Title: Optimization of factors affecting decolourization of sulphonated Azo Dye Red HE7B *in vitro* by *Bacillus* sp. Azo1.

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Abstract

Azo dyes are widely used organic molecules that are generally recalcitrant to biodegradation thus cause environmental pollution. 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. Azo1was determined. Decolourization of the dye was achieved under anaerobic. Optimum decolourization of Red HE&B was observed at 30°C and 50mg/L dye concentration where more than 90% decocolurization was achieved within 3days of inoculation. *Bacillus* sp. Azo1 could decolourize the dye through a range of pH 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.

Key words: Azo dye, Decolourization, Red HE7B, *Bacillus* sp.

Use of synthetic dyes and colourants are on increasing trend. These dyes and pigments find application in paper, textile, food, cosmetics and pharmaceutical industries. Azo dyes are the most extensively used synthetic dyes accounting more than 70% of total industrial dye demand[1]. Representatives of these dyes are recalcitrant to degradation due to a high degree of chemical, biological and photocatalytic stability[2] and has potential for persistence and accumulation in the environment[3]. They are considered as environmental pollutants and can pose threat to human health due to their role in carcinogenesis[4-6]. Textile industries accounts for 80% consumption of these dyes and are among the largest consumer of dyes and pigments[7]. Considerable portion of these dyes enters into the fresh water bodies through effluent discharge due to inefficient dyeing process and interfere with aquatic flora and fauna by impeding light penetration, 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 including nutrient cycling. Sulfonated azo dyes are difficult to remove from water using waste water treatment strategy due to its high water solubility. Red HE7B is a reactive sulfonated diazo dye extensively used in textile dyeing. Though, being a xenobiotic, many micro organisms 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] have been reported. In present investigation the factors affecting decolourization were optimized for maximum decolorization of Azo dye Red HE7B by *Bacillus* sp. Azo1 isolated textile mill effluent contaminated soil.

Materials and methods

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Bacterial culture: Bacterial culture *Bacillus* sp. Azo1 (NCBI gene bank accession no HQ640949) was previously isolated from textile mill effluent contaminated soil characterized and preserved in division of microbiology IARI, New Delhi.

Culture media: Nutrient broth (gL^{-1}) peptone-5.0 g, Beef extract-3.0g; Sodium Chloride-5.0g, pH- 7.0

Evaluation of different factors affecting decolourization of Red HE7B by Bacillus sp. Azo 1

Effect of Aeration - The culture was grown in nutrient broth for 24 hours on shaker at $30^{0}C$ followed by amending the medium with filter sterilized dye at 50mg/L concentration. One set of culture broth was incubated on orbital shaker at 200 rpm at $30^{0}C$ and other under static condition in BOD incubator at $30^{0}C$. Since maximum decolourization was observed under static conditions, further evaluation of other factors affecting decolourization of Red HE7B were carried out under static conditions.

Effect of temperature and dye concentration- 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, 25, 30, 35, 40 and $45^{0}C$ under static condition.

Effect of pH - The pH of nutrient broth media was adjusted to 3, 5, 6, 7, 8, 9 and 11 with 0.1N NaOH or HCl and incubated at $30^{0}C$.

Effect of different co-substrate on decolourization of Red HE7B

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 day of

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inoculation and at interval of 24 hours for 8 days at 543 nm using Perkin Elmer spectrophotometer (model Lambda E2201). Per cent decolourization was calculated as described by elsewhere[7].

Statistical analysis

Multiple comparisons of mean were done by Fisher's LSD using statistical software SAS 9.4.

Results

Effect of aeration on decolourization of Red HE7B

Maximum decolourization of Red HE7B was observed under static conditions and nearly 99% of the dye was decolourized by 8th day of incubation. Aeration did not have a positive effect on decolourization and no significant increase in decolourization was observed up to 4th days when incubation was done on shaker (Fig1).

Effect of different temperature and dye concentrations on decolourization of Red HE7B.

