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## Research Article

# Isolation and Identification of Microorganism to Study of Their Potential to Degrade Harmful Azo Dyes by the Enzyme Azoreductase Produced by the Microorganism

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**Abstract:** Azo Dyes are used commonly in different industries. Through the biotransformation of dye compounds, various dye precursor and carcinogenic aromatic amines are generated which leads water pollution and affect phytoplankton's, water animals and human beings. Thus there is an aurgent need to remove these dyes from various industrial effluents. Enzymatic degradation of dyes through microorganisms was an effective and eco friendly process. In this study different microbes that produce dye degrading enzymes were isolated and identified as *B. Brevis*, *B. Pantothenticus* (Bacteria) and *A. Niger*, *Fusarium* (Fungus). *B. Brevis* and *B. Pantothenticus* showed degradation for methyl red at gm/l dye concentration and for congo red (when the concentration was decreased). Percentage dye degradation of *B. Pantothenticus* was 91.66 (For methyl red) and 75.37 (For congo red). Percentage dye degradation through A. Niger was 85.71 (For methyl red).The enzyme responsible for dye reduction was purified by Ion exchange chromatography. Enzyme assay was performed for the purified enzyme, it is found that the optimum pH and temperature was 7 and 40ºC respectively. Purity of enzyme was determined by SDS-PAGE and the m.wt. of enzyme was64 KD.
**Keywords:** Azo Dyes, Congo red, Methyl red.

## INTRODUCTION

Today, more than 100,000 commercial dyes are available in market and nearly one million tons per annum are produced, whereas 10% of dyes are released in environment and natural resources as dye stuff waste. This production is increased day by day to meet the needs of growing population, also increases the release of dye effluent. Congo red is a carcinogenic direct used for the coloration of paper products. It is recalcitrant and found in effluents of paper factories. Azo dyes represent the largest class of organic colorants listed in the color Index (i.e., 60-70% of the total). They make up the vast majority of the dyes discharged [1]. Azo -dye is a synthetic dye that has the azo group of two nitrogen atoms (N=N) connecting aromatic ring compounds. Azo dyes may be direct, acid, or basic. Direct dyes are relatively large molecules with high affinity especially for fibers.

**Fig-1:Dye effluent generated by Industries which pollute water bodies**

**A Dyes** is a natural or synthetic colored substance that has an affinity to the substrate to which it is being applied. Dyes are classified on the basis of their application and chemical structure. They are composed of a group of atoms responsible for the dye colour, called chromophores, as well as an electron withdrawing or donating substituents that cause or intensify the colour of the chromophores, called auxochromes [2]. The most important chromophores are -C=C-, -C=N-, -C=O, -N=N-, $-NO_2$ and -NO

groups.Acid dyes are anionic compounds that are mainly used for dyeing, nitrogen containing fabrics like wood, polyamide, silk, and modified aryl. Basic dyes are cationic compounds that are used for dyeing acid group containing fibers, usually synthetic fibers like modified polyaryl [1]. Congo Red is an azo dye with a structure 3, 3′-((biphenyl)-4,4′- diylbis(azo))-bis(4-amino-1-naphthalenesulphonicacid) disodium salt. It is intended primarily for the coloration of paper products, used in medicine (as a biological stain) and as an indicator since it turns from red-brown in basic medium to blue in acidic, used to color textile and wood pulp. It is a recalcitrant and act as potent carcinogen and mutagenic because of the presence of aromatic amine group.

**Azo dyes as a Recalcitrant**

Azo Dyes are synthetic organic colourants that contain azo compound (R-N=N-R') in which R and R' can be either Aryl or Alkyl group. They are considered as xenobiotic compounds that are very recalcitrant to biodegradation processes [3,4].

The stability and their xenobiotic nature of reactive azo dyes make them recalcitrant. There are thousands of synthetic azo dyes used in the textile, pharmaceutical, cosmetics and food industries, of which more than 500 contain potentially carcinogenic aromatic amines in their chemical formulation[5]. During the dyeing processes about 10–90% of the dye stuff do not bind to the fibres and therefore, released into the sewage treatment system or the environment [3]. Aromatic amines, such as benzidine, induce urinary bladder cancer in humans and tumours in some experimental animals[5]. Azo dyes used in this study for their Degradation through enzymes produced by microbes are Methyl red & Congo red

**Role of Microbes in Azo dye degradation :**

Microorganism like bacteria, fungi have ability to degrade these recalcitrant compound and they have potential to degrade azo dyes under favorable condition. Lack of suitable condition, microorganism not able to degrade or mineralize these compounds as a result these compounds can persist in the environment and known as Recalcitrant. Degradation of recalcitrant compound can be achived by physical/chemical or biological methods.

**Mechanism of Reaction catalyzed by azoreductase:**

Azoreductase catalyze the reaction by Ping Pong Mechanism by using 2 equivalents of NAD(P)H to reduce one equivalent of the azo compound substrate. For example :- Reduction of one methyl red into two equivalent of Aniline.

$Ar–N=N–Ar' + 2(NAD(P)H + H^+) \rightleftharpoons Ar–NH_2 + NH_2–Ar' + 2NAD(P)^+$

Where, Ar = *p*-dimethylaniline
Ar' = *o*-benzoic acid.

It is the key enzyme expressed in azodye-degrading bacteria that catalyses the reductive cleavage of the azo bond. Azoreductases reduce the azo bond (N=N) in azo dyes to produce colorless amine products[17]. Azoreductase activity has been identified in several species of bacteria recently; such as *Caulobacter subvibrioides* C7-D, *Xenophilus azovorans* KF46F, *Pigmentiphaga kullae* K24, *Enterobacter agglomerans* and *Enterococcus faecalis* [6]. Azo reductases catalyze the reaction only in presence of reducing equivalents like FADH and NADH. Most of the azo dyes have sulphonate substituent groups and a high molecular weight and they are unlikely to pass through cell membranes. Therefore, the reducing activity referred to the dye is not dependant on the intracellular uptake of the dye [7]. Russ *et al.*[8] suggested that bacterial membranes are almost impermeable to flavin containing cofactors and, therefore, restrict the transfer of reduction equivalents by flavins from the cytoplasm to the sulphonated azo dyes. Thus, a mechanism other than reduction by reduced flavins formed by cytoplasmic flavin-dependent azoreductases must be responsible for sulphonated azo dye reduction in bacterial cells with intact cell membranes [8]. One such mechanism involves the electron transport-linked reduction of azo dyes in the extra-cellular environment. To achieve this, the bacteria must establish a link between their intracellular electron transport systems and the high molecular weight, azo dye molecules.

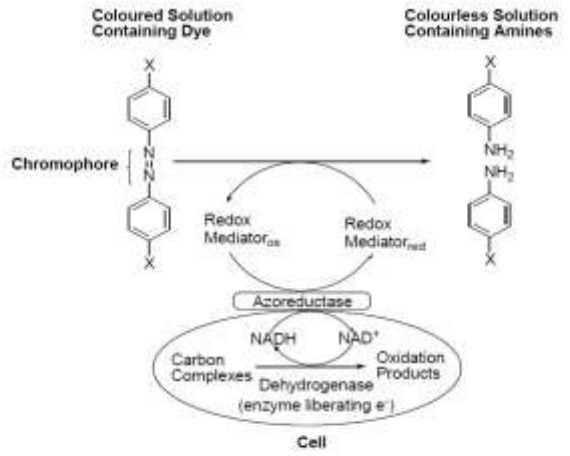



**Fig-4: Proposed mechanism for reduction of azo dyes by azo reductase (Courtesy: Keck *et al.*, 1997*)*.**

**OBJECTIVE**

- Isolation of bacteria.
- Screening to check their potential to degrade azo-dye.
- To calculate the Percentage dye degradation by the screened bacterial and fungal species.
- To study the activity of enzyme at different kinetic parameters.
- To purify and characterize the Azoreductase enzyme.

