

Optimization of factors affecting decolourization of sulphonated azo dye Red HE7B *in vitro* by *Bacillus* sp. Azo1

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Azo dyes are widely used organic molecules that are generally recalcitrant to biodegradation, thus causing environmental pollution. In this study, the effect of various growth conditions like temperature, pH, dye concentration, aeration and presence/absence of co-substrate on decolourization of azo dye Red HE7B by *Bacillus* sp. Azo1 has been determined. Decolourization of the dye was achieved under static condition. Optimum decolourization of Red HE7B was observed at 30°C and 50 mg/l dye concentration, where more than 90% decolourization was achieved within three days of inoculation. *Bacillus* sp. Azo1 could decolourize the dye through a range of pH values from 6 to 9. Presence of co-substrate limited the decolourization with glucose being the most potential inhibitor. Identification of factors influencing decolourization will help in rapid removal of these pollutants from contaminated habitat.

Keywords: Azo dye, *Bacillus* sp., co-substrate, decolourization.

USE of synthetic dyes and colourants is on an increase nowadays. These dyes and pigments find application in paper, textile, food, cosmetics and pharmaceutical industries. Azo dyes are the most extensively used synthetic dyes accounting for more than 70% of total industrial dye demand¹. Representatives of these dyes are recalcitrant to degradation due to a high degree of chemical, biological and photocatalytic stability², and have the potential for persistence and accumulation in the environment³. They are considered as environmental pollutants and can pose a threat to human health due to their role in carcinogenesis⁴⁻⁶. Textile industry accounts for 80% consumption of these dyes and is among the largest consumer of dyes and pigments⁷. Considerable portion of these dyes enters into the freshwater bodies through effluent discharge due to inefficient dyeing process and interferes with aquatic flora and fauna by impeding light penetration and reducing dissolved oxygen level, thus increasing biological oxygen demand of the freshwater ecosystem. In soil, entry of azo dyes through contaminated irrigation water can eventually upset the soil biological processes, includ-

ing nutrient cycling. Sulphonated azo dyes are difficult to remove from water using wastewater treatment strategy due to their high water solubility. Red HE7B is a reactive sulphonated diazo dye extensively used in textile dyeing. Though being a xenobiotic, many microorganisms have been found to decolourize these dyes by means of various oxidative and reductive enzymes. Biodecolourization of several azo dyes by *Aeromonas hydrophila*⁸, *Pseudomonas* sp.⁹, *Rhizobium radiobacter*¹⁰, *Bacillus subtilis*¹¹ has been reported. In the present study, the factors affecting decolourization have been optimized for maximum decolourization of Red HE7B by *Bacillus* sp. Azo1 isolated from a textile mill effluent contaminated soil.

Bacterial culture *Bacillus* sp. Azo1 (NCBI GenBank accession no. HQ640949) was previously isolated from textile mill effluent contaminated soil characterized and preserved at the Division of Microbiology, Indian Agricultural Research Institute (IARI), New Delhi.

Culture media include: nutrient broth (g l⁻¹) peptone (5.0 g), beef extract (3.0 g), sodium chloride (5.0 g) and pH (7.0).

The culture was grown in nutrient broth for 24 h on a shaker at 30°C, followed by amending the medium with filter sterilized dye at 50 mg/l concentration. One set of culture broth was incubated on an orbital shaker at 200 rpm at 30°C and the other under static conditions in BOD incubator at 30°C. Since maximum decolourization was observed under static conditions, further evaluation of other factors affecting decolourization of Red HE7B was carried out under these conditions.

The nutrient broth was amended with filter-sterilized dye at final concentration of 25, 50, 100, 200 and 500 mg/l. These flasks were incubated at 20°C, 25°C, 30°C, 35°C, 40°C and 45°C under static conditions.

The pH of nutrient broth medium was adjusted to 3, 5, 6, 7, 8, 9 and 11 with 0.1 N NaOH or HCl and incubated at 30°C.

Nutrient broth medium was supplemented with 1% glucose, sucrose, starch and citric acid. Unamended nutrient broth was used as control for comparison. Three replications for each treatment were maintained in all the experiments. Decolourization was recorded on the day of inoculation and at an interval of 24 h for 8 days at 543 nm using a spectrophotometer (Perkin Elmer model Lambda E2201). Percentage of decolourization was calculated as described elsewhere⁷.