At 20^{0}C temperature maximum decolourization up to 78% was observed at 25 mg/L dye concentration and least was observed 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 8th days 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 8th days of incubation at 25^{0}C. No significant difference in per cent decolourization was observed at 25 and 50 mg/L dye concentration 3rd day onwards at this temperature. At both these concentration >90%

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decolourization was observed at the end of 8th day. At 30 ^{0}C temperature, 100% decolourization of dye was observed on 8th day of incubation at dye concentration 25 and 50 mg/L. At these two concentrations 94% and 79% decolourization was recorded after only 24 hours 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 per cent decolourization at 24 hour intervals was not significantly different, however, significant difference in per cent decolourization between 1st day and 8th day was observed. At 50 mg/L and 100 mg/L dye concentration also on 4th and 5th day onwards no significant increase in per cent decolourization was observed at 24 hours interval. At 35^{0}C also more than 90% decolourization was observed at the end of 8th day at 25, 50 and 100 mg/L concentration of dye. No significant increase in per cent decolourization at 25 and 50 mg/L dye concentration was recorded at 24 hours interval after 3rd to 6th day 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^{0}C but with increase in dye concentration to 500 mg/L lower decolourization was noticed. With further increase in temperature to 45^{0}C there was very little increase in decolourization after 24 hours of incubation. Dye decolourized at a very high rate within 24 hours of incubation at 25 and 50 mg/L concentration only and more than 70% dye was decolourized in this time interval. At this temperature the decolourization at 25 and 50 mg/L dye concentration was at par to each other on different days. Here also lowest decolourization was observed at 500 mg/L dye concentration.

Results of above experiment showed that 100% decolourization occurred only at 25 and 50 mg/L dye concentration. Also, more than 85% decolourization at these two concentrations

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was noticed at all the temperatures except 20^0C. Maximum decolourization was observed after 24-48 hours in every case, beyond which decolourization rate was slow. Very little decolourization was observed at 500 mg/L dye concentration making it as limiting concentration for decolourization. Maximum decolourization for all the chosen dye concentrations was recorded at 30^0C, and it the most suitable temperature for decolourization. Based on the results of this experiment, 50 mg/L dye concentration and 30^0C temperature was found to be most appropriate for decolourization and hence this concentration and temperature was followed for rest of the experiment (Fig. 2a and Fig. 2b).

Effect of hydrogen ion concentration on decolourization of Red HE7B

Effect of pH on decolourization was determined at seven different pH. Very less decolourization was observed at pH 3 and as the pH increased, the per cent decolourization also increased. Though the decolourization was relatively slow with time at pH 11 compared to other pH nevertheless, the isolate could decolourized 89 to 95% of dye at pH 5 to 11. Rapid decolourization (>80%) was observed after 24 hours only at pH 6 to 9. Highly acidic pH did not favour decolourization, however, decolourization rate was not significantly affected at alkaline pH. No significant difference in rate of decolourization was observed form pH 6 to pH 9 and it was observed to be at par on throughout the incubation period (Fig 3).

Effect of different co-substrate on decolourization of Red HE7B

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 hours in

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nutrient broth. All the additional carbon sources had inhibitory effect on rate of decolololourization, however citric acid was found to be least inhibitory followed by starch and sucrose. Maximum inhibition was observed when glucose was used as additional carbon source (Table 1 and Fig 4).

Discussion

Different factors affecting biodecolourization of Red HE7B a sulfonated diazo dye by isolate *Bacillus* sp. Azo1 was investigated.

Decolourization of Red HE7B took place under static condition and oxygen had 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 bonds[12]. This process is generally inhibited by oxygen since it strongly competes with the azo group for electrons resulting lowered decolourization in 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 was also reported by Khan et al,[15].

Effect of different dye concentrations and temperature on decolourization indicated that though, decolourization occurred from 25 to 45^{0}C, maximum decolourization of highest dye concentration was achieved at 30^{0}C only. At lower temperature the decolourization rate was slow but as the temperature increased the decolourization was favoured and rapid decolourization was observed. This could be due to 30^{0}C being optimum temperature for its growth and with increase/decrease in temperature there was slowing down of the growth with the consequent decrease in the rate of decolourization. Very little decolourization at higher concentration of 500

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mg/L may be due to inhibitory effect of parent dye itself on the microbes. Azo dye containing one or more sulfonic acid groups on aromatic rings might act as detergent[16] or inhibit nucleic acid synthesis[17]or inhibits the cell growth of micro organism. Inhibitory effect of azo dye Methyl red was observed on growth of *Kocurea rosea*, *Pseudomonas aerugenosa* and *Azotobacter vinelandii*[18] support our findings.

