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# Determination of Potential of Halophilic *Bacillus* and *Alishewanella* Species for Decolorization of Acid Blue Dye

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## ABSTRACT

*Ecofriendly methods involving bacteria and fungi are finding utilities for treatment of textile industry effluents. A range of bacterial taxa have been introduced for decolorization of dyes most of which are mesophilic. Those bacteria reported so far might have limitation with regards to their survival in the nature as these have restrictions dealing with pH and temperature optima. As the textile effluent discharged in the environment possesses varied physical and chemical properties; it is important to extract those bacterial communities with properties that can withstand harsh environmental conditions. Present study aims in extraction of bacteria with such properties. Bacteria able to degrade DNA were carried further for their abilities to decolorize acid blue dye; one of the popular dye in textile industry. These bacteria were fully characterized by 16S rRNA sequencing. These halophilic bacteria have been extracted from Lonar Lake, Maharashtra, India. Of these two most efficient bacteria namely Alishewanella sp and Bacillus on the basis of efficiency of decolorization of acid blue dye were selected further. Both bacteria are able to grow over wide pH ranging from 5 to 13 and temperature ranging from 30$^0$C to 50$^0$C. Sufficient growth was also observed in nutrient media containing salt concentration ranging from 0.5% to 2.5% with optimum growth at 2%. The study suggests these bacteria would be suitable for decolorization of textile effluents possessing different physiological properties. The study was further extended on periodic studies on dye decolorization and it was found that 72h period was most suitbale for decolorization of acid bolue dye. A period exceeding this could enhance marginal difference in dye decolorization. Despite such growth optima the bacteria Alishewanella sp and Bacillus species could degrade 61.85% and 63.17% dye respectively. Accession number for Alishewanella sp and B. thuringiensis is JX298819 and JX298814 respectively.*

***Key words:*** *Alishewanella, Bacillus, acid blue, dye, decolorization.*

## INTRODUCTION

During the process of dyeing 10-15% dyes remains unused and is discharged in the water bodies with improper treatment. Globally the concentration of these dyes in the water bodies constitutes around 2,80,000 tons per year. Besides forming toxic compounds these also create anaerobic conditions and unavailability of light to the aquatic life[1]. However dye is not the only constituent of textile industries chemicals like dispersants, acids, bases, salts, detergents, humectants, and oxidants has to be used during processing which further deteriorates the water quality. Few of the dyes alone or in combination with hazardous chemicals may turn carcinogenic and may cause various health hazards. Therefore the treatment of dyes is a serious concern[2].

Because of these health hazards it is absolute requirement to remove these dyes from water bodies. The dyes mostly constitute azo (–N ═ N–) groups and are the largest class of synthetic dyes. Several methods are being employed for removal of these dyes from water bodies. For these purpose biological and non-biological systems are in effect. The biological systems are more preferred as these are eco-friendly and economical. The biological system includes growing and non-growing forms[3].

The non-biological systems constitute electrooxidation, coagulation/flocculation, photocatalysis and oxidation with ozonization[4,5,6,7]. Physical methods like adsorption, ion exchange and membrane filtration transfer dyes into another forms without complete degradation[8,9,10].

The non-growing biological forms constitute enzymes. Few important enzymes in this category are azoreductases, laccases, peroxidases and some lignolytic enzymes[11]. The efficiency of latex peroxidase and horseradish peroxidase from *Ficus sycomorus* in the decolorization of some synthetic dyes was compared by Abdel-Aty *et al*,[12]. The versatility of laccase enzymes lead them one of the popular enzymes for decolorization of azo dyes. Ligninolytic enzymes from white rot fungi *Phanerochaete chrysosporium* could degrade azo dye using lignin peroxidase and manganese peroxidase that too *in vitro* condition[14]. Recombinant *E. coli* having azoreductases have also been found effective for dye decolorization[15].

The biological forms for decolorization of dyes include fungi, algae and bacteria. Fungi particularly white rot fungi have been used because of their capabilities to degrade natural paints as well as decolorization of dyes. Fungi obviously have shown green solution over degradation of malachite green. Those fungi also degrade recalcitrant organic compounds. More than 96% of malachite green was decolorized by species of *Aspergillus flavus* and *Alternaria solani*[16]. Laccase from *Trametes hirsuta* and *Sclerotim rolfsii* was found to decolorize indigo dye[17]. Immobilization of fungi *Tramets pubescens* and *Pleurotus ostreatus* was done to study decolorization of dyes. The enzymes responsible were laccase, Mn-dependent and independent peroxidases, lignin peroxidase, and aryl-alcohol oxidase that were monitored regularly[18]. Synthetic dyes like orange G, amaranth, orange I, remazol brilliant blue R, Cu-phthalocyanin, Poly R-478, malachite green and crystal violet were decolorized by *Dichomitus squalens*. Of these dyes remazol was most efficiently decolorized[19]. Aniline blue and congo red were decolorized in the range of 40.9% to 70% by fungi *Aspergillus proliferans*. Colored effluents were also decolorized by these fungi[20].