Multiple comparisons of mean were done by Fisher's LSD using statistical software SAS 9.4. Maximum decolourization of Red HE7B was observed under static conditions and nearly 99% of the dye was decolourized by the eighth day of incubation. Aeration did not have a positive effect on decolourization and no significant increase in decolourization was observed up to four days when incubation was done on a shaker (Figure 1).

At 20°C temperature, maximum decolourization up to 78% was observed at 25 mg/l dye concentration and least

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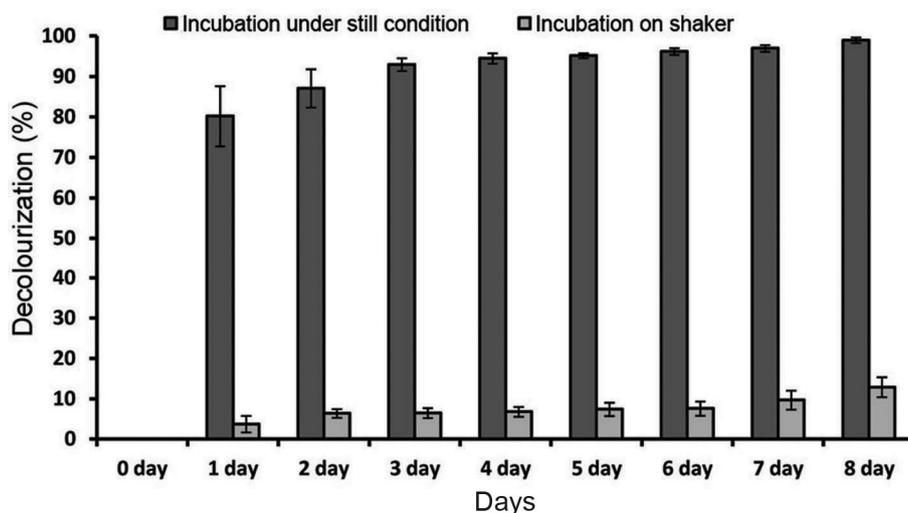


Figure 1. Effect of aeration on decolourization of sulphonated azo dye Red HE7B by *Bacillus* sp. Azol (error bar represents \pm standard deviation of three replications).

at 500 mg/l. With increase in dye concentration, there was significant decrease in decolourization efficiency; however, with increase in incubation time continuous increase in decolourization was recorded. Maximum decolourization was observed on day 8 of incubation. Higher dye decolourization rate was recorded in the initial days and a drop in the rate of decolourization was observed after three days of incubation. At 200 mg/l concentration, more than 75% decolourization of dye was recorded after eight days of incubation at 25°C. No significant difference in percentage of decolourization was observed at 25 and 50 mg/l dye concentration from the third day onwards at this temperature. At both these concentrations, >90% decolourization was observed at the end of eight days. At 30°C temperature, 100% decolourization of dye was observed on day 8 of incubation at dye concentration 25 and 50 mg/l. At these two concentrations, 94% and 79% decolourization respectively, was recorded after only 24 h of incubation. Decolourization as high as 89% at 100 mg/l, 71% at 200 mg/l and 39% at 500 mg/l dye concentration was observed at this temperature. Significant decrease in decolourization was observed with increase in dye concentration. At 25 mg/l dye concentration, increase in percentage of decolourization at 24 h interval was not significantly different; however, significant difference in decolourization percentage between day 1 and day 8 was observed. At 50 and 100 mg/l dye concentration on day 4 and day 5 onwards, no significant increase in decolourization percentage was observed at 24 h interval. At 35°C, more than 90% of decolourization was observed at the end of day 8 at 25, 50 and 100 mg/l concentration of dye. No significant increase in decolourization percentage at 25 and 50 mg/l dye concentration was recorded at 24 h interval after day 3 to day 6 of incubation. At this temperature as high as 80% decolourization was recorded at 200 mg/l dye concentration. Significant increase in decolourization with time was observed at 40°C, but with

increase in dye concentration to 500 mg/l, lower decolourization was noticed. With further increase in temperature to 45°C, there was very little increase in decolourization after 24 h of incubation. Dye decolourized at a high rate within 24 h of incubation at 25 and 50 mg/l concentration only and more than 70% of dye was decolourized in this time interval. At this temperature, the decolourization at 25 and 50 mg/l dye concentration was at par with each other on different days. Here also, the lowest decolourization was observed at 500 mg/l dye concentration.