## MATERIAL AND METHODS

### MATERIALS

**Reagents:**

70% Alcohol**,** Crystal violet, Grams iodine, Ethanol, Safranin, Lacto phenol cotton blue, NaOH( 0.1 N),HCL ( 0.1 N ), Hydrogen peroxide, Malachite green, VP Reagents, Phenol red, 2%$Na_2co_3$, 1% Potassium tartrate, 0.5% $cuso_4$, BSA Standard (200µg/ml), Folin Ciocalteau reagent, Acrylamide 30%, Bis-Acrylamide 0.8%, Tris HCL 1.875 M, Tris HCL 0.6M, APS 5%,TEMED, SDS 5%

**Chemicals**

Ammonium sulphate, Methyl red, Congo red, Cellulose, NADH, Arabinoseis, Tris HCl.

### METHODS

- **Collection of water samples:** Water sample was collect from Dye contaminated area in sterile Plastic bottle. Sample was stored in deep freezer at -4ºC.
- **Dyes:** Two commercially available dyes are used i.e. Congo red and Methyl red.
- **Stock Dye solution**: Stock solution of each dye was prepared by adding 1gm of dye in 1000 ml of sterilize D.W. Those stock solutions were used for inoculating the medium and the conc. of stock dye solution in the medium was 10 ml/l.

**Isolation of dye degrading bacteria and fungus:**

**Serial dilution**

**Microbes were isolated through serial dilution method**

Dye decolorizing Bacteria and fungi were isolated from textile dye effluent by serial dilution ranging from $10^{-1}$ to $10^{-6}$ dilution for bacteria and $10^{-1}$ to $10^{-4}$ dilution for fungus. After dilution 100 µl diluted sample were pellet on NAM media for bacterial isolation and in SDA media for fungal isolation by the spared plate method. The inoculated medium for bacteria was incubated in incubator at $37^0$C for 24 hrs, and for fungus medium was incubated at room temperature for 4 to 5 days. The bacterial pure culture was obtained by re-streaking different individual bacterial colony onto new agar medium and incubated at 37 °C. Pure culture of fungus was obtained by point inoculation of fungus into SDA media.

**Identification**

**Identification of bacteria**

All the Bacterial isolates were identified on the basis of colony morphology, Grams staining and biochemical test according to Bergey's Manual of Systematic Bacteriology.

**For Microscopic identification of Bacteria, Grams staining and Endospore Staining was performed.**

**BIOCHEMICAL TEST**

**For Bacterial identification following biochemical test was done.**

- CATALASE TEST
- STARCH HYDROLYSIS TEST
- CARBOHYDRATE FERMENTATION TEST
- MR/VP TEST
- CITRATE TEST
- MOTILITY TEST
- ARABINOSE TEST

**Identification of fungus**

Fungal species was identified by external appearance (i.e. color, shape) and by the staining with lactophenol cotton blue stain. Morphology was observed under microscope.

**Screening**

Selected isolates must be screened to know the ability of producing enzyme that are responsible for the dye degradation. For this, nutrient broth was used for screening of bacterial isolates and SDA broth for fungus.

**Inoculation of medium and dye for the screening**

10 ml of broth containing 100 µl of stock dye solution of methyl red was poured in 3 test tubes and then it was inoculated by a loop full culture of 3 different bacterial isolates and kept it in incubator at $37^o$ C for 1 week. Same method was applied for remaining dyes, bacterial and fungal isolates. Fungal inoculated medium were kept at room temp.

**Monitoring for Decolourization**

Spectrophotometric method was used to monitor the optical density of the decolorization. The standard isolates which gave instance decolorization of azo dyes was inoculated in 50 ml mineral salts basal media in which 500 µl of dye solution has been added after autoclaving. A control flask was also maintained. After inoculation 5 ml of the sample was taken out in sterile condition and centrifuged at 6000 rpm for 10 min. Supernatant was taken and its optical density was determined spectrophotometrically at 620 nm the absorbance maxima of azo dye methyl red, evan's blue and 500 nm of congo red being studied for decolourization. Thereafter the percent of dye decolourization on zero day was calculated by the formula [9].

% Dye decolorization =

$$\frac{O.D_{\text{zero day}} - O.D_{\text{sample}} \times 100}{O.D \text{ zero day}}$$

The inoculated media was then incubated in shaking incubator at 150 rpm. Percentage dye degradation was calculated every alternate day for seven days and the above described method and formula was used every time.

**Production of Enzyme**

For the assay of enzyme and their purification it is necessary to produce large quantity of enzyme.

**Production of bacterial enzyme**

Bacterial enzyme was produced in Large quantity by the inoculation in the production media containing limited nitrogen source after which azo dye was the only source of nitrogen. For bacteria, Production media made by adding per liter of D.W., Potassium Dihydrogen Phosphate 3.0 g, Disodium Hydrogen Phosphate- 6.0 g, Glucose- 2.0 g, Sodium Nitrate- 0.42g, NaCl- 100g, Dyes (stock) – 10 ml and the pH was 7.0. The pH of the medium was adjusted using 0.1 M HCl and NaOH. 50 ml production media was prepared by adding 500 µl of stock dye solution for each dye. Then it was inoculated with 1ml of bacterial nutrient broth to get the enzyme in large amount. The inoculated medium in PTC bottle was kept in the incubator for 10 days

**Production of fungal enzyme**

For the large production of fungal enzyme SDA broth production media was used and the pH 5.6 was adjusted. Further process was as same as bacterial enzyme production instead of the incubation. Bottle was kept at room temperature.

**Extraction of Enzyme**

The enzyme was extracted by centrifugation. The broth was taken at the end of incubation time and was centrifuged at 6000 rpm for 15 min. The supernatant was taken and was treated as crude extract.

**Enzyme Assay**

For assay of crude and purified enzyme, firstly the following materials are prepared for the azoreductase assay:-

- Potassium phosphate buffer 50mM (Reagent A).
- Azo dye solution 0.1%, (Reagent B)
- Enzyme solution (Reagent C)
- Reduced nicotinamide adenine dinucleotide sodium salt monohydrate (NADH) 2 mM (Reagent D).

The assay procedure is based on the principle that with the addition of NADH to the reaction mixture containing substrate, buffer and enzyme solution, the substrate azo dyes azo bond is degraded and there is a decrease in the absorbance of the dye after an initial lag phase.

**Unit Definition:**

One unit will reduce 1.0 µ mole of azo dye per minute in the presence of NADH using Millimolar extinction coefficient of azo dyes at pH 7.0 and 30°C temperature.

**Calculation**

Units/ml enzyme =

$$\frac{(AA\ 532nm / min\ Test - AA532nm / min\ Blank)\ (3)\ (df)}{A \times (0.1)}$$

3 = Total volume (in milliliters) of assay
df = Dilution factor
A = Millimolar extinction coefficient of azo dye
Methyl red = 23.36 mM-1 cm-1)
Congo red = 21.20 mM-1 cm-1)
0.1 = Volume (in milliliter) of enzyme used

**Procedure**:- The procedure adopted was based on the one done by Zimmermann etal. [15].

1. Pipetted (in milliliters) the following reagents into test tubes:

| | Test | Blank |
|---|---|---|
| Reagent A (Buffer) | 2.80 | 2.80 |
| Reagent B (Azo dye solution) | 0.05 | 0.05 |
| Reagent C (Enzyme solution) | 0.1 | 0.1 |
| (Distilled water) | | |
| Mixed by inversion and equilibrate at room temperature. Then add: | | |
| Reagent D (NADH) | 0.05 | 0.05 |

2. Immediately mixed by inversion and recorded the decrease in absorbance at 532 nm for approximately 5 min. obtained the ÄA 532nm/ minute for both the Test and Blank.
3. Enzyme activity was than calculated by the formula given above.