Algae are receiving greater attention as these are stable at fixed places in the environment. These algal species are versatile for degradation of range of dyes and are effective over a long term continuous operations. Malachite green was degraded by *Cosmarium* species a green algae. These efficient species had optimum pH of 9[21]. Same dye was decolorized by *Chlorella* species. The mechanism involved was biosorption. The dye was rapidly removed in agitated experiment which was dependent on concentration of dead algal biomass and methyl green. Biosorption techniques likewise are effectively and economically employed over conventional activated carbon[22]. Methyl green was also degraded by *Chara* species. Both live and dead cells were undertaken for degradation of methyl green. The reusability of these cells was also studied[23]. A brown macroalga namely *Stoechospermum marginatum* was examined for acid orange dye adsorption. When biomass was modified by propylamination it was found absorption was double enhanced[24]. Biosorption studies have also been carried using *Spirulina platensis*. When compared with absorption by activated carbon, a marginal difference was found between these two techniques i.e. 94.4–99.0% and 93.6–97.7% as removed by *S. platensis* and activated carbon[25].

The unicellular prokaryotic forms i.e. bacteria are widely studied for decolorization of dyes. *Acinetobacter junii* is studied for reactive red dye decolorization[26]. The bacterium is also capable of simultaneous removal of dyes and reduction of chromium. A range of bacteria is recently reviewed by Imran et al, (2014) i.e. *Enterobacter* sp., *Pseudomonas putida*, *Bacillus* sp., *Lactobacillus casei*, *Lactobacillus casei*, *Caulobacter subvibrioides* strain and *Sphingomonas* sp[27]. The species of *Bacillus* i.e. *Bacillus cereus* and *Bacillus megaterium* could degrade 95% and 98% dye. Optimization of media and physiological conditions had also been carried out[28]. *Lysinibacillus fusiformis* could decolorize >70% azo dye which was isolated from the effluent[29]. Among other dye decolorizing bacteria includes *Pseudomonas* sp, *Alteromonas* sp, *Enterococcus* sp, *Serratia* sp and *Enterobacter* sp[30]. The bacterial consortium composed of *Sphingomonas paucimobilis*, *Bacillus* sp. and *Staphylococcus epidermidis* was one of the

highly effective method for decolorization. In this study it was observed that 100% decolorization was achieved and 75.76% COD was reduced. *Lysobacter* species could decolorize more than 80% congo red dye and yellow HEGR[31].

Acid blue 29 is an anionic azo dye used in the textile, paints, inks, plastics and leather industries[32]. In this study bacterial decolorization of acid blue dye from halophiles is studied as these are capable of growth at varied physiological conditions. The textile effluent discharged in the environment consists of wide range of chemicals differing in physiological properties as well. The salt tolerant bacteria are thought to withstand such conditions and would be effective in growth and decolorization of dyes.

## MATERIALS AND METHODS

**Sample Collection:**

Samples were collected from meteorite impact crater lake i.e. Lonar Lake, situated in Buldhana district, Maharashtra. These bacteria were previously studied for abilities to degrade DNA. Having studied for this purpose around thirty bacteria were undertaken to decolorize acid blue dye.

- **Decolorization of dye:**

The medium employed was modified Zhou and Zimmermann (ZZ) agar containing (yeast extract-5 g/L, glucose-5 g/L, $(NH_4)_2$ $SO_4$-0.5 g/L, $KH_2PO_4$-2.66 g/L, $Na_2HPO_4$-4.32 g/L, agar-20 g/L and acid blue dye – 0.1 g/L) and pH 7.0. Around 30 bacteria were inoculated on this media and incubated on shaker for $30^0$C.

- **Optimization of cultural conditions:**

Two bacteria were selected on the basis of highest decolorization among the 30 isolates. Optimization of cultural conditions was studied afterwards. Bacteria were incubated at different temperatures ranging from 25 to 45 $^0$C. At optimized temperature those were also grown at varied pH ranging from 3 to 10. Effect of salt concentration on the growth was also studied by incorporating 0.5%, 1%, 1.5%, 2% and 2.5% salt into the nutrient solution.

