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Original Article

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Isolation of Potential Azo dye Degrading/Tolerating Bacteria from Polluted Environment

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Various industries including the food and textile industries result in the release of azo dyes into the environment. These dyes are known to exert toxic effects on humans, animal and plants. Biological methods of azo dye bioremediation is a solution for the detoxification of azo dye in the environment. The present study was undertaken to isolate bacteria with potential for azo dye bioremediation. From five polluted environment samples, eight bacteria were isolated in azo dye supplemented Nutrient agar. Six isolates (75%, n=8) decolorized azo dye completely following 24 hours of incubation. The control tube with no bacterial inoculation remained blue, indicating that only microbial biotransformation processes were taking place. In each case, a blue-colored ring remained at the top, indicating the anoxic nature of the dye decolorization process. All isolates grew in presence of 400 ppm, 60% tolerated 600 ppm and 20% tolerated 800 ppm, whereas 1000ppm was inhibitory for growth of all isolates. Dead autoclaved cells of three representative isolates were tested for biosorption potential. Only one isolate turned the methylene blue supplemented nutrient broth colorless. This explains that this isolate was not metabolizing methylene blue rather it bound the dye to the cell structure. Two isolates did not show biosorption abilities indicating that their mechanism of bioremediation was enzymatic reduction. 16s rDNA sequencing identified two of the isolates as *Lysinibacillus capsici* and *Stenotrophomonas muris*.

Key words: Azo dye, degradation, bacteria, bioremediation

Introduction

Azo dyes are artificially colored compounds employed in numerous industries, including the textile, food, leather, and paper sectors, as well as in other sectors as additives in various goods [1]. Up to 40% of the dye pollution in developing nations like Bangladesh and India comes from these colors [2]. Azo dyes are widely used in the food business because they give processed meals a noticeable appearance and make them appealing to consumers [3]. In laboratories azo dyes are used as pH indicators or biological stains. In laboratories azo dyes are used as pH indicators or biological stains. In the textile industry, only 85 % of dye attach to clothes while the remaining 15 % is wasted in water as an effluent [4]. Some examples of azo dyes are Trypan Blue, Methyl Red, Allura Red, Sunset Yellow and Phenylazophenol. The degradation and reduction of azo dyes produce compounds that are harmful and mutagenic in nature [5]. They pose harmful effects like genotoxicity, mutagenicity, and carcinogenicity to humans along with other animals [6]. Oral exposure of azo dyes to human results in aromatic amines formation with carcinogenic properties by intestinal microflora and liver azo reductases. In highly industrial countries, intestinal cancer is prevailing, so there is a large connection between high cancer cases and huge azo dye uses in these countries. Beside this many other human maladies have also been observed [7]. Azo dyes can also pose toxic effects on plants, animals and other life forms [8].

Many different approaches (physical, chemical and biological techniques) are now being used by scientists for dyes removal from wastewater [9]. Various microorganisms are capable of degrading azo dyes, although the degradation efficiency is dependent on the strain [8]. Microbial biodegradation is cheap and economical with less sludge production [10]. Decolorization of azo dyes by different mixed and pure cultures of bacteria is reported under anoxic conditions[11]. The present study was aimed at isolation and identification of bacteria capable of azo dye biotransformation from the polluted environment.

Materials and Methods

Sample

A total of five samples were collected from Buriganga and Shitalakkha river and tannery and dyeing industry effluents in Hazaribag and Narayangonj.

Azo dye

Methylene blue dye was used as a representative azo dye in this study.

Isolation of bacteria

To isolate azo dye tolerant bacteria, 200 ppm methylene blue was added to nutrient agar plate. The environmental sample was diluted and spread on the plates and incubated at 37°C for 18-24 hours.

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Bacterial growth and dye decolorization

Dye decolorization was studied in 50 ppm methylene blue supplemented nutrient broth incubated at 37°C for 48h in static incubator. The tubes were observed at regular intervals to determine the decolorization efficiency of the isolates and to identify the microorganism with the fastest decolorizing ability.