**Purification of enzyme**

- **Ammonium salt precipitation**

**Protocol**

- 5.6 gm. Ammonium sulphate was added in 10 ml ml. of crude sample, stirrer slowly. Process was carried out at cooled condition for 45 min.
- Then sample was kept in overnight at 4`c.
- Sample was centrifuged at 10,000rpm for 10 min.
- Pellet was collected and dissolved in 10 ml of 50 mM phosphate buffer.

**Dialysis**

**Activation of Dialysis membrane**

- Took 100 ml of D.W. And boiled it.
- Dialysis membrane was added into boiling water.
- Boiled it for 10 min.
- Added 2% $NaHCO_3$, boiled it for 10 min.
- Take another 100 ml of D.W. and boiled it.
- Transferred the dialysis membrane into this boiling water and boiled it for 10 min.

- Took out the membrane with the help of forceps.
- Tied the one side of the membrane.
- 10 ml of enzyme suspension was added in to the dialysis membrane.
- Tied another side of the membrane.
- Placed it in beaker contained phosphate buffer.
- Placed the beaker in refrigerator.
- Dialysis membrane placed in another phosphate buffer for 2-3 times.

**Ion exchange chromatography**

**Column preparation:-**

- 2 gm of cellulose weighed and it dissolved in 50 ml of phosphate buffer pH-7, then poured in column and equilibrated with phosphate buffer, checked the pH 7.
- After the column preparation the dialyzed sample was poured onto the column from sides without disturbing the DEAE bed, kept for 45 min.
- Enzyme was eluted using the first eluting buffer i.e. 25 mM Tris HCl and 25 mM NaCl. Elute was collected in test tube.
- The same process of elution was carried out using 50, 75, 100,125 and 150 mM Nacl respectively.

**Protein estimation (By Lowry method)**

**Protocol**

- Prepared the 2%$Na_2CO_3$ in 0.1N NaOH (Reagent A) and 0.5% copper sulphat in 1% Potassium sodium tartrate (reagent B)
- After that prepared the Alkaline copper solution by mixing 50 ml of reagent A and 1ml of reagent B prior to use as reagent C. FC reagent was prepared by adding 1ml of FC reagent in 4ml of D.W.
- BSA sample was prepared by adding BSA in D.W. and the concentration of BSA was 200µg/ml. Poured 0.2, 0.4, 0.6, 0.8 and 1.0 ml BSA sample into 5 test tube and labeled them as Standard 1 2, 3, 4, 5 respectively.
- In other test tube 0.2 ml sample of each crude, ammonium salt precipitation crude, dialysis crude and elutes of I.E.C (which shows the higher enzyme activity) was poured.
- All the sample were makeup up to 1ml by distilled water. A blank test tube also maintained in which 1ml distilled water was poured instead of any sample.
- 5-5ml of Alkaline copper solution was added in each test tube including blank and incubated at room temperature for 10 min.
- After incubation 0.5 ml FC reagent was added in all the test tubes and let them to incubate at dark for 30 min.
- After incubation, OD was taken at 660 nm against blank.

**Enzyme Kinetics:-**

The main factor which influence the rate of reactions include:

- The physical state of reactants.
- The concentration of reactants/substrate.
- Effect of temperature or the temperature at which the reaction occurs: As the temperature rises, molecular motion -and hence collisions between enzyme and substrate – speed up. There is an optimum temperature at which enzymes are highly active and after optimum temperature there is an upper limit beyond which the enzyme becomes denatured and ineffective.

To study the effect of temperature four different temperature i.e. 4ºC, 10ºC, 40ºC, 60ºC was selected and the process was carried out as same as the enzyme assay but at different temperature.

Effect of pH: The conformation of a protein is influenced by pH . All enzymes have optimum pH in which they show higher activity. Enzyme activity increases with increase pH, but after optimum pH, enzyme activity decreases with increase pH. In this study enzyme activity at five different pH i.e. pH 3,5,7,9 and has been studied. For this Phosphate buffer of particular pH was prepared and the further process was same as enzyme assay.

**SDS-PAGE**

Extracted enzyme was purified by SDS-PAGE. A very common method for separating proteins by electrophoresis uses a discontinuous polyacrylamide gel as a support medium and sodium dodecyl sulfate (SDS) to denature the proteins. The method is called sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

**Observation and Result**

**Sample collection:-**

Mallapur Industrial Area was selected for the sample collection because it has textile industries and nearby the dye contaminated effluent was present. So the effluent sample was collected from such contaminated sites.

**Isolation of microorganism**

**Serial dilution**

Dye decolorizing Bacteria and fungi were isolated from textile dye effluent by serial dilution ranging from $10^{-1}$ to $10^{-6}$ dilution for bacteria and $10^{-1}$ to $10^{-4}$ dilution for fungus. After the incubation period of serially diluted sample, 3 different bacterial colonies (figure 6) which was named as $S_1$ $S_2$ $S_3$ and 2 fungal species (figure 5) was obtained.

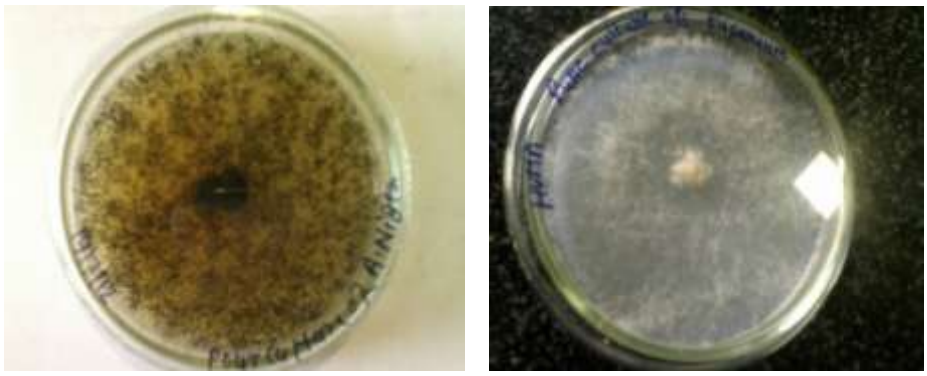


**Fig-5: Isolated fungal species**

**Fig-6: Isolated Bacterial colonies.**

**Identification of microorganism:**

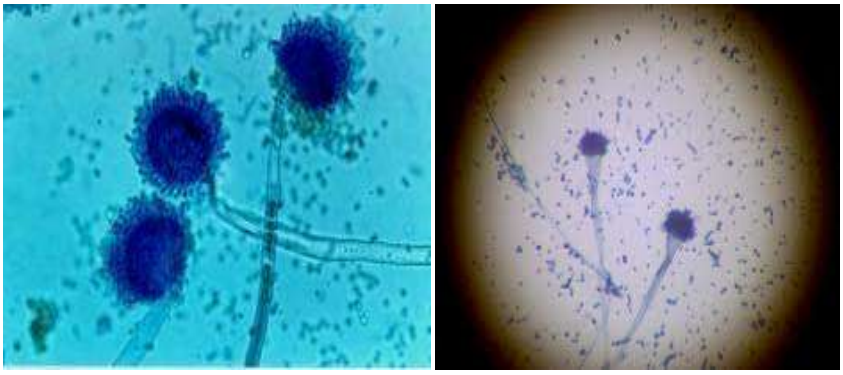
**Identification of fungus:**

**i) Morphological characteristic**

In two fungal colonies, one is dense small black colony and Large visible hyphae was clearly observed. Another fungal colony is long wooly, white to pink.(figure 8).

