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# Bioremediation and Detoxification of Trypan Blue by *Bacillus* sp. Isolated from Textile Effluents

**P. Jeevitha, D. Manjula, I. Ramya and J. Hemapriya***

*Department of Microbiology, DKM College for women, Vellore, India*

**Corresponding author*

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**A B S T R A C T**

Azo dyes are commonly used in many commercial industries. 16 bacterial isolates were isolated from textile effluents, of which 4 isolates (HB1, HB2, HB3 and HB4) showed ability to decolorize Trypan blue dye. Based on the standard morphological and biochemical characteristics, HB3 isolate that showed maximum decolorization of Trypan blue was identified as *Bacillus* sp. HB3 isolate showed 96.6 % decolorization of Trypan blue within 24 h of incubation. Maximum decolorization of Trypan blue was found to be achieved at 35 °C, neutral pH in the presence of glucose (Carbon source) and Yeast extract (Nitrogen source). The activity of azo reductase, lignin peroxidase, tyrosinase, manganese Peroxidase was investigated for their role in biodegradation of Trypan blue. Specific activity of the azoreductase enzyme was found to be 0.46 U mg $^{-1}$ protein. The crude protein extract subjected to SDS-PAGE resulted in the formation of a clear band (original band) against blue back ground which indicated the location of active azoreductase enzyme

## Introduction

Azo dyes are the largest group of synthetic chemicals that are widely employed in the textile, leather, cosmetics, food coloring and paper production industries.

The chemical structure of these compounds features substituted aromatic rings that are joined by one or more azo groups (–N=N–). The annual world production of azo dyes is estimated to be around one million tons (Pandey *et al.*, 2007) and more than 2000 structurally different azo dyes are currently in use (Vijaykumar *et al.*, 2007). During the dyeing process, approximately 10-15 % of the used dye is released into wastewater (Asad *et al.*, 2007). Moreover, many azo dyes and their degradation intermediates such aromatic amines are mutagenic and carcinogenic and discharge of them into surface water obstructs light penetration and oxygen transfer into bodies of water, hence affecting aquatic life (Ozturk and Abdullah, 2006).

Most of dyes have a synthetic origin and complex aromatic molecular structure, which make them stable and difficult to biodegrade. Reactive dyes differ from all other dye classes in that they bind to textile fibers, such as cellulose and cotton, through covalent bonds (O'Mahony *et al.*, 2002). Reactive dyes are

typically azo-based chromophores combined with various types of reactive groups, which show different reactivity. The recalcitrance of azo dyes has been attributed to the presence of sulfonate groups and azo bonds, two features generally considered as xenobiotic (Rieger *et al.,* 2002). Some of the azo dyes are difficult to treat by conventional wastewater treatment methods. Compared with physical and chemical methods, biological techniques are preferable because of economical advantages and eco safety.

Many microbial strains have been isolated to degrade this kind of aromatic compound (Rajaguru *et al.,* 2000; Stolz, 2001). Most of the metabolic studies have been limited to bacterial genera; however, since azo dyes are considerably recalcitrant (Pagga and Brown, 1986). The reduction of azo dyes leads to formation of aromatic amines which are known mutagens and carcinogens.

Further it is difficult to degrade these aromatic amines containing waste water by conventional treatment processes. Hence, economical and eco-friendly approaches are needed to remediate dye-contaminated wastewater from various industries.

Among the various bioremediation technologies, decolorization using microbial cells has been widely used. The anaerobic reduction of azo linkages converts the azo dyes to usually colorless but potentially harmful aromatic amines. The anaerobic reduction of the produced aromatic amines can be converted into non-harmful products by several bacterial strains under aerobic condition by their reductive mechanisms. From this is evident that bacteria are rarely able to decolorize azo compound in the presence of oxygen (Chang *et al.,* 2001). This study was an attempt to isolate the bacterial strains which could decolorize the azo dyes even in aerobic condition.

## Materials and Methods

### Sampling sites and Textile dyes used

The sampling area was the textile industries and dyeing units located in and around Gudiyatham, Vellore District, Tamil Nadu, India. Trypan Blue used in this study was procured commercially. Stock solution was prepared by dissolving 1 g of the dye in 100 ml distilled water.