There was rapid decolourization of the dye within 24 hours of the dye addition however in all the cases decrease in the rate of decolourization observed beyond 24 hours. This could be due to accumulation of degradation products of dye which could have adverse effect on the growth of micro organism. 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 medium did not significantly influence the decolourization, as decolourization occurred from pH 5 to 11. Reduction in rate of decolourization at pH less than 5 could be due to lower initial cell mass (OD at 24 hrs of inoculation was 0.3). At pH 5 initially there was slow decolourization but at the end of experiment 89% decolourization was recorded. The utilization of peptone and beef extract could have produced alkaline products like ammonia[20] and brought the pH of the media in favourable range of decolourization. At higher pH once again low cell mass at 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 easily assimilable carbon source and in presence of a readily assimilable carbon source the metabolism of complex carbon source like peptone and beef extract will be hindered[21]. Consequently regeneration of NADH, a crucial

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decolourization rate determining factor, will be slowed down resulting in the lower rate of decolourization[22]. 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 dyes[23]. Metabolisms of peptone or beef extract lead to regeneration of NADH that can acts as electron donor for reduction of azo bonds[24]. Complex carbon and energy sources like yeast extract might act as source of redox mediators like riboflavin and niacin which can accelerates the transfer of biologically generated reducing equivalents[25] to reduce azo dyes to the corresponding amines, thus leading to decolourization of the dye. Reduced decolourization in presence of glucose might be due to the glucose repression that turns off expression of azoreductase as reported by Chang et al,[26].

Conclusion

In this study evaluation of different factors affecting the decolourization of diazo dye Red HE7B by *Bacillus* sp. Azo1, isolated from textile mill effluent contaminated soil. Optimum decolourization was recorded under static condition. The decolourization of dye was observed at temperature range from 25 to 45^{0}C but optimum temperature for decolourization was found to be 30^{0}C. At this temperature 100% decolourization up to 50 mg/L dye concentration and 71% decolourization up to 200 mg/L dye concentration was observed. Optimum concentration for dye was 50 mg/L and 500 mg/L was most inhibitory concentration. The decolourization of Red HE7B took place at wide range of pH from 5 to 11 with optimum decolourization at 7 pH. Nutrient broth i.e peptone and beef extract was best media substrate for decolourization and presence of additional carbon sources did not favour decolourization. The study will have implication in rapid removal of colours from dye contaminated effluents under optimized biological system before its discharge.

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

Treatments	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

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Figure legends

Figure 1. Effect of Aeration on decolourization of Sulphonated azo dye Red HE7B by *Bacillius* sp. Azo1 (error bar represents ± standard deviation of three replication)

Figure 2. Individual (a) and interactive (b) effect of Dye concentration, temperature and incubation time on decolourization of Sulphonated azo dye Red HE7B by *Bacillius* sp. Azo1

Figure 3. Effect of pH on decolourization of Sulphonated azo dye Red HE7B by *Bacillius* sp. Azo1

Figure 4. Effect of presence/absence of additional carbon sources on decolourization of Sulphonated azo dye Red HE7B by *Bacillius* sp. Azo1

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Figure 1.