**Microscopic characterization after lactophenol cotton blue staining**

Conidia, conidiophores and mycelium was observed for black fungal colonies under 45x magnification thus it was confirmed as *A.niger(*figure 10*)*.



A.niger Fusarium

**Fig-7: Microscopic characterization of identified fungal colonies**

**Identification of bacteria**

- Colony morphology- According to colony morphology, all the bacterial isolates were circular in shape, all of them having flat elevation, smooth texture, and their appearance was cream & shiny. They were differ in their size and margin (table 1)

**Table-1: Colony morphology of bacterial isolates**

| Character | $S_1$ | $S_2$ | $S_3$ |
|---|---|---|---|
| Shape | Circular | Circular | Circular |
| Margin | Entire | Curled | Entire |
| Elevation | Flat | Flat | Flat |
| Size | Punctiform | Small | Moderate |
| Texture | Smooth | Smooth | Smooth |
| Appearance | Cream & Shiny | Cream & Shiny | Cream & Shiny |

**Gram staining**

All the bacterial cells retain the crystal violet stain and appeared in purple colour and rod shaped thus all the bacteria were gram positive and bacillus species (table 2.)

**Table-2: Grams staining of bacterial isolates**

| Character | $S_1$ | $S_2$ | $S_3$ |
|---|---|---|---|
| Gram's reaction | Positive | Positive | Positive |
| Shape | Rod | Coco bacillus | Rod |

**Screening**

**Screening of methyl red**

In the screening of methyl red it was found that bacterial species $S_2$ was showed the intense decolorization just after 1 day and reduction of methyl red was clearly observed as compare to other microbes. After 7 days, bacterial species S1, S2 and fungal species *fusarium* and *niger* was showed intense decolourization for methyl red degradation (figure 8&9).

**Screening of congo red**

After 7 days all the bacterial and fungal species were unable to degrade congo red dye at gm/l dye concentration (figure10).

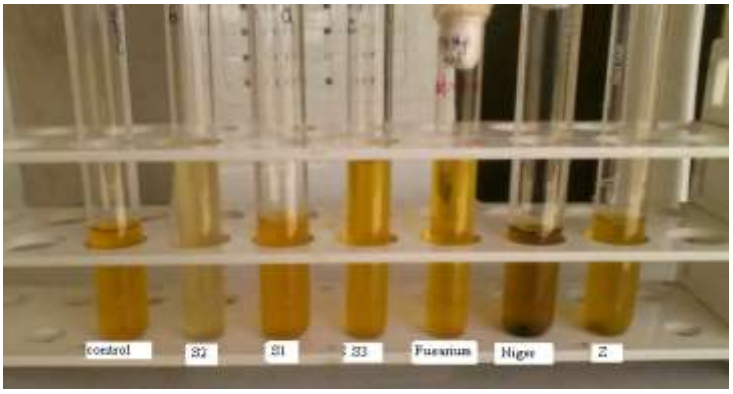



**Fig-8:Screening for methyl red dye degradation. results after 1 day.**

**Fig-9:Screening for methyl red dye degradation, results after 7 days.**

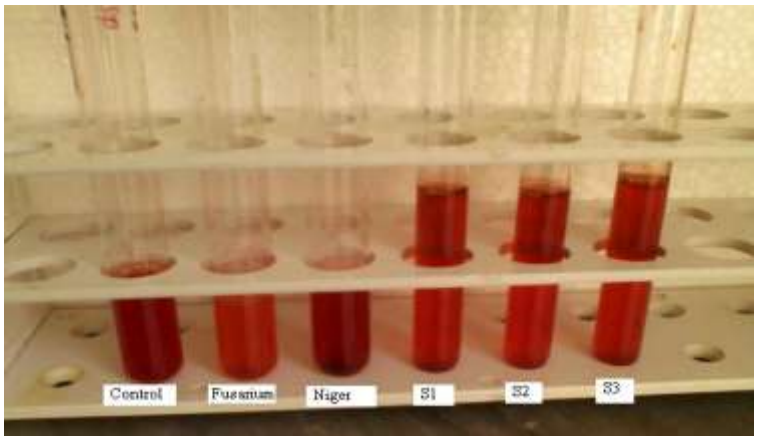



**Fig-10: Screening for Congo red dye degradation, results after 7 days.**

**Screening of congo red with decreased dye concentration**

As shown in figure 15 all the microbes were unable to degrade congo red at 1g/l dye concentration so when the dye concentration of congo red was reduced that was just half of the previous concentration, intense decolorization were showed by $S_1$ $S_2$ within 7 days.

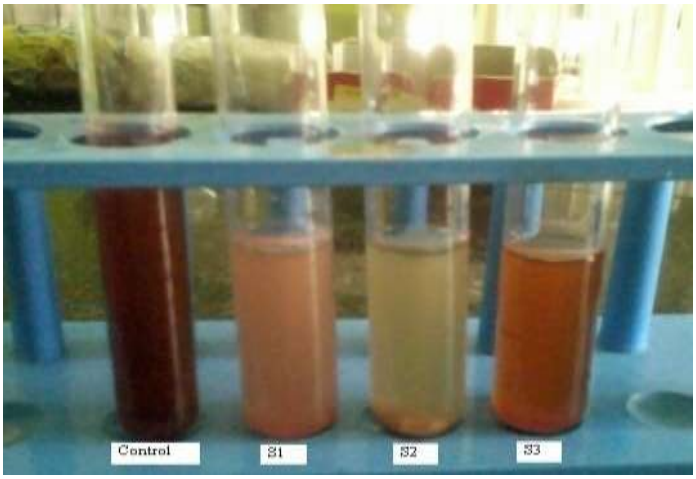



**Fig-11: Screening for congo red dye degradation with decreased dye concentration, results after 7 days.**

**Decolorization of congo – red dye:**

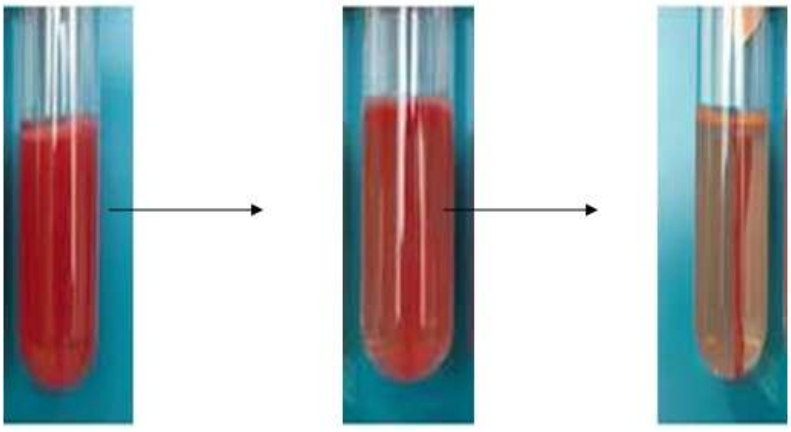


**Fig.12: Degradation of congo red with increased day of incubation.**

**Selection of organism**

During or after the incubation period all the test tubes were taken and compare with control. Dye decolourization was ranked on the basis of visual identification as Intense (+++), Moderate (++), Slight (+), No decolourization (-), *The A.niger, Fusarium* and $S_1$ $S_2$ organisms containing test tubes showing intense or moderate decolourization were selected. Thus after the screening of all the dyes, $S_1$ $S_2$ was selected for their identification.

