate uptake of Methyl Orange during decolorization by *Pseudomonas* spp. ETL-1982

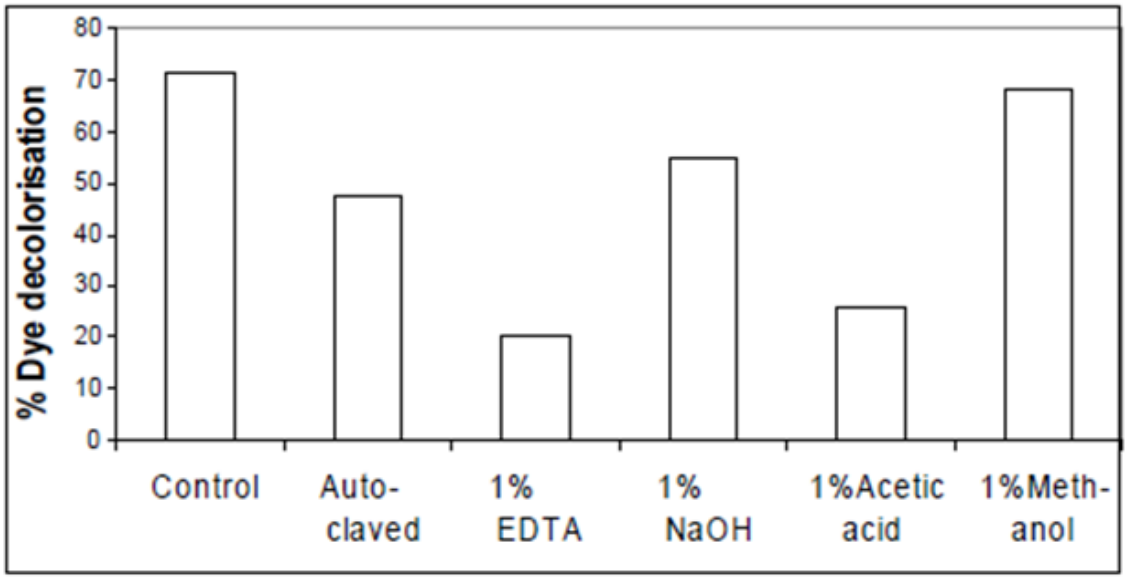

Figure 11. Dye decolorization by native and pretreated biomass of *Pseudomonas* spp. ETL-1982