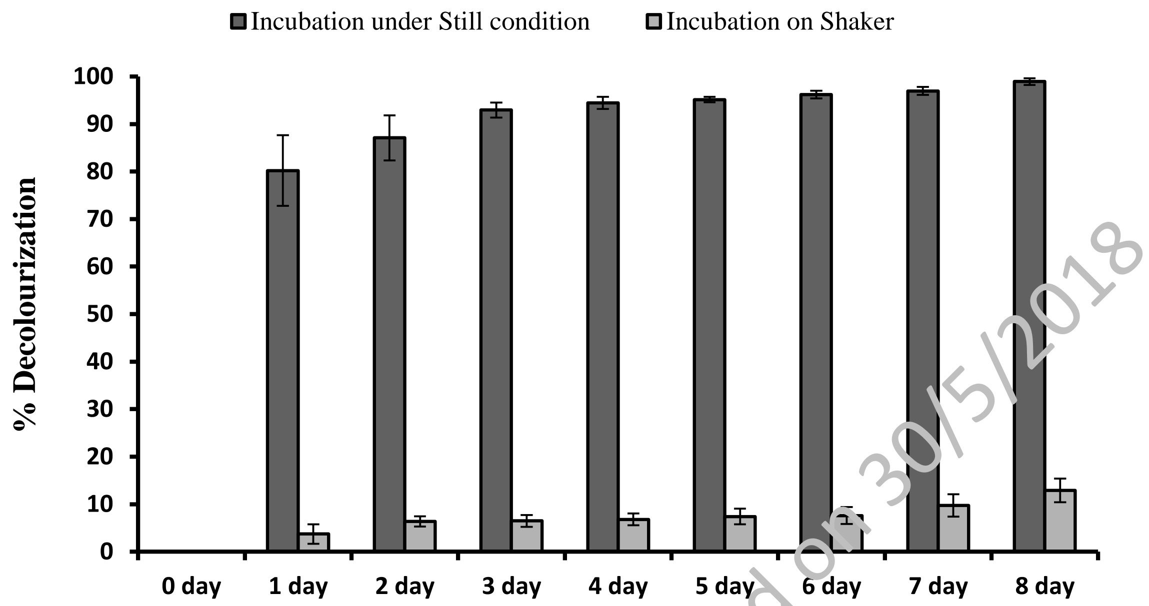

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Figure 2a.

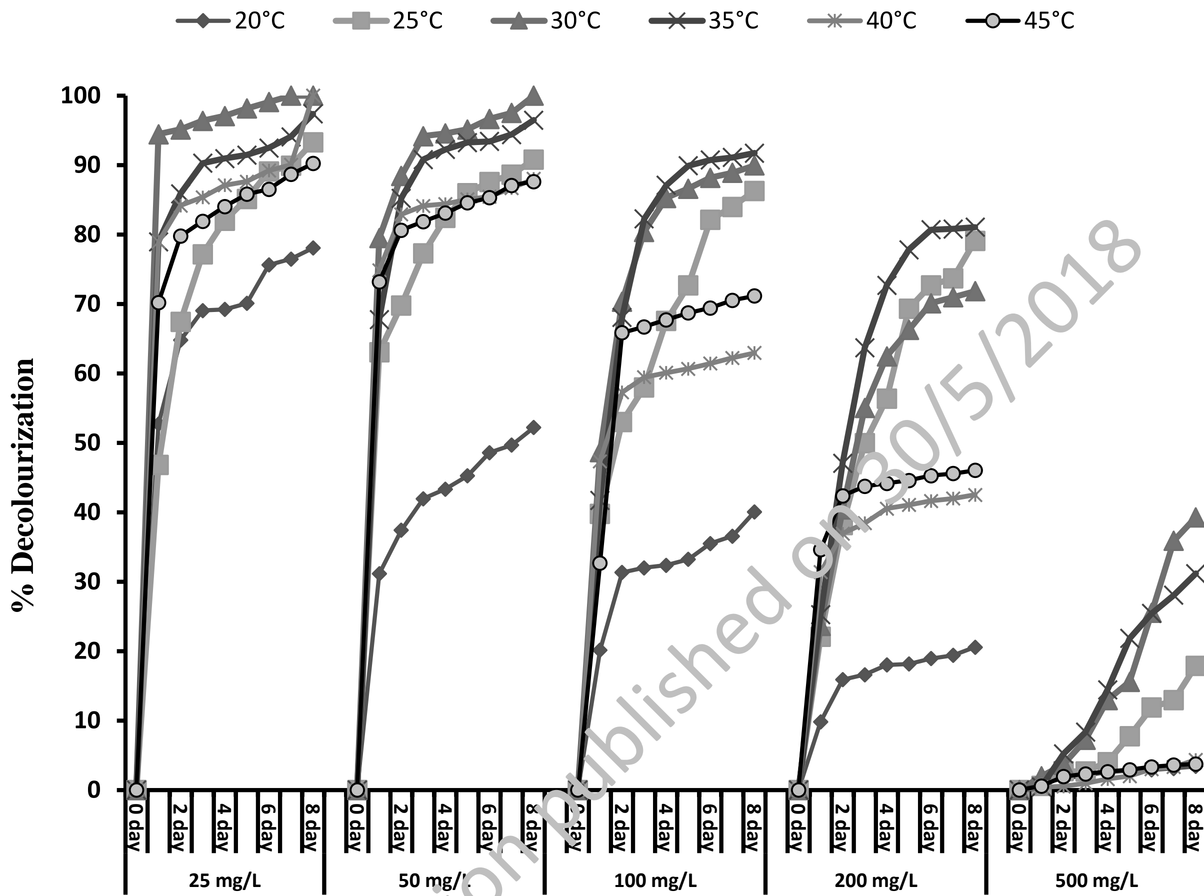

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Figure 2b.