**Biochemical test of $S_1$ $S_2$ bacteria**

According to Bergey's Manual different biochemical test was performed and both the species were identified (table 3).

**Table-3. Biochemical test of bacterial isolates**

| Test | $S_1$ | $S_2$ |
|---|---|---|
| Catalase test | + | + |
| Starch hydrolysis test | + | + |
| VP test | - | - |
| Endospores test | + | + |
| Glucose fermentation | - | + |
| 6.5 % NaCl growth | + | - |
| Motility test | Motile | Non motile |
| Citrate test | + | - |
| Acid via Arabinose | - | - |
| MR test | - | Not performed |
| Identified species | *B. brevis* | *B. pantothenticus* |

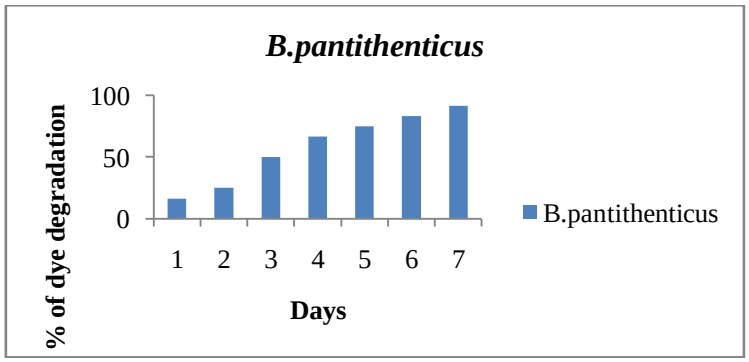
**Degradability Assay**

**For Methyl red**

Bacteria *B. Pantothenticus* and fungus *A.niger* amd *Fusarium* was selected for degradability assay on the basis of instance decolorization. *B. Pantothenticus* shows 91.66% degradation of methyl red within 7 days and the degradation percentage was increased after day by day (Table 4). *A.niger* degraded 92.85% and fusarium degraded 85.71% of methyl red within 7 days (Table 5).*Fusarium* shows intense decolorization of methyl red as compare to other micro organism.

**Table-4: The degradability assay for the dye Methyl red through *B. pantothenticus***

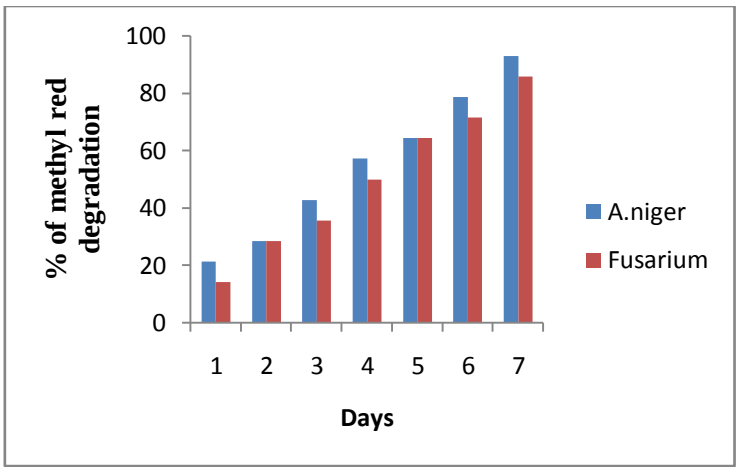
| Day | O.D ( At 620 nm ) | | %Dye degradation of sample |
|---|---|---|---|
| | Blank | Sample | |
| 0 | 0.12 | 0.12 | 0.00 |
| 1 | 0.12 | 0.10 | 16 |
| 2 | 0.12 | 0.09 | 25 |
| 3 | 0.12 | 0.06 | 50 |
| 4 | 0.12 | 0.04 | 66.66 |
| 5 | 0.12 | 0.03 | 75 |
| 6 | 0.12 | 0.02 | 83.33 |
| 7 | 0.12 | 0.01 | 91.66 |




**Graph-1: Percentage of methyl red degradation against days through *B.pantothenticus***

**Table-5: The degradability assay for the dye Methyl red through *A. niger and Fusarium***

| Day | O.D ( At 620 nm ) | | | | |
|---|---|---|---|---|---|
| | Blank | Sample | | | |
| | | *Niger* | %Dye degradation of sample | *Fusarium* | %Dye degradation of sample |
| 0 | 0.14 | 0.14 | 0.00 | 0.14 | 0.00 |
| 1 | 0.14 | 0.11 | 21.42 | 0.12 | 14.28 |
| 2 | 0.14 | 0.10 | 28.57 | 0.10 | 28.57 |
| 3 | 0.14 | 0.08 | 42.85 | 0.09 | 35.71 |
| 4 | 0.14 | 0.06 | 57.14 | 0.07 | 50 |
| 5 | 0.14 | 0.05 | 64.28 | 0.05 | 64.28 |
| 6 | 0.14 | 0.03 | 78.57 | 0.04 | 71.42 |
| 7 | 0.14 | 0.01 | 92.85 | 0.02 | 85.71 |



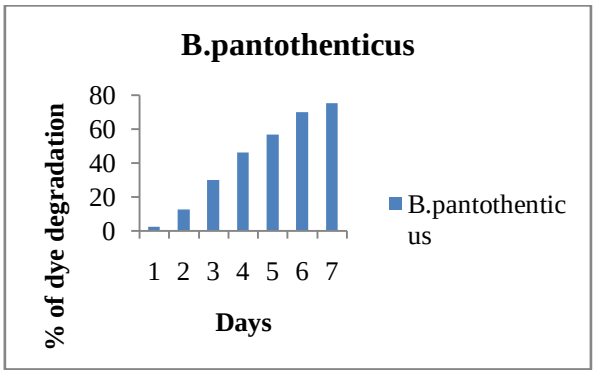

**Graph-2: Percentage of methyl red degradation against days through *A.niger* and *A.fusarium***

**For Congo red**

Congo red was 75.37% degraded by B. pantothenticus after 7 days. But the degradation takes place at decreased dye concentration i.e. 500µg/l (Table 6).

**Table-6: The degradability assay for the dye Congo red through *B. pantothenticus* (At decreased dye concentration 500mg/l )**

| Day | O.D ( At 500 nm ) | | Dye degradation ( % ) |
|---|---|---|---|
| | Blank | Sample | |
| 0 | 1.34 | 1.34 | 0.00 |
| 1 | 1.34 | 1.31 | 2.23 |
| 2 | 1.34 | 1.17 | 12.68 |
| 3 | 1.34 | 0.94 | 29.85 |
| 4 | 1.34 | 0.72 | 46.26 |
| 5 | 1.34 | 0.58 | 56.71 |
| 6 | 1.34 | 0.40 | 70.14 |
| 7 | 1.34 | 0.33 | 75.37 |




**Graph-3: Percentage of congo red degradation against days through *B.pantothenticus***

**Enzyme assay**

**For methyl red degradation**

It was found that enzyme activity of *B. pantothenticus* for crude was highest and it was decreased after ammonium salt precipitation and after dialysis. Elute 1 shows the highest enzyme activity i.e. 0.07725 after purification thus elute 1 was selected for further work (Table 7).

Crude extract of *A.niger* and *fusarium* was shows the highest enzyme activity. Enzyme activity was decreased after ammonium salt precipitation but dialysis crude shows the higher activity as compare to ammonium salt crude and I.E.C crude. After purification elute 1of *A.niger* and elute-6 of *fusarium* shows the highest activity as compare to other elutes thus elute-1 and elute-6 were selected for the further work (Table 8).