### Isolation and Screening of Bacterial Strains Decolorizing Azo dye

The effluent and sludge samples were serially diluted and spread over minimal agar medium containing 50 ppm of Trypan Blue. pH was adjusted to 7.0 before autoclaving and incubated at 37°C for 5 days. Colonies surrounded by halo (decolorized) zones were picked and streaked on minimal agar medium containing azo dye. The pure cultures were maintained on dye-containing nutrient agar slants at 4°C.

### Decolorization Assay

Loopful of bacterial culture was inoculated in Erlenmeyer flask containing 100 ml of nutrient broth and incubated at 150 rpm at 37 °C for 24 h.

Then, 1 ml of 24 h old culture of the bacterial isolates were inoculated in 100 ml of nutrient broth containing 50 ppm of Trypan Blue and re-incubated at 37 °C till complete decolorization occurs. Suitable control without any inoculum was also run along with experimental flasks. 1 ml of sample was withdrawn every 24 h and centrifuged at 10,000 rpm for 15 min. Decolorization extent was determined by measuring the absorbance of the culture supernatant at 547 nm using UV-visible spectrophotometer, according to Hemapriya *et al.,* (2010).

$$\text{Decolorization efficiency (\%)} = \frac{\text{Dye (i)} - \text{Dye (r)}}{\text{Dye (i)}} \times 100$$

Where, Dye (i) refers to the initial dye concentration and Dye (r) refers to the residual dye concentration. Decolorization experiments were performed in triplicates.

## Optimization of Culture Conditions for Dye Decolorization by *Bacillus* sp. HB3

### Effect of Temperature, pH and Dye Concentration

The effect of temperature, pH and dye concentration on dye decolorizing ability of the isolate was studied. This was carried out by incubating the bacterial strains at different temperature (25-45°C), pH (5-9) and various dye concentrations (100-500 ppm).

### Effect of Carbon and Nitrogen source on Dye Decolorization

To investigate the effect of various carbon and nitrogen sources, different carbon sources such as, glucose, lactose, and sucrose (1%) and different nitrogen sources like yeast extract, beef extract, and peptone (1%) were added as a supplement individually to Nutrient broth medium for decolorization of Tryphan Blue.

### Enzyme Assays

Assay was carried out in cuvettes with a total volume of 1 ml. One unit per enzyme activity was defined as the amount of enzyme that transformed 1µ mol of substrate per minute (1 unit = 1U).

### Preparation of Cell Free Extract

The bacterial strain HB3 was inoculated in Nutrient Broth containing Azo dye (Trypan Blue) and incubated at 37 °C. The cells were harvested by centrifugation at 7000 rpm for 30 min in cooling centrifuge, washed with 50 mM phosphate buffer (pH 7.0) and resuspended in the same buffer.

Then, the cells were disturbed and cell debris was removed by centrifugation at 4 °C. The resultant supernatant was used as the source of crude protein / enzyme.

### Laccase Activity Assay

Laccase activity was determined using 2,2'-azino-di-(-3-ethylbenzo-thiazoline-6-sulfonic acid) (ABTS) as the substrate.

5µl of 50 mM citrate buffer (pH 4.0) was mixed with 430µl of distilled water and 20µl of laccase.

The reaction was started by addition of 50µl of 6 mM ABTS and increase in absorbance at 547 nm was monitored. The enzyme activity was calculated using an extinction coefficient of ABTS of $\varepsilon_{436} = 36$ m $mol^{-1}$ $cm^{-1}$ (Ander and Messner, 1998).

### Tyrosinase assay

Tyrosinase activity was determined in reaction mixture of 2 ml containing 500 µl of 0.01% catechol in 500 µl of 0.1 M phosphate buffer (pH 7.4) and 1ml of cell free culture at 495 nm (Zhang and Flurkey, 1997).

### Lingnin peroxidase (LiP) assay

LiP (Lingnin Peroxidase) activity was determined by monitoring the formation of propanaldehyde at 547 nm in a reaction mixture of 2.5 ml containing 500 µl of 100 mM n-propanol, 500 µl of 250 mM tartaric acid, 500 µl of 10 mM $H_2O_2$ and 1 ml of cell free culture (Shanmugam *et al.,* 1999) at 547 nm.