Results of the experiment showed that 100% decolourization occurred only at 25 and 50 mg/l dye concentration. Also, more than 85% of decolourization at these two concentrations was noticed at all the temperatures, except 20°C. Maximum decolourization was observed after 24–48 h in every case, beyond which the decolourization rate was slow. Very little decolourization was observed at 500 mg/l dye concentration, making it the limiting concentration for decolourization. Maximum decolourization for all the chosen dye concentrations was recorded at 30°C; this was most suitable temperature for decolourization. Based on the results of this experiment, 50 mg/l dye concentration and 30°C temperature were found to be most appropriate for decolourization, and hence these were followed for rest of the experiment (Figure 2a and b).

Effect of pH on decolourization was determined at seven different pH values. Very less decolourization was observed at pH 3 and as the pH increased, the percentage of decolourization also increased. Though the decolourization was relatively slow with time at pH 11 compared to other pH values, nevertheless, the isolate could decolourize 89% to 95% of the dye at pH 5–11. Rapid decolourization (>80%) was observed after 24 h only at pH 6–9. Highly acidic pH did not favour decolourization, however, the decolourization rate was not significantly affected at alkaline pH. No significant difference in rate of decolourization was observed from pH 6 to 9, and it was

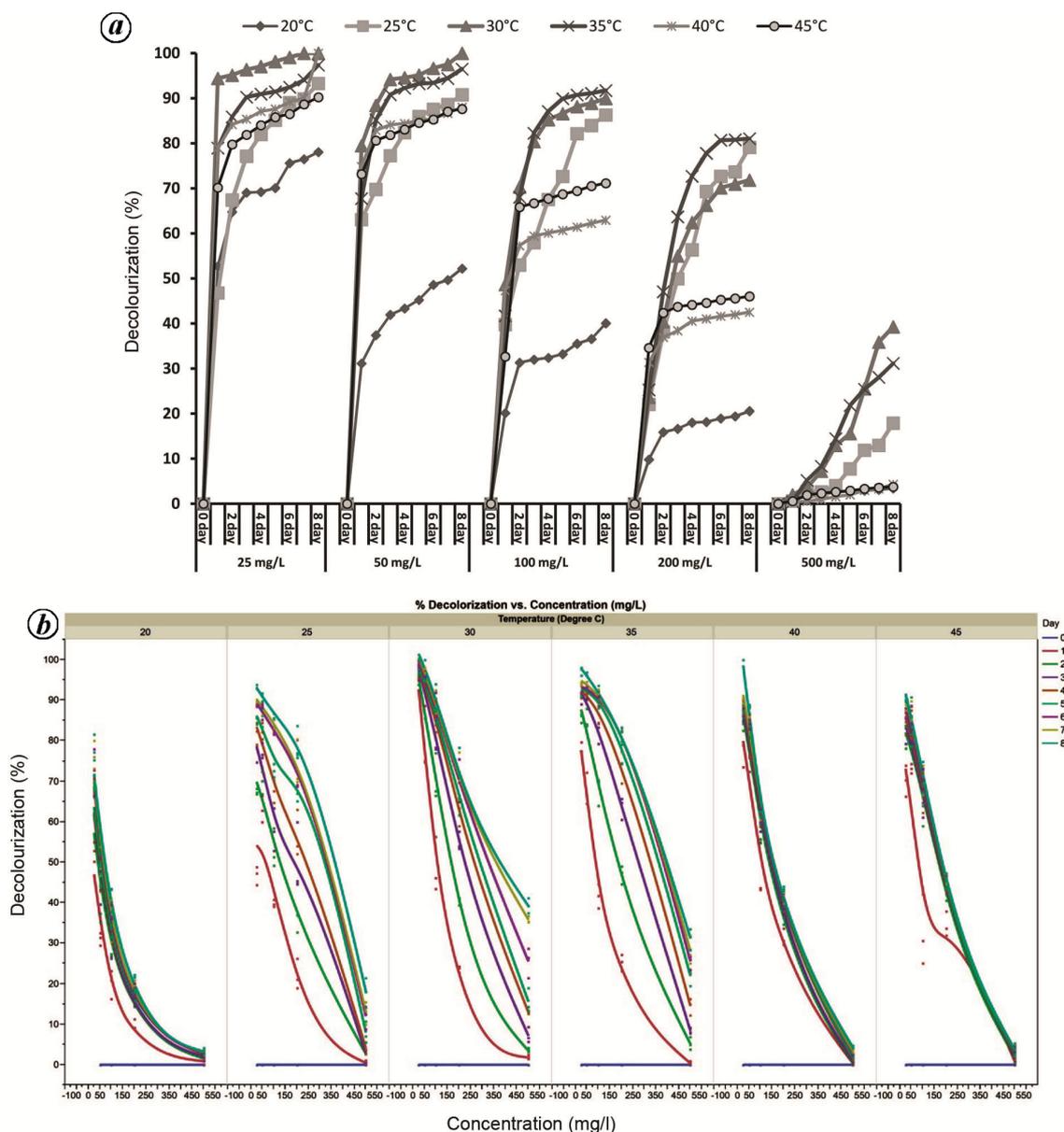