**Table-7: Enzyme activity of crud and purified enzyme of *B. pantothenticus* (For methyl red degradation)**

| S. No. | Tubes | OD at 532nm | Enzyme activity units/ml |
|---|---|---|---|
| 1 | Blank | 0.24 | - |
| 2 | Crude Extract | 0.39 | 0.19313 |
| 3 | Ammonium salt ppt$^{n}$ crud | 0.33 | 0.11588 |
| 4 | Dialysis crud | 0.30 | 0.07725 |
| 5 | I.E.C. crud | 0.29 | 0.06438 |
| 6 | Elute-1 | 0.30 | 0.07725 |
| 7 | Elute-2 | 0.28 | 0.0515 |
| 8 | Elute-3 | 0.28 | 0.0515 |
| 9 | Elute-4 | 0.25 | 0.01288 |
| 10 | Elute-5 | 0.27 | 0.03863 |
| 11 | Elute-6 | 0.25 | 0.01288 |

**Table 8. Enzyme activity of crud and purified enzyme of *A.niger* and *fusarium* (For methyl red degradation)**

| S. No. | | *A.niger* | | *Fusarium* | |
|---|---|---|---|---|---|
| | Tubes | OD at 532nm | Enzyme activity units/ml | OD at 532nm | Enzyme activity units/ml |
| 1 | Blank | 0.24 | - | 0.24 | |
| 2 | Crud Extract | 0.38 | 0.18026 | 0.40 | 0.20601 |
| 3 | Ammonium salt ppt$^{n}$ crude | 0.31 | 0.09013 | 0.35 | 0.14163 |
| 4 | Dialysis crude | 0.33 | 0.11588 | 0.32 | 0.103 |
| 5 | I.E.C. crude | 0.32 | 0.103 | 0.27 | 0.03863 |
| 6 | Elute-1 | 0.32 | 0.103 | 0.26 | 0.02575 |
| 7 | Elute-2 | 0.30 | 0.07725 | 0.26 | 0.02575 |
| 8 | Elute-3 | 0.29 | 0.06438 | 0.31 | 0.09013 |
| 9 | Elute-4 | 0.30 | 0.07725 | 0.31 | 0.09013 |
| 10 | Elute-5 | 0.26 | 0.02575 | 0.33 | 0.11588 |
| 11 | Elute-6 | 0.26 | 0.02575 | 0.35 | 0.14163 |

**For Congo red degradation**

The crude extract of *B.pantothenticus* for congo red degradation shows 0.25472 enzyme activity which was highest as compare to other samples. Enzyme activity was continuously decreased after ammonium salt precipitation and after dialysis. Elute-6 was selected for further work because it shows higher enzyme activity as compare to other elutes of I.E.C. and enzyme activity of elute-6 was similar to Dialysis crude (Table 9).

**Table-9:Enzyme activity of crud and purified enzyme of *B. patothenticus* (For Congo red)**

| S. No. | Tubes | OD at 532nm | Enzyme activity units/ml |
|---|---|---|---|
| 1 | Blank | 1.00 | - |
| 2 | Crud Extract | 1.18 | 0.25472 |
| 3 | Ammonium salt ppt$^{n}$ crude | 1.10 | 0.14151 |
| 4 | | | |
| 5 | Dialysis crude | 1.08 | 0.11321 |
| 6 | I.E.C. crude | 1.04 | 0.0566 |
| 7 | Elute-1 | 1.03 | 0.04295 |
| 8 | Elute-2 | 1.03 | 0.04295 |
| 9 | Elute-3 | 1.05 | 0.07075 |
| 10 | Elute-4 | 1.06 | 0.08491 |
| 11 | Elute-5 | 1.06 | 0.08491 |
| | Elute-6 | 1.08 | 0.11321 |

**Protein Estimation**

**For methyl red degrading Enzyme**

The used concentration of 5 standard sample was 20, 40, 60, 80, and 1.00µg. When the graph for standard sample was plotted, straight line with three points was obtained. On the basis of standard graph, concentration of other sample was gained. Protein concentration in elute1 for methyl red degrading Enzyme by *B. Pantothenticus* was 29 µg/ml (Table 10).Concentration of protein in elute 1 of *A.niger* was 38 µg/ml and 31 µg/ml in elute 6 of *Fusarium* (table 11).

**Table-10: Protein Estimation of methyl red degrading Enzyme ( by *B.Pantothenticus*)**

| S. No. | Test tube | BSA (ml) (mM) | D.W. (ml) | Reagent C (ml) | FC reagent (ml) | OD at 660 nm | Concentration of protein µg/ml |
|---|---|---|---|---|---|---|---|
| 1 | Blank | - | 1.00 | 5 | 0.5 | 0.00 | - |
| 2 | Stabdered-1 | 0.2 | 0.8 | 5 | 0.5 | 0.09 | 20 |
| 3 | Standered-2 | 0.4 | 0.6 | 5 | 0.5 | 0.16 | 40 |
| 4 | Standered-3 | 0.6 | 0.4 | 5 | 0.5 | 0.27 | 60 |
| 5 | Standered-4 | 0.8 | 0.2 | 5 | 0.5 | 0.39 | 80 |
| 6 | Standered-5 | 1.00 | - | 5 | 0.5 | 0.45 | 100 |
| 7 | Crud extract | 0.2 | 0.8 | 5 | 0.5 | 0.26 | 57 |
| 8 | Ammonium salt ppt$^{n}$ crude | 0.2 | 0.8 | 5 | 0.5 | 0.21 | 46 |
| 9 | Dialysis crud | 0.2 | 0.8 | 5 | 0.5 | 0.18 | 40 |
| 10 | I.E.C. | 0.2 | 0.8 | 5 | 0.5 | 0.19 | 42 |
| 11 | Elute-1 | 0.2 | 0.8 | 5 | 0.5 | 0.13 | 29 |

**Table-11: Protein Estimation of methyl red degrading enzyme (By *A.Niger and Fusarium*)**

| S. No. | Test tubes | *A.niger* | | *fusarium* | |
|---|---|---|---|---|---|
| | | OD at 660 nm | Concentration of protein µg/ml | OD at 660 nm | Concentration of protein µg/ml |
| 1 | Blank | 0.00 | - | 0.00 | - |
| 2 | Crud extract | 0.31 | 69 | 0.30 | 67 |
| 3 | Ammonium salt ppt$^{n}$ crude | 0.24 | 53 | 0.15 | 33 |
| 4 | Dialysis crud | 0.24 | 53 | 0.13 | 29 |
| 5 | I.E.C. | 0.20 | 44 | 0.13 | 29 |
| 6 | Elute-1 | 0.17 | 38 | - | - |
| | Elute-6 | - | - | 0.14 | 31 |

**For Congo red degrading enzyme**

Protein concentration in elute 6 for Congo red degrading Enzyme by *B. Pantothenticus* was 44 µg/ml but the concentration of protein was higher in crude extract i.e. 53 µg/ml (Table 12)

**Table-12: Protein Estimation of Congo red degrading enzyme (By *B.Pantothenticus*)**

| S. No. | Test tubes | OD at 660 nm | Concentration of protein µg/ml |
|---|---|---|---|
| 1 | Blank | 0.00 | - |
| 2 | Crud extract | 0.24 | 53 |
| 3 | Ammonium salt ppt$^{n}$ crude | 0.21 | 46 |
| 4 | Dialysis crude | 0.21 | 46 |
| 5 | I.E.C. | 0.18 | 40 |
| 6 | Elute-6 | 0.20 | 44 |