### MnP (Manganese Peroxidase) assay

The reaction mixture contained 500 µl of 50 mM sodium malonate buffer (pH 4.5), 25µl of 20 mM $MnCl_2$ solution, 415µl of distilled water and 50 µl pf MnP. The reaction was started by adding 20 µl of 10 mM $H_2O_2$. The extinction of the solution was measured photometrically at the wavelength 547 nm ($\varepsilon_{270}$= 11.59 $mmol^{-1}$ $cm^{-1}$) (Wariishi *et al.,* 1992).

### Azo Reductase Assay

Assay was carried out in cuvettes with a total volume of 1 ml using colorimeter. The reaction mixture consists of 400 µl of potassium phosphate buffer with 200 µl of sample and 200 µl of reactive dyes (500 mg/l). The reaction was started by addition of 200µl of NADH (7mg/ml) and was monitored photometrically at 547 nm. The linear decrease of absorption was used to calculate the azoreductase activity. One unit of azoreductase can be defined as the amount of enzyme required to decolorize 1µ mol of Trypan Blue per minute.

### SDS PAGE of Azoreductase

SDS was excluded from both electrophoresis system and sample buffer. Native gel was cast with 12 % resolving gel and 4% stacking gel. 0.1% Carboxy methyl cellulose was added to the resolving gel to facilitate binding of Trypan Blue dye. Crude protein extract was mixed with sample buffer (without SDS and β-mercaptoethanol) and run on the gel under native conditions. Azoreductase enzyme was located on the gel by activity staining. For this, the gel was washed two to three times in 50 mM phosphate buffer (pH 7) and stained with 100 µM Tryphan Blue. The gel was then transferred to phosphate buffer containing 2 mM NADH. Appearance of colorless band against blue background (original band) in 15-20 min indicated the location of active azoreductase enzyme. (Ausubel *et al.,* 1987).

## Results and Discussion

### Isolation, Screening and Identification of Bacterial Strains Decolorizing Textile Dyes

The results shown in Table.1 revealed that 04 bacterial isolates, designated as HB1 to HB4 were found to be capable of decolorizing Trypan Blue (Fig. 1). Out of 04 isolates, HB3 was found to be the superior strain with the highest decolorization efficiency (96.60 %). Morphological, cultural and biochemical characteristics of HB3 strains were tabulated in Table.3. On the basis of the above mentioned characteristic features and by the comparison with "Bergey's manual of Determinative Bacteriology", the isolate HB3 was identified as *Bacillus* sp. Strain HB3 (Table. 2). The extent of dye decolorization of Trypan Blue by the bacterial isolates (HB1 to HB4) is shown in Fig. 2.

### Optimization of Dye Decolorizing Ability of HB3 Isolate

### Effect of Incubation Time

Dye decolorization by *Bacillus* sp. Strain HB3 was found to be growth dependent, since considerable dye decolorization was noticed in the fermentation broth as soon as the bacterial strains entered the late exponential phase and the activity reached the maximum level in stationary phase after 24 h of incubation (Fig. 3).

### Effect of Temperature

The influence of incubation temperature on the decolorization of Trypan Blue by *Bacillus* sp. HB3 was studied at temperatures ranging from 25-45 °C. The color removal efficiency of the bacterial isolate (HB3) achieved highest

levels (94.02 %) at 35°C, after 24 h of incubation. However, incubation at temperatures below 30°C and above 40°C was found to be down regulating the decolorization percentage of the isolate (Fig. 4).

## Effect of pH

Dye decolorization efficiency of *Bacillus* sp. HB3 against Trypan Blue was detected over a broad range of pH (5.0-9.0), with optimum decolorization of (87.54 %) being exhibited at neutral pH (7.0). At slightly alkaline pH (8.0), decolorization efficiency of the isolate was found to be effective (69.54%) (Fig. 5).

## Effect of Dye Concentration

The results revealed that the decolorization rate of the isolates was optimized in the presence of initial dye concentration of 100 ppm (Fig. 6).

As the dye concentration increased in the culture medium, a gradual and directly proportional decline in color removal was attained.

At high concentration (500 ppm), Trypan Blue greatly suppressed decolorization ability of *Bacillus* sp. HB3.