Figure 2. Individual (a) and interactive (b) effect of dye concentration, temperature and incubation time on decolourization of Red HE7B by *Bacillus* sp. Azol1.

observed to be at par throughout the incubation period (Figure 3).

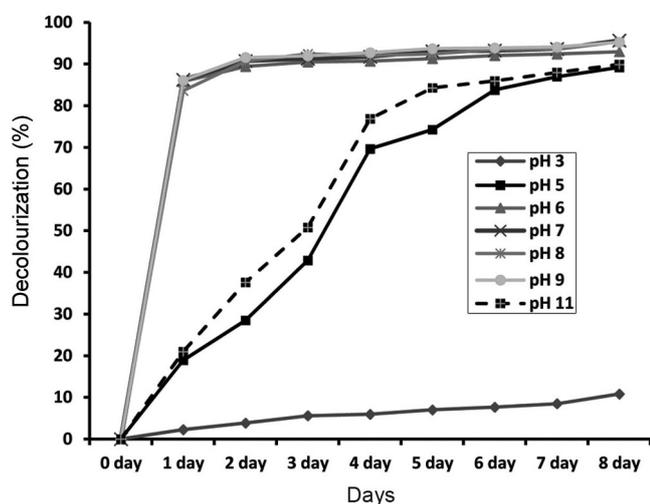
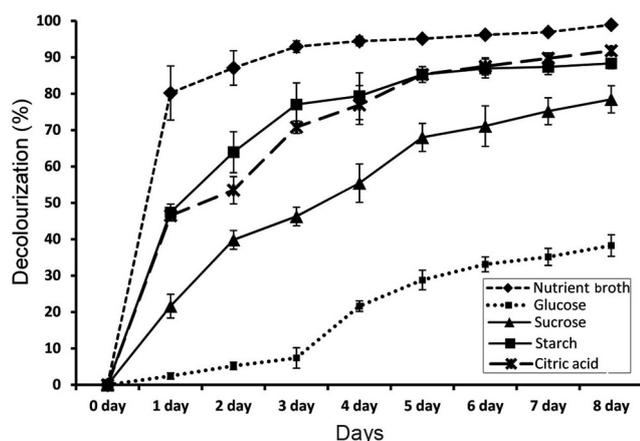
Different carbon sources were evaluated for their effect on rate of decolourization of Red HE7B. Rate of dye decolourization was observed to be faster in the absence any additional carbon source. Significant difference was observed in decolourization when different carbon sources were added to the nutrient broth. There was fast decolourization up to 80% within 24 h in nutrient broth. All the additional carbon sources had inhibitory effect on the rate of decolourization; however, citric acid was found to be least inhibitory followed by starch and sucrose. Maximum inhibition was observed when glucose was used as the additional carbon source (Table 1 and Figure 4).

Decolourization of Red HE7B took place under static conditions and oxygen showed inhibitory effect on decolourization. Bacteria generally decolourize azo dyes under reducing condition using flavins, quinines or external redox mediators like azoreductase enzymes for reduction of azo bonds¹². This process is generally inhibited by oxygen since it strongly competes with the azo group for electrons, resulting in lowered decolourization in the presence of oxygen^{8,13,14}. Two-fold increase in activity of azoreductase was observed during decolourization of Red HE7B7. Decolourization of monoazo dye reactive Red 195 under microaerophilic condition has been reported by Khan *et al.*¹⁵.