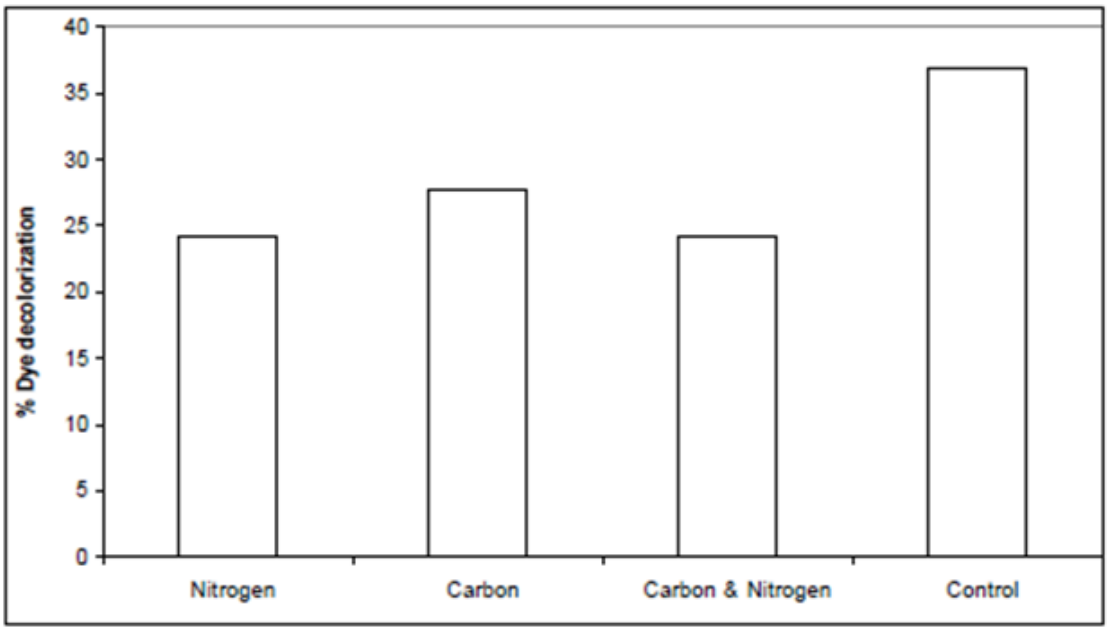

Figure 12. Effect of dye assimilation as carbon and nitrogen source by *Pseudomonas* spp. ETL-1982

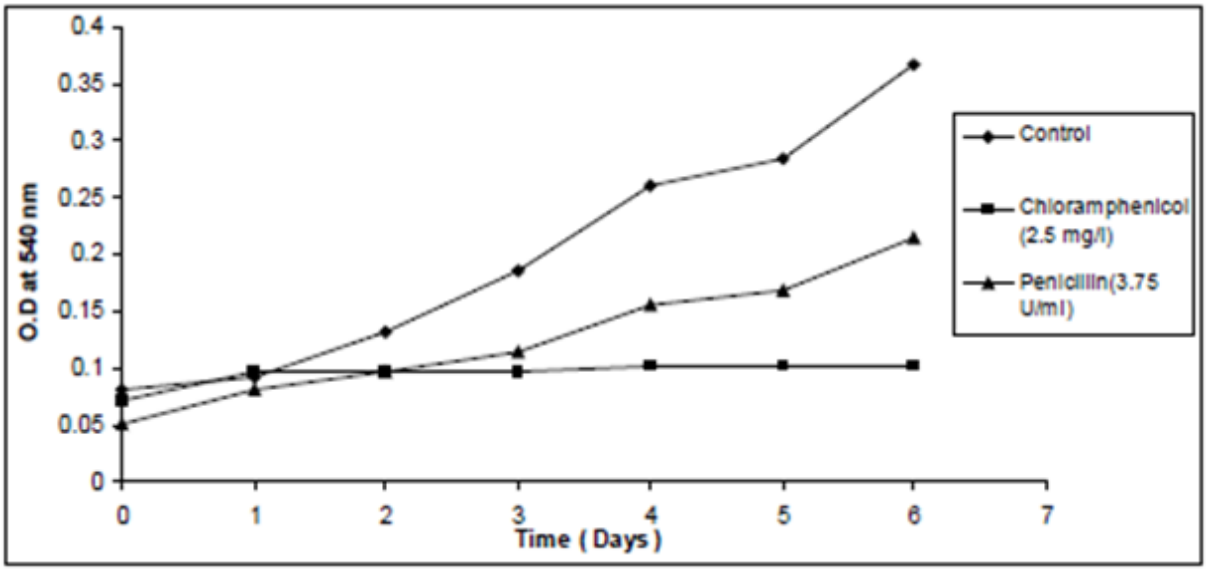

Figure 13. Effect of antibiotics on cell growth of *Pseudomonas* spp. ETL-1982 in screening medium