- **Analysis of Decolorization Rate:**

The efficiency of those bacteria was determined on the basis of decolorization of the acid blue dye. Suitable control was kept without inoculation. Nearly 2 ml of the sample was taken for spectrophotometric estimation of decolorization. Prior to this sample was centrifuged for 7,500 rpm for 15 minutes and clear supernatant was taken for decolorization studies. The percent decolorization was determined according to the following formula[28].

$$D=[(A0-A1)/A0]\times100$$

Where,

D=% of decolorization;

A0=initial absorbance;

A1=Final absorbance

- **Periodic Studies on Dye Decolorization:**

Four day period was selected to determine the maximum decolorization. About 5ml sample was withdrawn after time intervals of 0hr, 24hr, 48hr, 72hr and 96hr. This was then centrifuged and supernatant was taken for spectrophotometric studies at 595 nm.

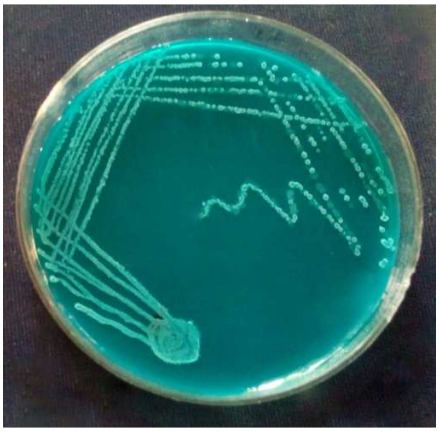
## RESULTS

**Decolorization of dye:**

In a broth medium as defined earlier; around thirty halophilic bacteria were inoculated of which two efficient bacteria selected on the basis of dye decolorization were used further. Prior to this, bacteria were also inoculated on similar agarized medium to determine resistance for acid blue dye (Fig.1). Almost all bacteria could grow on this media but efficient growth on this media was displayed by selected two

cultures i.e. *Bacillus thuringiensis* and *Alishewanella* sp. The accession number for *Alishewanella* sp and *B. thuringiensis* is JX298819 and JX298814 respectively.

**Fig.1: Growth of *Bacillus* species on acid blue dye containing media**



Species of *Alishewanella* sp are gram negative non-motile rods. Those species isolated from Lonar Lake could not degrade any of the sugar studied i.e. dextrose, mannitol, lactose, trehalose, arabinose, galactose, sorbitol, rhamnose, salicilin, xylose, aesculin and mallic acid. Whereas *Bacillus thuringiensis* degraded dextrose, trehalose and sorbitol.

**Optimization of physiological conditions:**

**Table1. Optimization of pH**

| ***Alishewanella* species** | | ***Bacillus thuringiensis*** | |
|---|---|---|---|
| **pH** | **Growth** | **pH** | **Growth** |
| 3 | - | 3 | - |
| 5 | - | 5 | + |
| 7 | ++ | 7 | ++ |
| 9 | +++ | 9 | +++ |
| 10 | +++ | 10 | +++ |
| 11 | +++ | 11 | +++ |
| 13 | +++ | 13 | +++ |
| Note: (+++) Maximum Growth, (++) Moderate Growth, (+) Low Growth, (-) No growth | | | |

Optimum pH for both the bacteria was found to be 9 but both bacteria displayed luxuriant growth till pH 13. There was no growth at pH 5 and below for *Alishewanella* sp (JX298819) species however a low degree of growth was shown by *B. thuringiensis* (JX298814) at pH 5 (Table1).

**Table2. Optimization of temperature**

| ***Alishewanella* species** | | ***Bacillus thuringiensis*** | |
|---|---|---|---|
| **Temperature ($^0$C)** | **Growth** | **Temperature ($^0$C)** | **Growth** |
| 10 | - | 10 | - |
| 30 | +++ | 30 | +++ |
| 40 | +++ | 40 | ++ |
| 50 | ++ | 50 | ++ |
| Note: (+++) Maximum Growth, (++) Moderate Growth, (+) Low Growth, (-) No growth | | | |

Optimum growth for both *Alishewanella* sp and *B. thuringiensis* was found at temperature 30$^0$C. *Alishewanella* species displayed excellent growth at 40$^0$C also and moderate growth at 50$^0$C as well. Moderate growth was found at 40$^0$C and 50$^0$C by *B. thuringiensis* (Table 2).