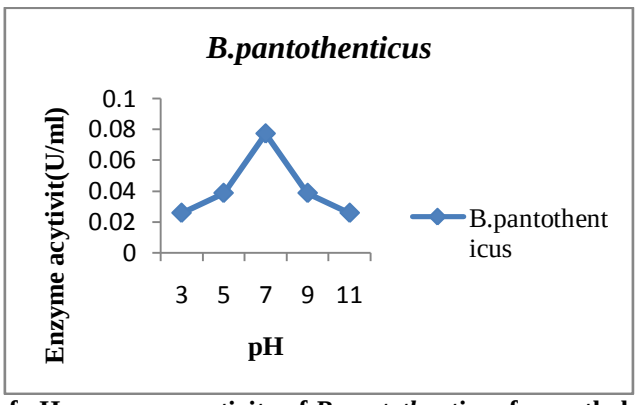
**Enzyme Kinetics**

**Effect of pH on Enzyme activity**

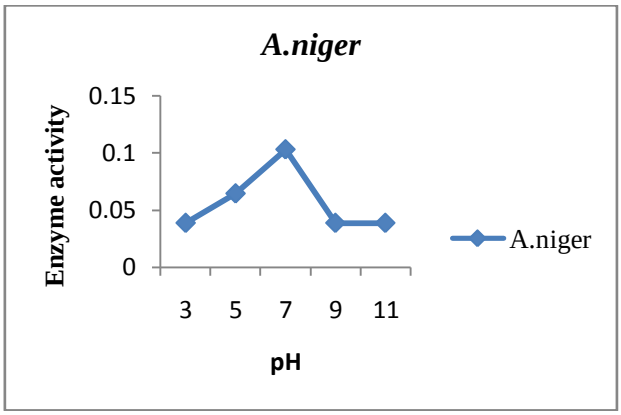
It was found that enzyme activity for all the dye degradation was higher at pH 7 as compare to pH 3,5,9 and 11 (Table 13). Differences in enzyme activity can be easily seems by plotting graph by taking enzyme activity against different pH (Graph 4 to 7).

**Table-13: Effect of pH on Enzyme activity**

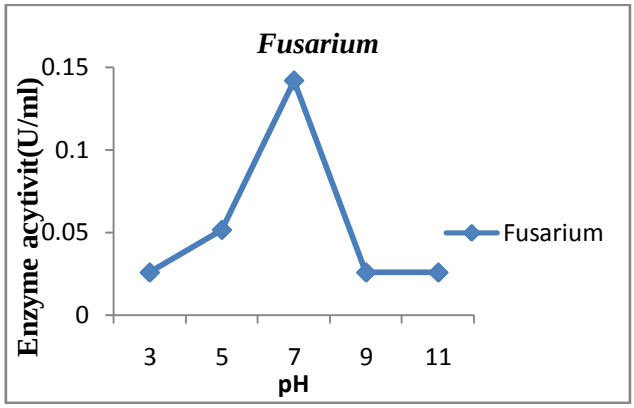
| Test tube | pH 3 | | pH5 | | pH7 | | pH 9 | | pH 11 | |
|---|---|---|---|---|---|---|---|---|---|---|
| | OD at 532 nm | Enzyme activity | OD at 532 nm | Enzyme activity | OD at 532 nm | Enzyme activity | OD at 532 nm | Enzyme activity | OD at 532 nm | Enzyme activity |
| Blank1 (Methyl red) | 0.28 | - | 0.36 | - | 0.24 | - | 0.20 | - | 0.12 | |
| Test(T)-a Methyl red (B.patithenticus) | 0.30 | 0.0257 | 0.39 | 0.0386 | 0.30 | 0.0772 | 0.23 | 0.0386 | 0.14 | 0.0257 |
| T-b Methyl red (A. niger) | 0.31 | 0.0386 | 0.41 | 0.0644 | 0.32 | 0.103 | 0.23 | 0.0386 | 0.15 | 0.0386 |
| T-c Methyl red (A. fusarium) | 0.30 | 0.0257 | 0.40 | 0.0515 | 0.35 | 0.142 | 0.22 | 0.0257 | 0.14 | 0.0257 |
| Blank-2 (Congo red) | 1.30 | - | 1.12 | | 1.01 | | 1.07 | | 1.04 | |
| T-d Congo red (B.pantothentics ) | 1.33 | 0.0424 | 1.18 | 0.0849 | 1.10 | 0.1273 | 1.11 | 0.0566 | 1.06 | 0.0283 |



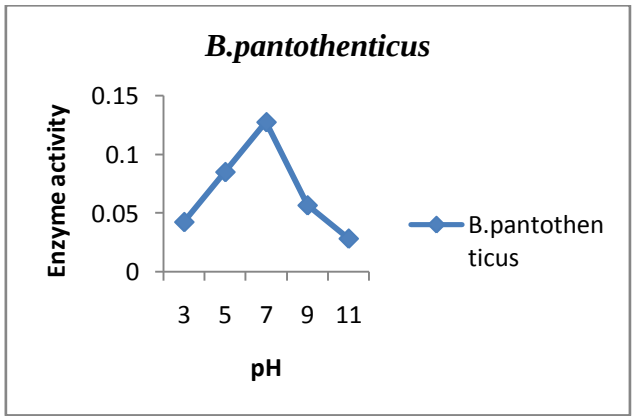

**Graph-4:Effect of pH on enzyme activity of *B.pantothenticus* for methyl red degradation.**




**Graph-5: Effect of pH on enzyme activity of *A.niger* for methyl red degradation.**




**Graph-6: Effect of pH on enzyme activity of *A.fusarium* for methyl red degradation.**



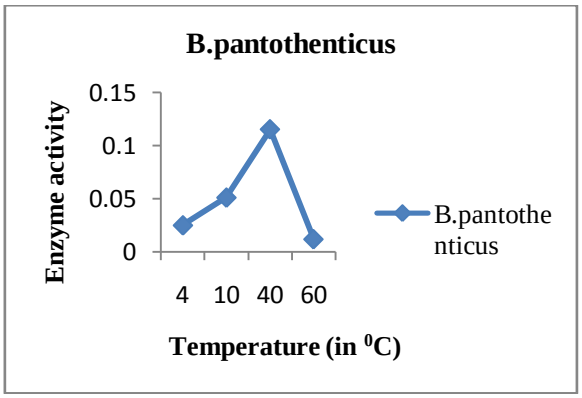

**Graph-7: Effect of pH on enzyme activity of *B.pantothenticus* for congo red degradation.**

**Effect of Temperature on enzyme activity**

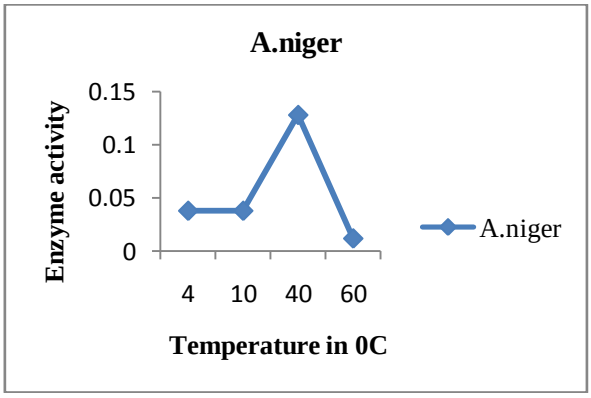
It was found that enzyme activity was higher and good at temperature between 37 to 40$^0$C and activity of enzyme was decreased at other temperature i.e. 4, 10, and 60$^0$C (Table 14). Differences in enzyme activity can easily seem by plotting graph by taking enzyme activity against different temperature (Graph 8 o 11).