**Table.1** Bacterial Strains Decolorizing Trypan Blue (HB1- HB4)

| S. No | Isolates | % of Decolorization |
|---|---|---|
| **1.** | HB1 | 64.66 % |
| **2.** | HB2 | 60.64 % |
| **3.** | HB3 | **96.60 %** |
| **4.** | HB4 | 53.13 % |

**Table.2** Morphological, Cultural and Biochemical Characteristics of HB3 Strain

| S. No | Test characteristics | Bacterial Isolate (HB3) |
|---|---|---|
| **I.** | **Morphological characteristics** | |
| **1.** | Colony morphology | Smooth, large, translucent. |
| **2.** | Cell morphology | Rod shape |
| **3.** | Gram reaction | +ve |
| **4.** | Motility | +ve |
| **II.** | **Physiological characteristics** | |
| **5.** | Growth under aerobic condition | +ve |
| **6.** | Growth under anaerobic condition | -ve |
| **7.** | Growth in Liquid medium | Turbid |
| **III.** | **Biochemical characteristics** | |
| **8.** | Catalase | +ve |
| **9.** | Oxidase | -ve |
| **10.** | IMVIC | (- + + +) |
| **11.** | Triple Sugar Iron test | AK/AK, no $H_2S$ & no gas |
| **12.** | Urease test | +ve |
| **13.** | Nitrate reduction test | -ve |