**Table3. Effect of salt on bacterial growth**

| ***Alishewanella* species** | | ***Bacillus thuringiensis*** | |
|---|---|---|---|
| **Salt conc. (%)** | **Growth** | **Salt conc. (%)** | **Growth** |
| 0.5 | + | 0.5 | + |
| 1. | +++ | 1. | +++ |
| 1.5 | +++ | 1.5 | +++ |
| 2.0 | +++ | 2.0 | +++ |
| 2.5 | ++ | 2.5 | ++ |
| Note: (+++) Maximum Growth, (++) Moderate Growth, (+) Low Growth, (-) No growth | | | |

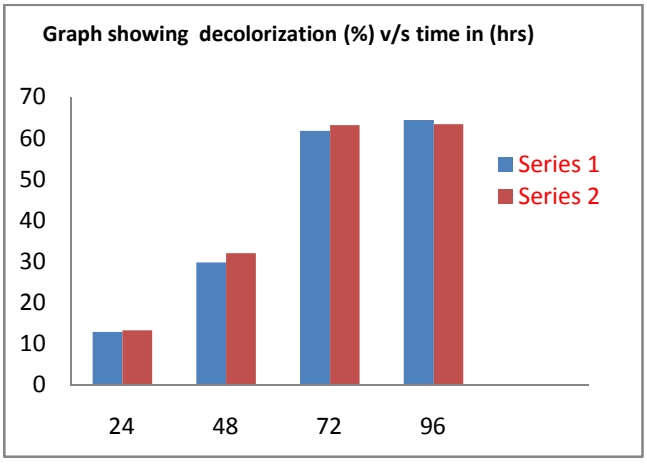
It was essential to determine growth at various salt concentrations. Both *Alishewanella* species and *B. thuringiensis* grew well at normal salt concnentrations used in case of mesophilic bacteria. These species have shown growth at quite higher levels than normally employed that is 2%. Moderate growth at 2.5% salt concentration was found in case of both bacteria indicating the survivability of these bacteria at higher salt concentrtaions (Table3).

**Table 4: Percentage Dye Decolorization By *Bacillus thuringiensis, Alishewanella* spp.**

| **Isolate** | **Time (hrs)** | **Absorbance (OD)** | **Decolourization (%)** |
|---|---|---|---|
| *Alishewanella* sp | 24 | 0.730 | 12.99% |
| *B. thuringiensis* | 24 | 0.727 | 13.34% |
| *Alishewanella* sp | 48 | 0.589 | 29.79% |
| *B. thuringiensis* | 48 | 0.570 | 32.06% |
| *Alishewanella* sp | 72 | 0.320 | 61.85% |
| *B. thuringiensis* | 72 | 0.309 | 63.17% |
| *Alishewanella* sp | 96 | 0.298 | 64.48% |
| *B. thuringiensis* | 96 | 0.307 | 63.40 |
| Initial O. D. was 0.839 | | | |

Periodic studies on dye decolorization were carried using *Alishewanella* species *and Bacillus thuringiensis*. After 24h only 12.99% and 13.34% of dye was decolorized respectively by *Alishewanella* species *and Bacillus thuringiensis*. Whereas dye was decolorized 29.79% and 32.06% after second day. Maximum dye was decolorized after 72h; in case of *Alishewanella* species it was 61.85% and that of *Bacillus thuringiensis* was 63.17%. Marginal differences were observed after 96h the enhancement in the decolorization was upto 64.48% and 63.40%. This suggests 72h period can be considered suitable for decolorization of acid blue dye (Table4 and graph1).

**Graph 2: Percent Dye Decolorization By *Alishewanella* sp. and *Bacillus thuringiensis***



## CONCLUSION

Bacteria in confined extreme environment have wide range of biotechnological potentials. Hence the study was undertaken to test the efficiency of thirty halophilic bacteria from Lonar Lake. The most efficient species were that of *Alishewanella* sp and *Bacillus thuringiensis*. Previous reports on bacterial dye decolorization deals with mesophilic bacteria that have limitations to growth optima. On the contrary halophiles in present study are able grow at wide range of pH and temperature optima and being salt tolerant can grow efficiently in textile dye possessing different chemical compositions. These bacteria have shown growth over 5 to 13 pH and temperature $30^0$C and $50^0$C. The dye decolorizing abilities have been studied over a four day period where we found that 72h period was most suitable to achieve maximum decolorization. The acid blue decolorized by *Alishewanella* sp and *Bacillus* species was 61.85% and 63.17% dye respectively.

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