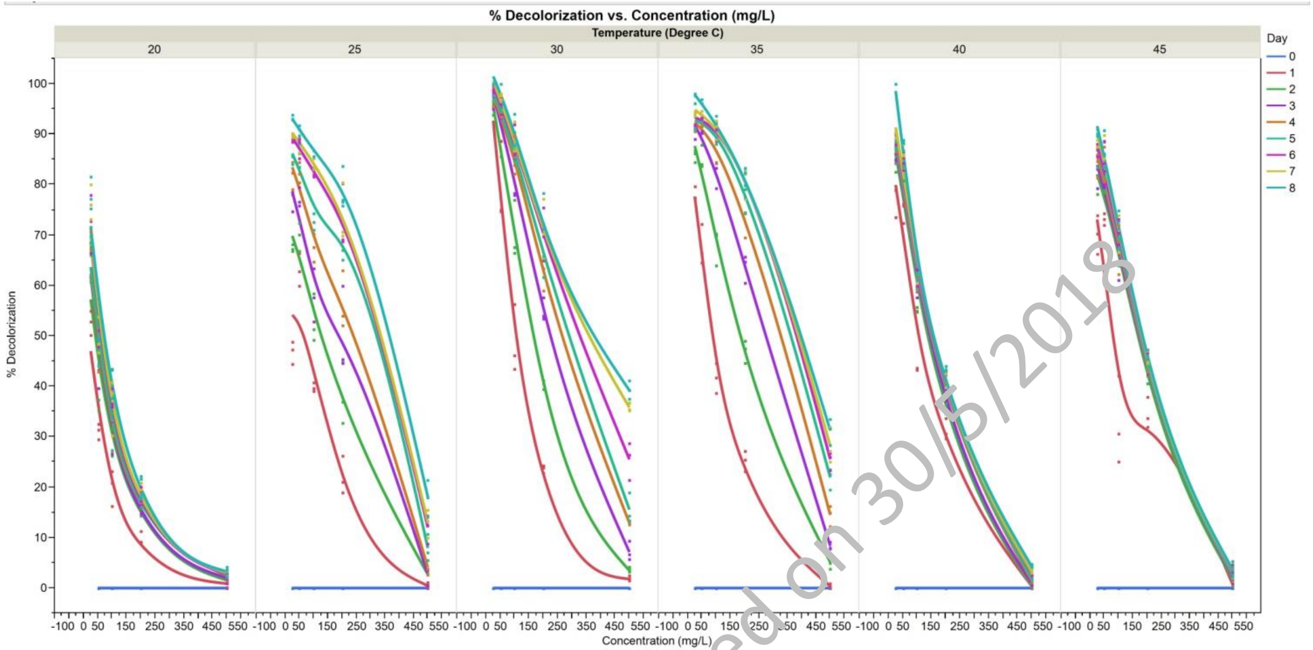

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Figure 3.