Minimum concentration of methylene blue for growth inhibition

The isolates were inoculated on different concentrations of methylene blue supplemented agar plate to determine the minimum concentration of dye that totally inhibits microbial growth. Dye was added in concentrations of 400, 600, 800 and 1000 ppm to nutrient agar plates. Isolates were inoculated and incubated at 37°C for 96 hours. The plates were checked at regular intervals.

Azo dye biosorption assay

Equal volumes of living and dead (autoclaved) cells were inoculated into nutrient broth supplemented with 100 ppm azo dye and incubated at room temperature for 24 hours to check for decolorization. Dead cells that decolorize the broth indicate that the dye binds to the cell outer layer and not transformed.

Identification of microorganism by 16s rRNA gene sequencing

16s rDNA was amplified using primers reported earlier[12]. The amplicon was purified, sequenced and BLAST was used to identify the organism.

Results

Isolation of dye tolerant bacteria

A total of 8 bacteria were isolated from the 5 samples added to nutrient agar supplemented with 200 ppm of methylene blue. Figures 1 shows a representative plate and colonies growing in the presence of azo dye.

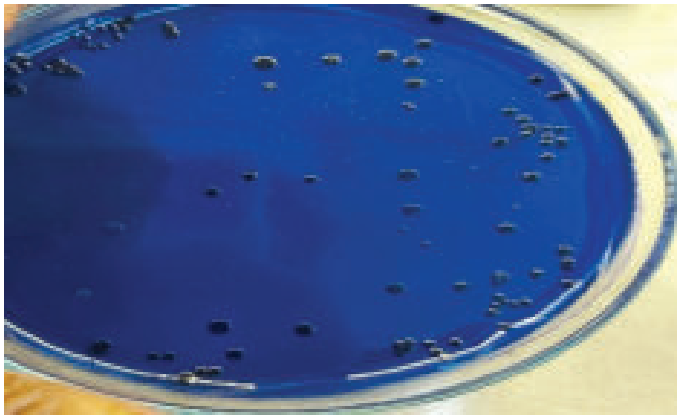

Figure 1. *A representative plate showing growth of bacteria in Nutrient Agar containing azo dyes*

The isolation rate from the five different samples is outlined in Table 1.

Table 1. *Isolation of eight bacteria from the environmental samples*

Sample no	Colonies
Sample 1	1A, 1B
Sample 2	2A
Sample 3	3A, 3B, 3C
Sample 4	No growth was observed
Sample 5	5A, 5B

Decolorization ability

Following 24 hours of incubation, six isolates (75%, n=8) decolorized azo dye completely. The remaining two isolates decolorized azo dye after 48 hours. A control tube with no bacterial inoculation remained blue, indicating that only microbial biotransformation processes were taking place. Figure 2 shows the time taken for decolorization by different bacteria. In each case, a blue-coloured ring remained at the top, indicating the anoxic nature of the dye decolorization process.

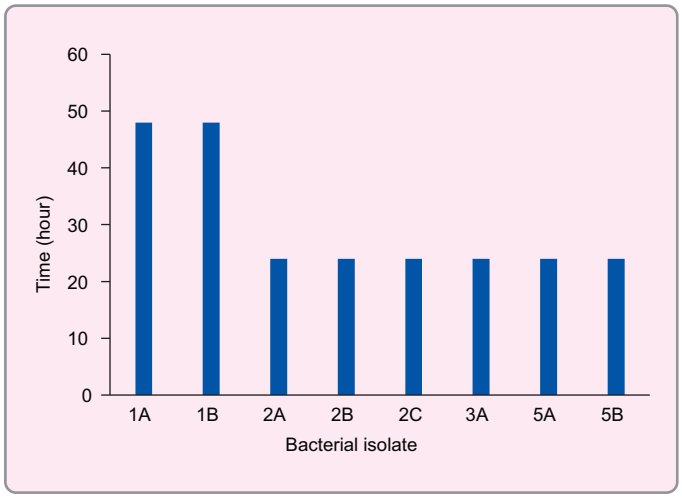

Figure 2. *Decolourization time taken by different isolated bacteria. The result of the control tube which did not decolorize is not shown.*

Tolerance of methylene blue

All the isolates were subcultured on 400 ppm, 600 ppm, 800 ppm and 1000 ppm methylene blue supplemented nutrient agar plate. The results are represented in Table 2. A concentration of 400 ppm was conducive for all isolates, whereas 1000ppm was inhibitory for growth of all bacteria isolated.