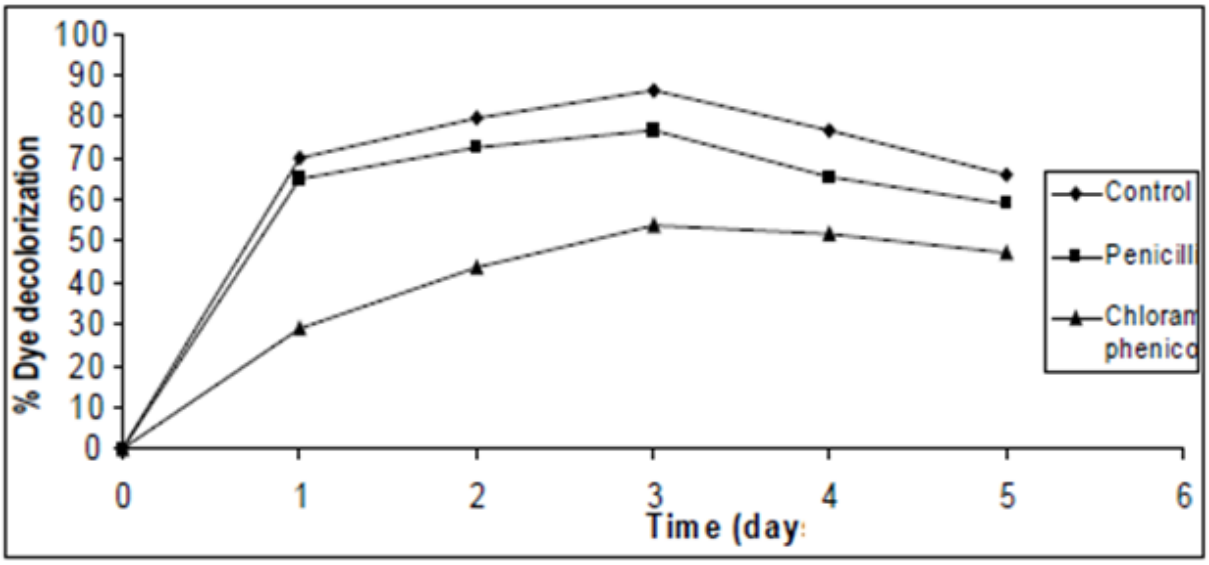

Figure 14. Effect of penicillin and chloramphenicol on dye decolorization by *Pseudomonas* spp. ETL-1982 in screening medium

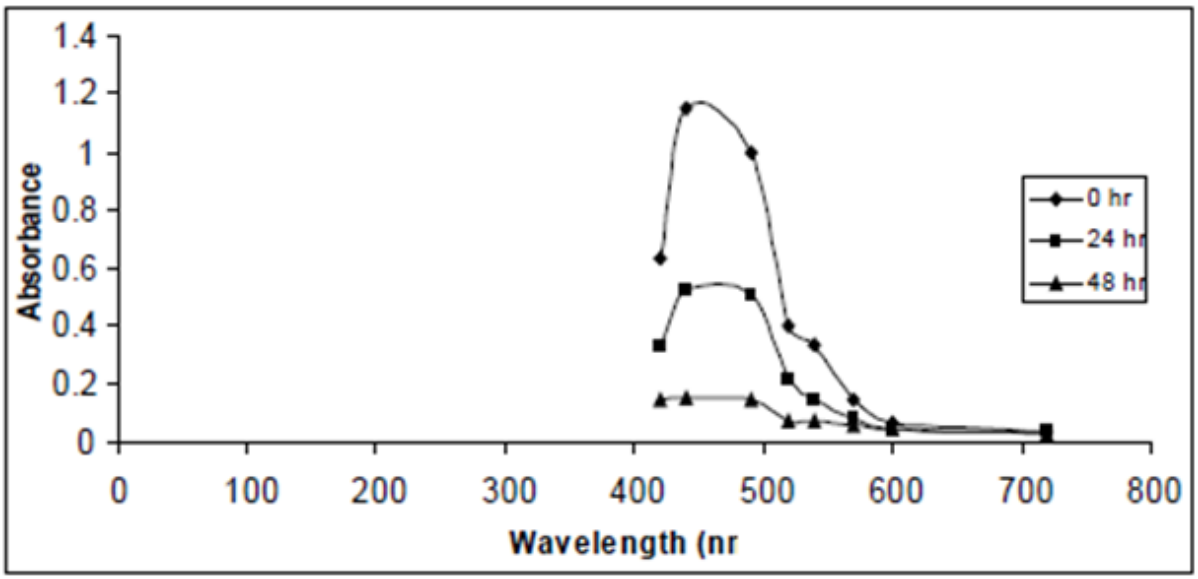

Figure 15. Spctrophotometric analysis of Methyl Oragne dye at 0hrs, 24hrs 7 48hrs decolorization by *Pseudomonas* spp. ETL-1982