**Table-14: Effect of Temperature on Enzyme activity**

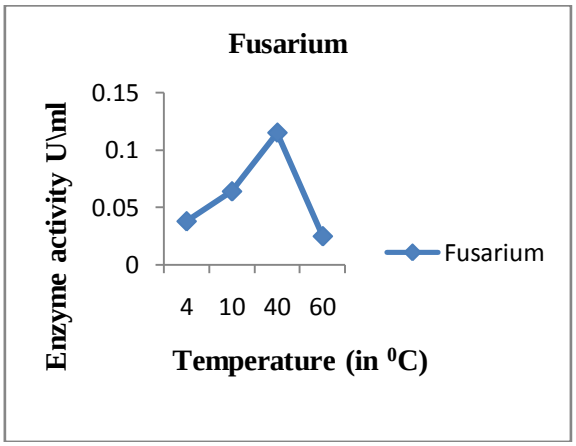
| Test tube | Temperature | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | $4^0$C | | $10^0$C | | $40^0$C | | $60^0$C | |
| | OD at 532 nm | Enzyme activity | OD at 532 nm | Enzyme activity | OD at 532 nm | Enzyme activity | OD at 532 nm | Enzyme activity |
| Blank1 (Methyl red) | 0.23 | - | 0.23 | - | 0.23 | - | 0.23 | - |
| T-a Methyl red (B.patithenticus) | 0.25 | 0.025 | 0.27 | 0.051 | 0.32 | 0.115 | 0.24 | 0.012 |
| T-b Methyl red (A. niger) | 0.24 | 0.012 | 0.26 | 0.038 | 0.33 | 0.128 | 0.26 | 0.038 |
| T-c Methyl red (A. fusarium) | 0.26 | 0.038 | 0.28 | 0.064 | 0.32 | 0.115 | 0.25 | 0.025 |
| Blank-2 (Congo red) | 0.98 | - | 0.98 | - | 0.98 | - | 0.98 | - |
| T-d Congo red (B.pantothentics) | 1.00 | 0.028 | 1.03 | 0.070 | 1.33 | 0.495 | 1.02 | 0.056 |



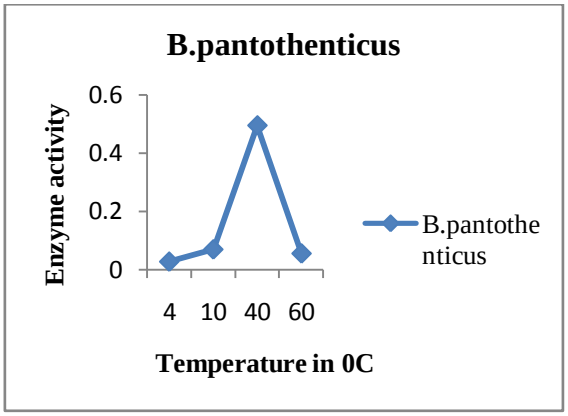

**Graph-8: Effect of temperature on enzyme activity of *B.pantothenticus* in methyl red degradation**




**Graph-9: Effect of temperature on enzyme activity of *A.niger* in methyl red degradation**




**Graph 10. Effect of temperature on enzyme activity of *A.fusarium* in methyl red degradation**




**Graph-11: Effect of temperature on enzyme activity of *B.pantothenticus* in congo red degradation.**

**DISCUSSION**

This study was carried out to estimate the Enzymatic degradation hazardous azodyes i.e. methyl red, congo red through the microbes. In this study degradation of azodyes has been carried out by *B.pantothenticus, A.niger and Fusarium.*

Previous study have sown that strain of staphylococcus sp., isolated from soil in a textile effluent treatement, were able to decolorized the sulphonate azo dye congo red[10] and Microbial degradation of Congo red by *Gliocladium virens* [11]. Various hazardous dyes likes, Congo red, Acid red, Basic blue and Bromophenol blue, Direct green by the fungus *Trichoderma harzianum* [12] has been investigated earlier but there is no literature on congo red degradation with *B.pantithenticus*. *B.pantothenticus* degrade 75.37% congo red that is the new literature for congo red degradation and the enzyme is oxygen insensitive and show intense decolorization.

*Van der Zee* [1] demonstrated that biological treatment strategy based on anaerobic reduction of the azo dyes, followed by aerobic transformation of the formed aromatic amines, holds promise to remove azodyes from waste water. Chang *et al* [16] demonstrated that presence of oxygen normally inhibit the azo bond reduction activity since aerobic respiration may dominate use of the NADH this impeding electron transfer from NADH to the azo bond. Percentage dye degradation by the isolated Bacillus megaterium was found to be 64.89% Jahir Alam Khan [13]. I have done degradation of azodyes in under aerobic condition and different from their results conclutions I found that in an aerobic condition degradation rate was as good at anaerobic condition.In an aerobic condition *B. pantothenticus* shows 91.66% methyl red degradation, 75.37% congored degradation, *A.niger* and *A.fusarium* shows 92.85% and 85.71% methyl red degradation respectively within 7 days.

Mane, U. V. [14] isolated Actinomycete, *Streptomyces krainskii*, SUK -5 was found to decolorize and degrade textile dye Reactive blue–59.This azo dye was decolorized and degraded completely by *Streptomyces krainskii* SUK–5 at 24 h in shaking condition in the nutrient medium at pH 8. But Gurulakshmi. M et al [6] has been isolated *Bacillus subtilis* which was able to to decolorize the Acidblue113. The bacterial culture exhibited 90% decolorization ability within 50 h. which is different from my work and the degradation percentage was higher than the percentage which I have done it might be due the difference of dyes. Optimum dye decolorizing activity of the culture was observed at pH 7.0 and incubation temperature of $37^0$C Gurulakshmi.

M et al [6] and it was similar to my work in which enzyme shows higher activity at pH 7 and at $40^0$C.Maximum, dye-decolorizing efficiency was observed at 200 mg/l concentration of Acidblue113 Gurulakshmi. M *et al* [6] but in this study maximum dye decolorization was observed at g/l concentration cxcept congo red which was 500mg/l. He confirmed decolorization by UV-VIS spectrophotometer but in this study decolorization were confiremed by colorimeter.

In this studyAzoreductase enzyme was purified by ion exchange chromatography and the m.wt. and purity was checked by SDS-PAGE and the similar procedure of purification was used by Jahir Alam Khan [13].

## CONCLUSION

Different industrial effluent containing dyes which produce aromatic amines are harmful for animal, plants and also cause the water pollution. On the basis of this study it was concluded that *B.pantothenticus*, *B.bravis* (Bacteria) and *A.niger, A.fusarium* (Fungus) have ability to produced Azoreductase enzyme which catalyzed the degradation of azodyes i.e. methyl red, congo red. It was found that particular microbial enzyme have capable to degrade specific kinds of dyes and all the isolated microbes was not able to degrade all dyes which was used in this study. Enzyme produced by *B.pantothenticus* have ability to degrade methyl red and congo red dye where the *A.niger* and *fusarium* are capable to degrade methyl red except the congo red. Enzymes used NADH for the electron transfer, which leads the azo bond reduction. Degradation of dyes and the enzyme activity is depends upon the concentration of dye. Activity of enzyme increases with decrease dye concentration. Around pH 6 to 8 and the temperature between 30 to 40. Azoreductase purified from all isolated microbes here is oxygen insensitive thus treatment of effluent can be performed at very low cost at the industrial site as compare to the anaerobic treatment which required large input.

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