Effect of different dye concentrations and temperatures on decolourization indicated that though decolourization

Table 1. Individual and interactive effect of additional carbon source and day (time) on decolourization of azo dye Red HE7B by *Bacillus* sp. Azo1 at $P = 0.05$

Treatment	DF	Sum of squares	Mean squares	F	Pr > F
Carbon source	4	63415.7230	15853.9307	1643.0573	<0.0001
Day (time)	8	76901.0944	9612.6368	996.2270	<0.0001
Carbon source * day	32	10961.6113	342.5504	35.5010	<0.0001

**Figure 3.** Effect of pH on decolourization of Red HE7B by *Bacillus* sp. Azo1.**Figure 4.** Effect of presence/absence of additional carbon sources on decolourization of Red HE7B by *Bacillus* sp. Azo1.

occurred from 25°C to 45°C, maximum decolourization of highest dye concentration was achieved at 30°C only. At lower temperatures, the decolourization rate was slow, but as the temperature increased decolourization was favoured and rapid decolourization was observed. This could be due to 30°C being the optimum temperature for its growth, and with increase/decrease in temperature there was slowing down of growth with consequent decrease in the rate of decolourization. Very little decolourization at higher concentration of 500 mg/l may be due to inhibitory effect of the parent dye itself on the

microbes. Azo dye containing one or more sulphonic acid groups on aromatic rings absorbs to cell wall of microbes¹⁶, or inhibit nucleic acid synthesis¹⁷, thus adversely affecting the growth of microorganisms. Inhibitory effect of azo dye methyl red was observed on the growth of *Kocurea rosea*, *Pseudomonas aeruginosa* and *Azotobacter vinelandii*¹⁸, thus supporting our findings.

There was rapid decolourization of the dye within 24 h of its addition; however, in all the cases decrease in the rate of decolourization was observed beyond 24 h. This could be due to accumulation of degradation products of dye, which could have an adverse effect on the growth of microorganisms. The degradation product of the dye Reactive Black had greater toxicity than the parent form when assayed against bioluminescent bacterium *Vibrio fischeri*¹⁹.

The pH of the medium did not significantly influence decolourization, as the processes occurred from pH 5 to 11. Reduction in the rate of decolourization at pH less than 5 could be due to lower initial cell mass (OD at 24 h of inoculation was 0.3). At pH 5 initially there was slow decolourization, but at the end of the experiment 89% of decolourization was recorded. The utilization of peptone and beef extract could have produced alkaline products like ammonia²⁰ and brought pH of the medium in the favourable range of decolourization. At higher pH, once again low cell mass at the beginning could have attributed to slow decolourization.

Higher rate of decolourization of the dye was observed when peptone and beef extract were used as carbon sources. Supplementation of medium with additional carbon sources decreased the rate of decolourization. Carbohydrates are an easily assimilable carbon source and in the presence of a readily assimilable carbon source, the metabolism of a complex carbon source like peptone and beef extract will be hindered²¹. Consequently, regeneration of NADH, a crucial decolourization rate determining factor, will be slowed down resulting in the lower rate of decolourization²². Supplementation of the medium containing complex carbon source like peptone, beef extract or yeast extract with simpler carbon sources like glucose, sodium citrate, etc. has been reported to decrease the rate of decolourization of azo dyes²³. Metabolisms of peptone or beef extract lead to regeneration of NADH that can act as an electron donor for reduction of azo bonds²⁴. Complex carbon and energy sources like yeast extract might act as a source of redox mediators like riboflavin and niacin, which can accelerate the transfer of

biologically generated reducing equivalents to reduce azo dyes to the corresponding amines, thus leading to decolorization of the dye²⁵. Reduced decolorization in the presence of glucose might be due to the glucose repression that prevents expression of azoreductase, as reported by Chang *et al.*²⁶.

In this study, we have evaluated different factors affecting the decolorization of diazo dye Red HE7B by *Bacillus* sp. Azo1, isolated from textile mill effluent contaminated soil. Optimum decolorization was recorded under static conditions. The decolorization of dye was observed at temperature range from 25°C to 45°C, but optimum temperature for decolorization was found to be 30°C. At this temperature, 100% decolorization up to 50 mg/l dye concentration and 71% decolorization up to 200 mg/l dye concentration was observed. Optimum concentration for dye was 50 mg/l and 500 mg/l was the most inhibitory concentration. The decolorization of Red HE7B took place at a wide pH range from 5 to 11, with optimum decolorization at pH 7. Nutrient broth, i.e. peptone and beef extract was the best media substrate for decolorization, and presence of additional carbon sources did not favour decolorization. This study will have implications in rapid removal of colours from dye-contaminated effluents under optimized biological system before they are discharged.

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ACKNOWLEDGEMENTS. J.K.T. thanks ICAR-IARI, New Delhi, for providing the infrastructure facilities and Council of Scientific and Industrial Research, New Delhi, for providing financial support in the form of fellowship.

Received 21 October 2017; accepted 3 May 2018

doi: 10.18520/cs/v115/i3/505-509