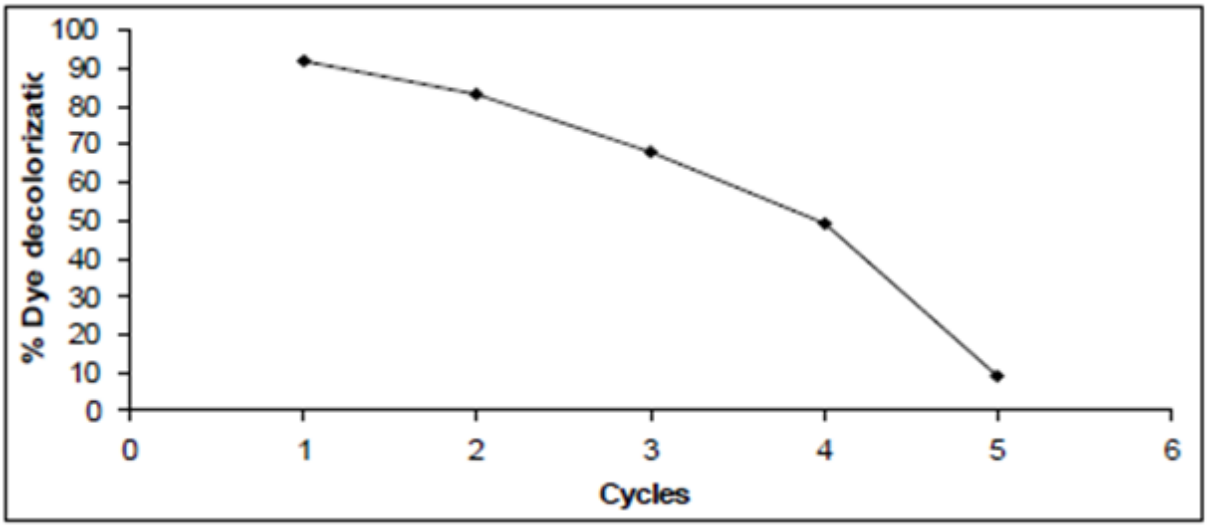

Figure 16. Fed batch declorizaton of Methyl Orange dye by *Pseudomonas* spp. ETL-1982