**Fig.1** Trypan Blue before and after decolorization in Nutrient Broth


**Fig.2** Decolorization Efficiency of Bacterial Isolates towards Trypan Blue

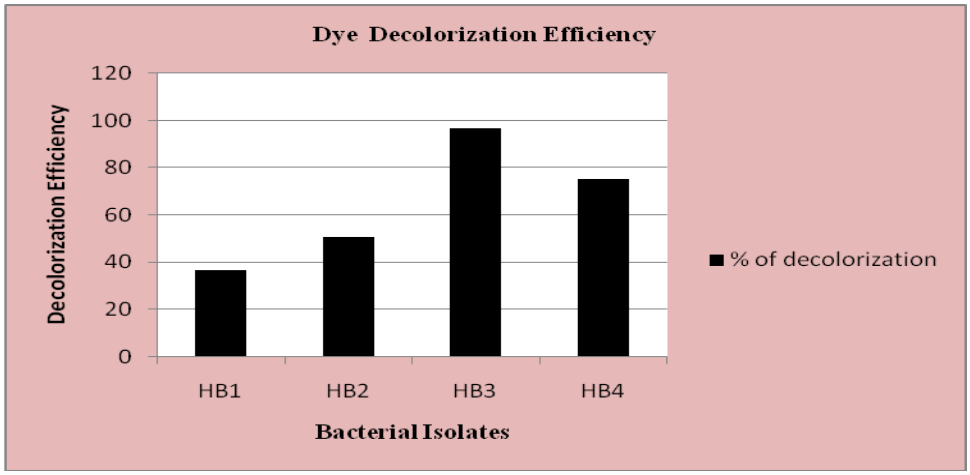



**Fig.3** Effect of Incubation Time on Decolorization of Trypan Blue by *Bacillus* sp. HB3

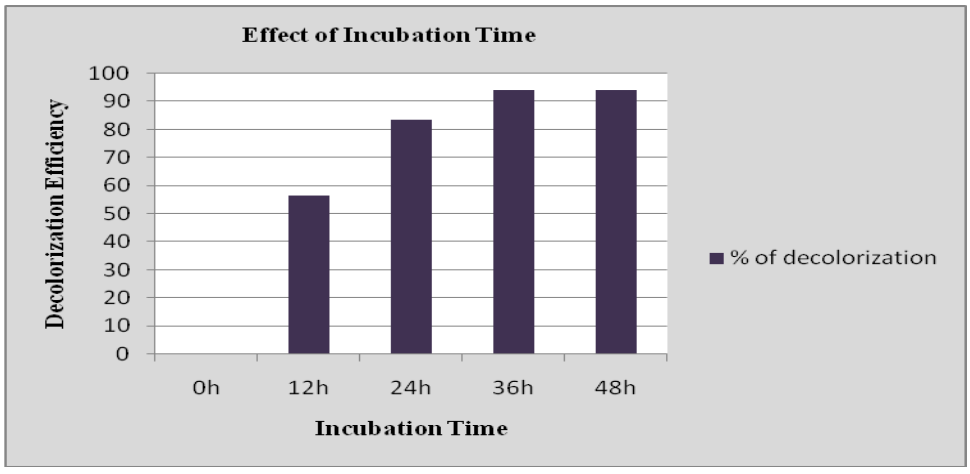