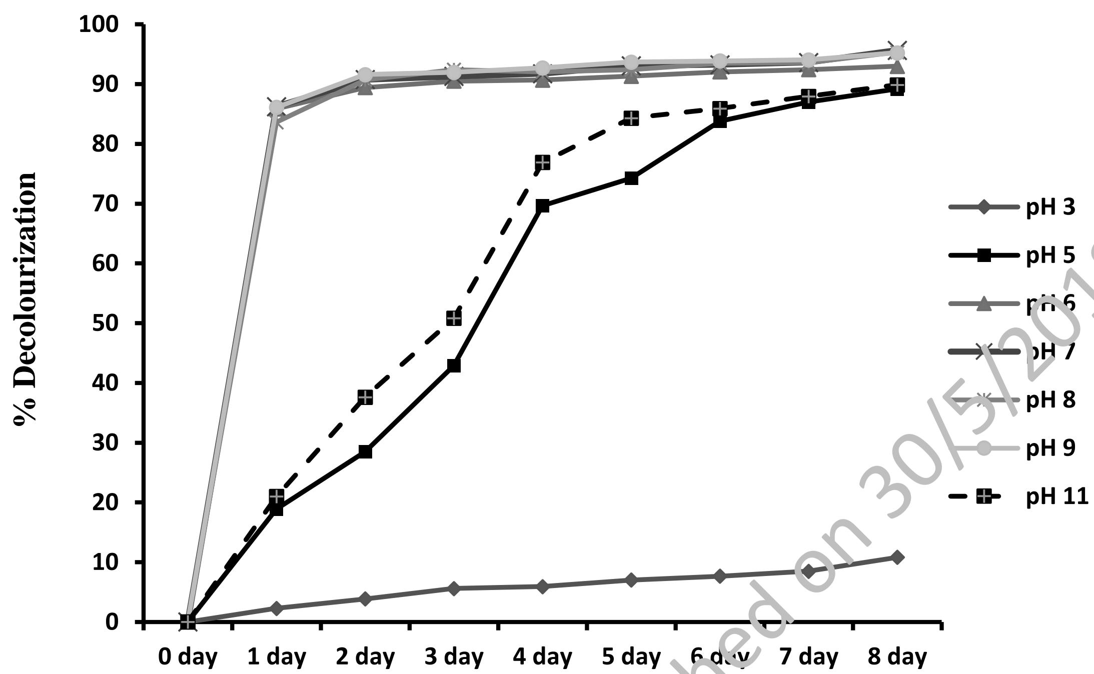

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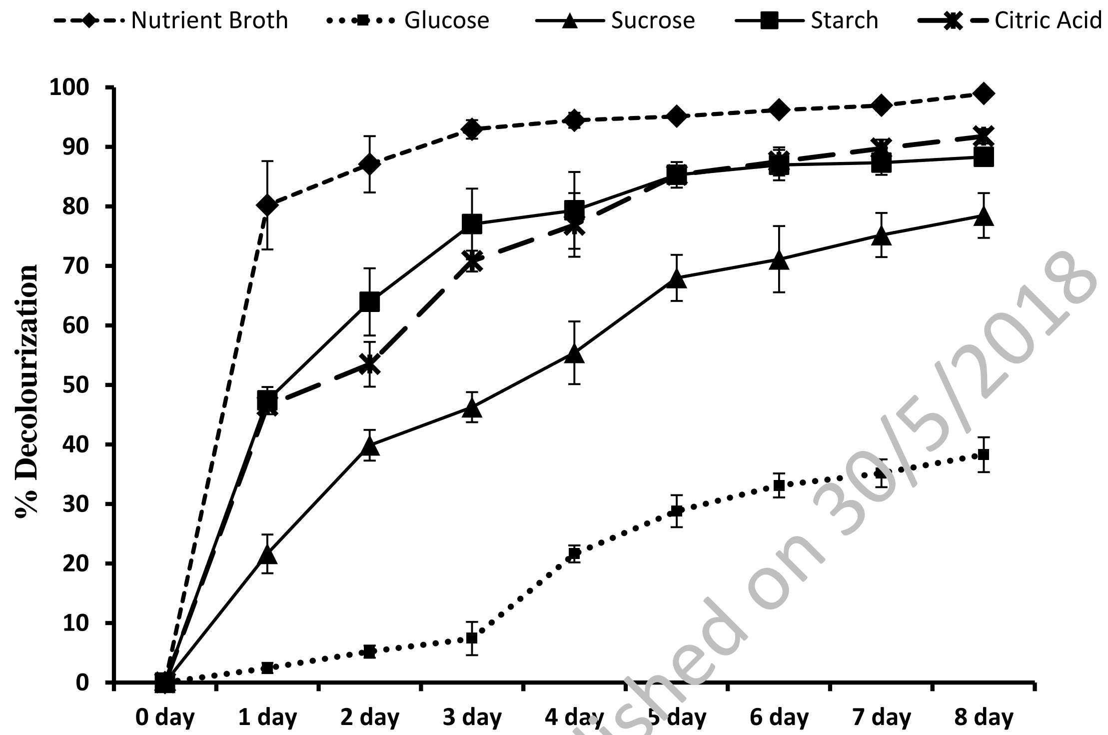
Figure 4.



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