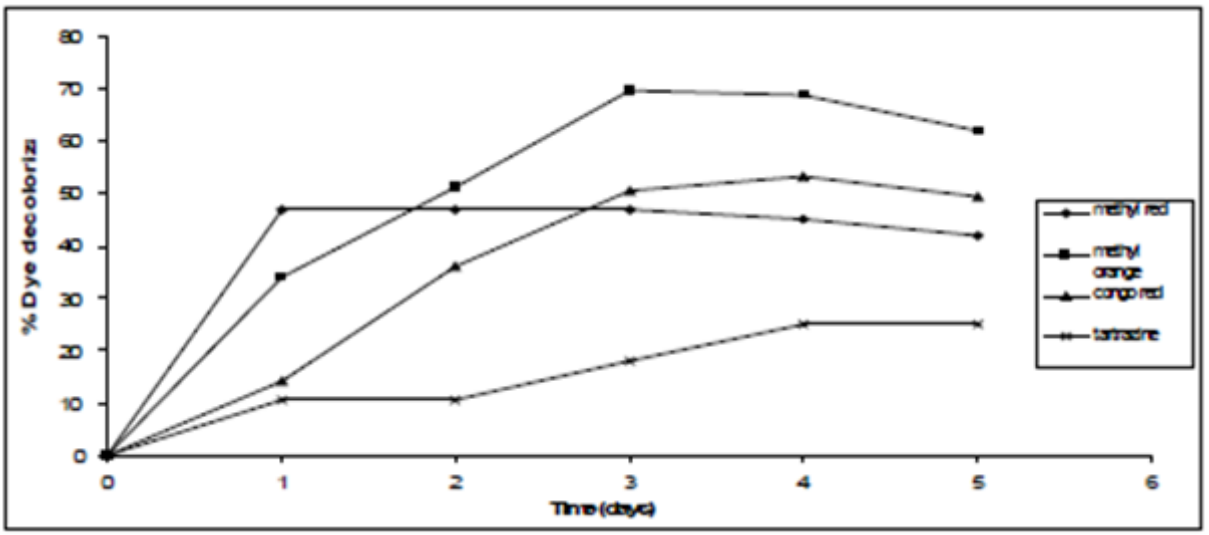

Figure 17. Decolorization of different azo dyes by *Pseudomonas* spp. ETL-1982

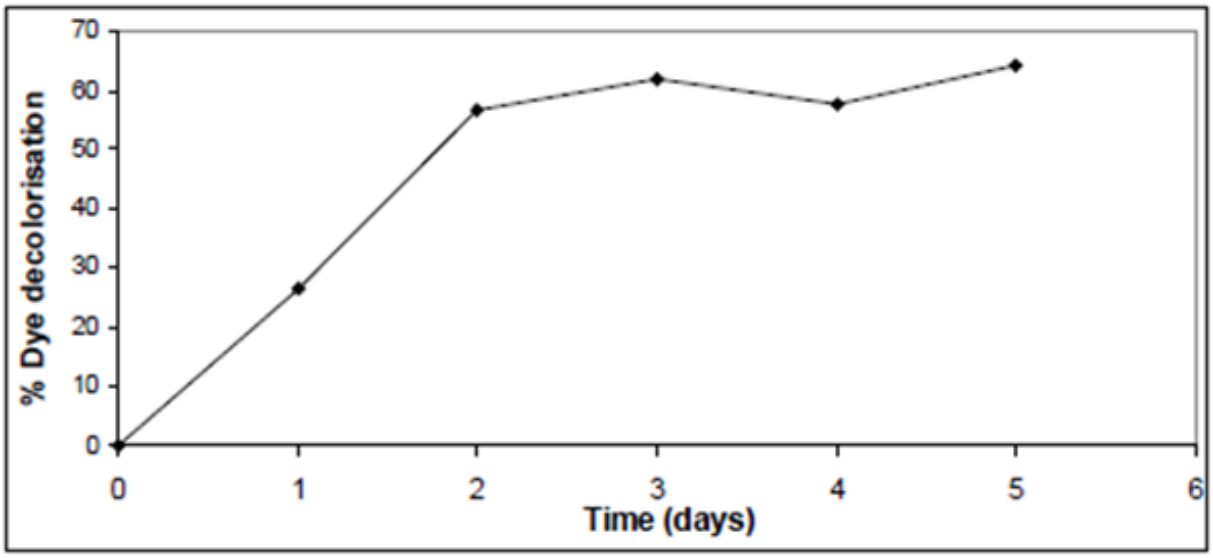

Figure 18. Decolorization of azo dyes in consortium (Methyl red, Methyl orange, Congo red and Tartrazine) by *Pseudomonas* spp. ETL-1982

4. Conclusion

A novel strain having a high capacity for rapid decolorization of Methyl orange as a azo dye was isolated from a site near to textile industrial outlet. The isolate was identified as *Pseudomonas* spp. ETL-1982. The effects of oxygen, pH, Temperatures, and dye concentration on the decolorization of methyl orange 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 Methyl orange dye by *Pseudomonas* spp. ETL-1982 is now in progress.

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