**Fig.4** Effect of Temperature on Decolorization of Trypan Blue by *Bacillus* sp. HB3

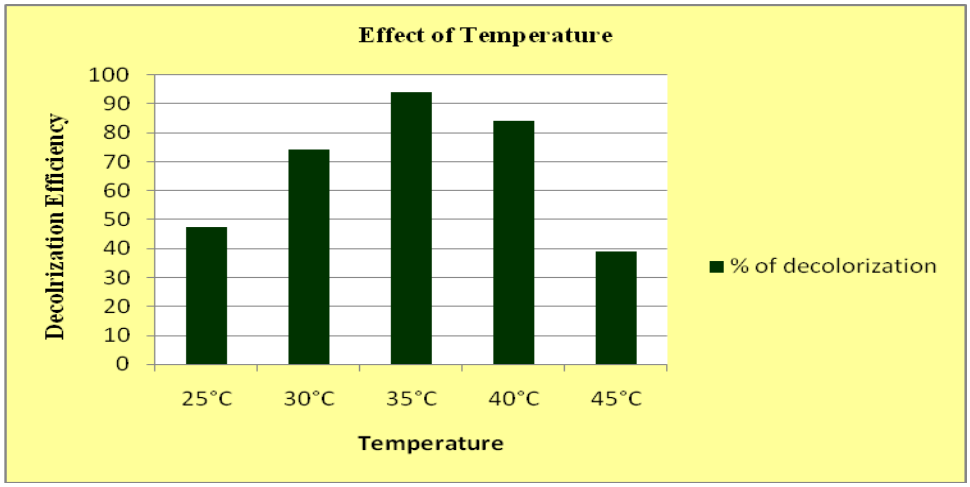



**Fig.5** Effect of pH on Decolorization of Trypan Blue by *Bacillus* sp. HB3

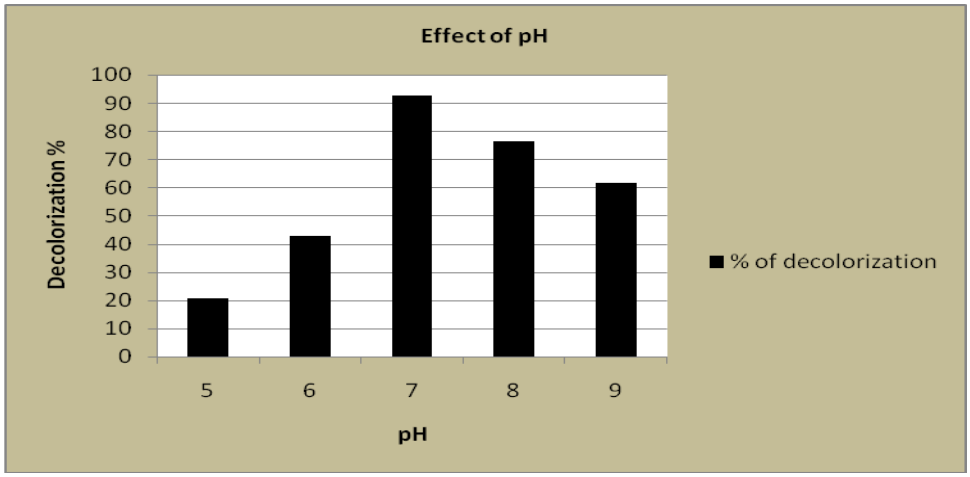



**Fig.6** Effect of Dye Concentration on Decolorization of Trypan Blue by *Bacillus* sp. HB3

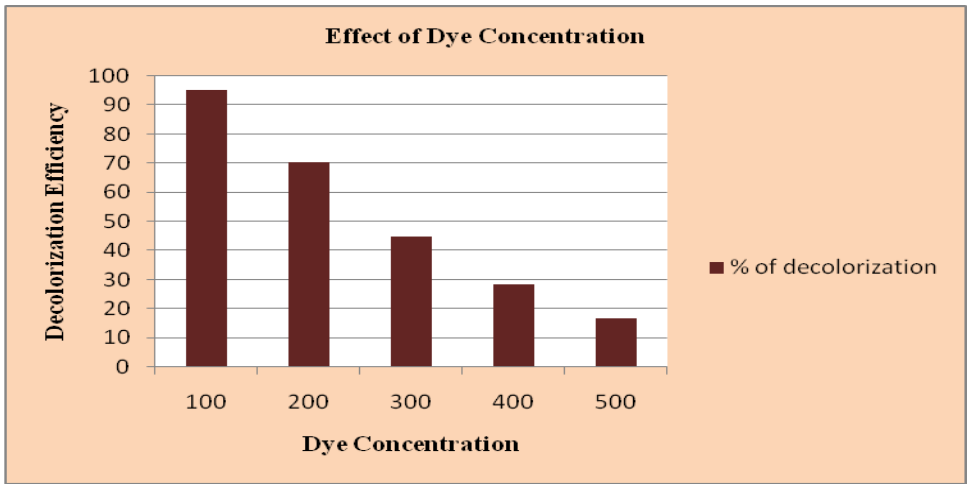