Table 2. *Tolerance of azo dye by different isolated bacteria*

Concentration of methylene blue (azo dye) (ppm)	Growth of bacteria (%)	Time taken (hours)
400	100	48
600	60	72
800	20	72
1000	No growth	-

Biosorption potential

This assay was done to determine the level of Methylene Blue adsorption onto microbial cells. Autoclaved cells of the isolates 5A, 3A, 3B, which showed fast decolorization potential, were grown in Nutrient broth supplemented with 50 ppm Methylene Blue for 24 hours to determine whether dead cells decolorized the dye, indicating to biosorption as the mechanism of decolorization. It was observed that isolate 5A turned the methylene blue supplemented nutrient broth colorless. This explains that this isolate was not metabolizing methylene blue rather it bound the dye to the cell structure. But the broth containing 3A and 3B remained blue indicating the possibility that they metabolized methylene blue.

Identification of microorganism by 16s rDNA gene sequencing

16s rDNA sequencing identified two of the isolates as *Lysinibacillus capsici* and *Stenotrophomonas muris*.

Discussion

Both bacteria and fungi are capable of azo dye degradation. Several bacteria have been reported to degrade azo dye[8]. In the case of fungi, adsorption/reduction or both are involved in the remediation of azo dye[13]. In the present study, one of the isolated bacteria demonstrated adsorption as its mode of bioremediation of azo dye. Two others indicated an enzymatic mode as dead cells were unable to decolourize the dye, indicating that enzymatic degradation, hence living cells, were needed for decolorization.

One of the isolates that involved enzymatic biodegradation was identified to be *Lysinibacillus capsici*. According to earlier research[14], *Lysinibacillus* spp. has been reported to effectively decolorizes manmade dyes. They have been shown to detoxify the azo dyes used in the dyeing processes. Textile effluent non-adapted *Lysinibacillus sphaericus* MTCC 9523 was observed to decolorize selective reactive azo dyes under optimal operating conditions. Visual color alterations using Reactive Yellow F3R (RYF3R) and Joyfix Red RB (JR) Fourier Transform Infrared (FTIR) decolorization of 96.30% and 92.71%, respectively. The functional groups of the degraded metabolites were also revealed by spectroscopy, and the lack of the peak for the azo group indicated that the azo link had broken. Difference in control dye retention time compared to treated sample on High-Performance Liquid Chromatography (HPLC) analysis, with peaks at 2.413 and 2.895 min for RYF3R. UV-visible spectrophotometric examination revealed biodegradation by this organism and confirmed dye decolorization with times of 2.466 min and 1.640 min for Joyfix Red RB[14].

Another isolate was identified to be *Stenotrophomonas muris* in the present study. In a previous study, *Stenotrophomonas maltophilia* was found to be capable of decolorizing pigments after being isolated from the composted neem oil-seed cake[15]. The effectiveness of the aforementioned isolate to breakdown malachite green was demonstrated by the kinetic analysis of dye degradation. Additionally, the influence of substrate concentration, pH, temperature, and agitation on the isolate's ability to decolorize malachite was investigated. At 28–30 °C and 150 rpm, the MG degradation rate was somewhat higher in a neutral pH range. The dye sample was analyzed using UV, HPLC, and FTIR techniques before and after the bacteria were added, and the results showed that *S. maltophilia* TPDM-1 had a high capacity for removing malachite green from the media. The genetic analysis of the isolate using PCR amplification and sequencing revealed the presence of the laccase and triphenylmethane reductase genes, respectively, "lac" and "tmr"[15]. Due to time constraints, genetic characterization of the isolates was not performed in the current investigation. Future research can focus on genetic profiling and metabolite identification to further understand the mechanisms underlying dye reduction.

The present study can be extended to determine environmental factors which are optimum for azo dye degradation and determine whether metabolites are produced upon incubation with bacteria to specify enzymatic degradation. The finding of potential bacteria capable of azo dye degradation points to possible natural biodegradation occurring in the environment.

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