**Fig.7** Effect of Carbon Sources on Decolorization of Trypan Blue by *Bacillus* sp. HB3

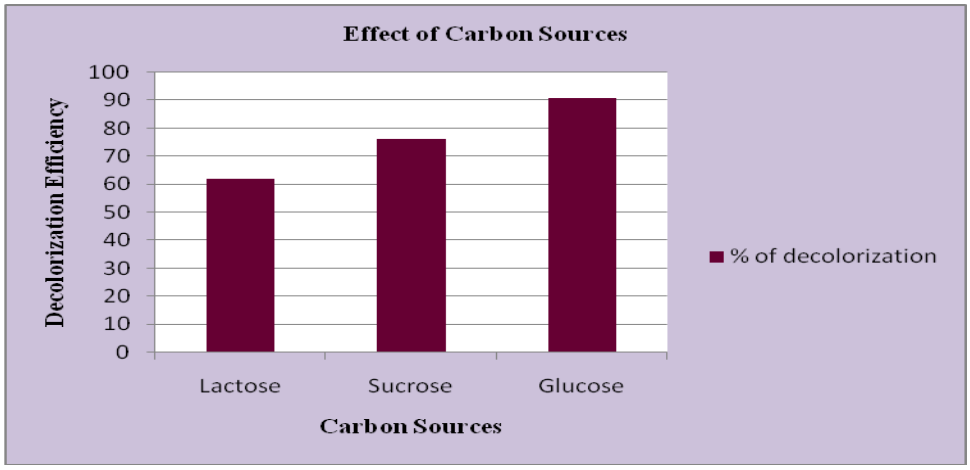



**Fig.8** Effect of Nitrogen Sources on Decolorization of Trypan Blue by *Bacillus* sp. HB3

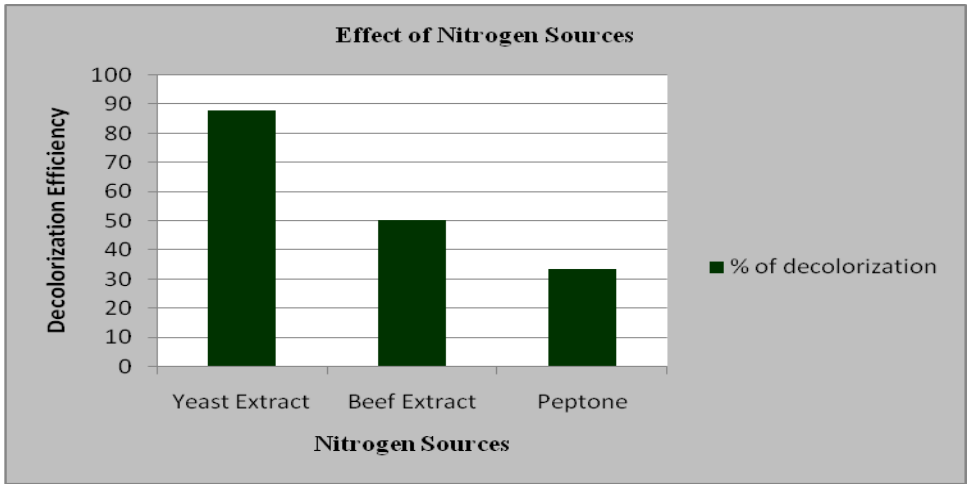



**Fig.9** Extracellular Decolorizing Enzyme production by *Bacillus* sp. HB3

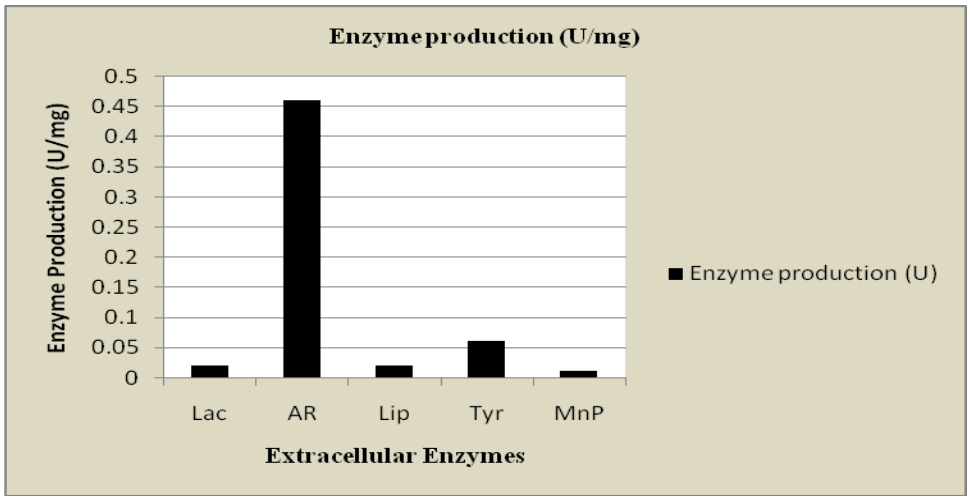
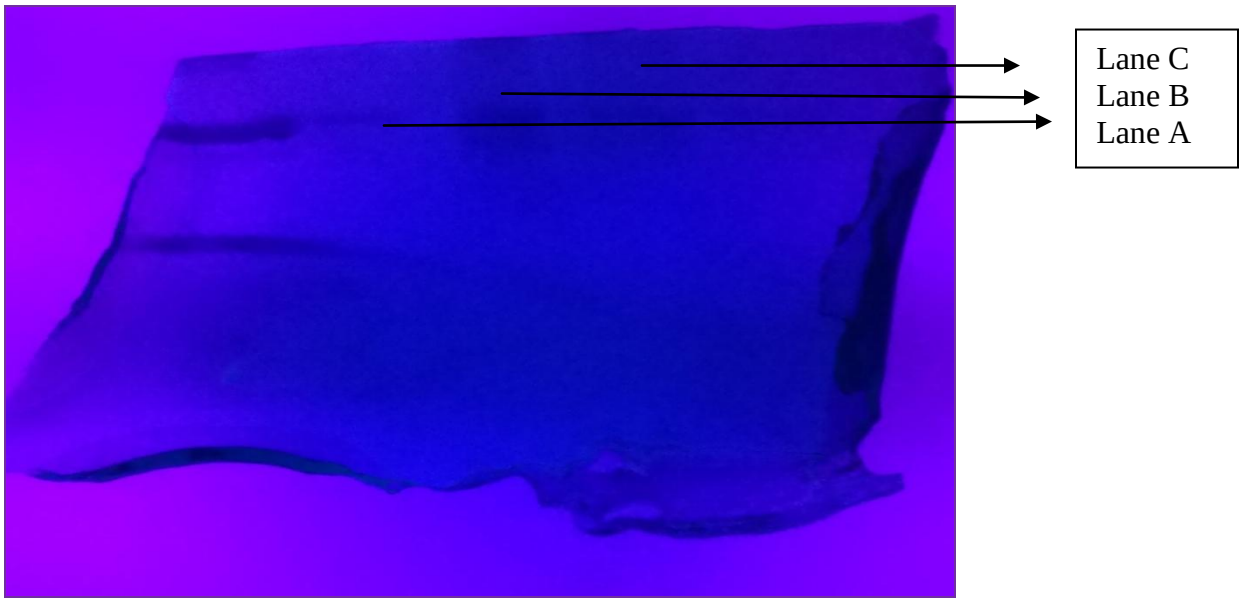


**Fig.10** Azo reductase Enzyme Activity of Bacterial Isolate (HB3) on SDS PAGE




Lane A: SDS-PAGE Molecular Weight Standards
Lane B: Sample
Lane C: Bovine Serum Albumin

## Effect of Carbon and Nitrogen Sources

The bacterial isolate HB3 was able to utilize most of the carbon sources tested, whereas glucose instigated maximum decolorization efficiency (90.93 %) (Fig. 7).

Among the various nitrogen sources tested, yeast extract was found to be the superior source in maximizing decolorizing ability (87.90%) (Fig. 8).

## Enzymatic assay for decolorization of Trypan Blue

The culture supernatant of HB3 cells that mediated the decolorization of Trypan Blue was screened for the presence of dye decolorizing enzymes such as Azoreductase, Laccase, Tyrosinase, Lignin Peroxidase, MnP (Manganese peroxidase). Azoreductase was found to be the dominant enzyme (0.46 U mg $^{-1}$ protein), whereas Laccase, Tyrosinase, Lignin peroxidase, MnP (Manganese Peroxidase were found to be secreted in very trace amounts (0.02, 0.06, 0.02, 0.01 U mg $^{-1}$ protein respectively) (Fig. 9).

## Azo Reductase Assay and SDS PAGE analysis

Crude protein extract obtained from *Bacillus* sp. HB3 cells was found to decolorize Trypan Blue dye using NADH as electron donor. Specific activity of the azoreductase enzyme was found to be 0.46 U mg $^{-1}$ protein (Fig. 13). The crude protein extract subjected to SDS-PAGE resulted in the formation of a clear band (original band) against blue back ground which indicates the location of active azoreductase enzyme (Fig. 10).

Environmental biotechnology is constantly expanding its efforts in the biological treatment of colored textile effluents, which is an environmental friendly and low-cost alternative to physico-chemical decomposition processes. The textile industries are multi-chemical utilizing concerns, of which various dyes are of

importance. During the dyeing process substantial amount of dyes and other chemicals are lost in the wastewater (Vaidya and Datye, 1982). An important element in guiding the direction and development of decolorization technology should logically depend upon a sound scientific knowledge, which undoubtedly warrants for further research. In view of the need for a technically and economically satisfying treatment technology, a flurry of emerging technologies are being proposed and tested at different stages of commercialization. Broader validation of these new technologies and integration of different methods in the current treatment schemes will most likely in the near future, render these both efficient and economically viable. The presence of dyes imparts an intense color to effluents, which leads to environmental as well as aesthetic problems (Singh and Singh, 2006).

Temperature variation had a significant effect on the decolorization of Trypan Blue by *Bacillus* sp. strain HB3. The rate of decolorization was found to be optimized at 35°C after 24 h of incubation.

The rate of decolorization decreased with the decrease in temperature. This fact implies that the local temperature in the micro-environment of the effluent samples has a very significant effect on the decolorization activity (Moosvi *et al.*, 2005). Decolorization activity of *Bacillus* sp. strain HB3 was significantly suppressed at temperatures more than 40°C, which might be due to the loss of cell viability or denaturation of the enzymes responsible for the decolorization at elevated temperatures. The most biologically feasible pH for the decolorization of Trypan Blue by *Bacillus* sp. strain HB3 was found to be 7.0. In contrast, optimal pH values for the decolorization of Reactive Red RB by a microbial consortium was found to be 8.0 (Cetin and Donmez, 2006). The foregoing results suggest the potential of utilizing *Bacillus* sp. strain HB3 to degrade textile effluent containing synthetic textile dyes via; appropriate bioreactor operation.

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