



Decolourization of azo dye by indigenous bacteria and its impact on seed germination

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Abstract

Textile dyes which are released in environment directly without proper treatment are a potential threat to living organisms. Hence, the present study was designed to isolate azo dye degrading bacteria from a dyes contaminated environment and assessing their ability to degrade reactive dyes into non-toxic product. Three bacteria isolated from textile effluents were identified by 16S rDNA sequencing as *Enterobacter* sp. S20112, *Bacterium* MJ20 and *Enterobacter aerogenes* strain HK20-1. It was found that pH 7.0 and temperature 28°C was suitable for decolourization of Red 3B dye by isolated bacteria. Among these three isolates, *Enterobacter* sp. decolourized 31.85% and 21.52%, *Bacterium* MJ20 decolourized 36.58% and 23.72% and *Enterobacter aerogenes* strain HK 20-1 decolourized 56.58% and 36.58% of 100 ppm and 200 ppm dye respectively. However, the decolourization rate was increased up to 93.44% by yeast extract supplementation. Similarly, the decolourization rate was augmented when *Bacterium* MJ20 and *Enterobacter aerogenes* strain HK 20-1 was used together. The effect of dyes from textile industry on seed germination of three leguminous crops pea (*Pisum sativum* L.), lentil (*Lens esculentum* L.) and gram (*Cicer arietinum* L.) were studied. Results revealed that textile dye Red 3B had inhibitory effects on seed germination and seedling growth on test crops. But, deleterious effects of Red 3B dye on seed germination and seedling growth were reduced when dye was used after treatment with isolated bacteria. Thus, these bacterial isolates showed some potentiality to be utilized for the bioremediation of textile effluents into non-toxic form to plants.

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Introduction

Textile dyes are a potential hazard to living organisms (Padhi, 2012). Recently, there are more than ten thousand dyes available commercially and seven lakh tons of dyes are produced annually (Zollinger, 1987). Among them, Azo dyes are the largest and most versatile class of dyes and account for more than 50% of the dyes produced annually.

A large percentage of the synthetic dye does not bind and is lost to the waste stream during the coloration process (Weber and Adams, 1995). Approximately 10-15% dyes are released into the environment during dyeing process making the effluent highly colored and aesthetically unpleasant (Saratale *et al.*, 2011).

The textile and clothing manufacturing is one of the industries with the largest representation in the industrial structure of Bangladesh and has always taken a prominent role in terms of employment and weight in the national economy (Islam *et al.*, 2013).

The textile industries consume the largest amount of dyestuffs, at nearly 60-70% and include a wide range of activities, from preparation of raw material to pretreatment, dyeing and finishing of textile material. In Bangladesh, it is common to discharge textile dyeing effluents to agriculture crop areas without treatment of water (Saha *et al.*, 2017).

The effect of dyeing industry effluent on different plants has been investigated by several workers (Dayama, 1987; Sujatha *et al.*, 1992; Himabindu and Reddy, 2005). However, the adverse effects of textile effluents on plants depend on the type of species, stage of life cycle of plant affected by, and types and concentrations of toxic materials in the effluent.

In view of such perspectives, the present investigation was conducted to evaluate the impact of textile effluent on germination in three leguminous crops *viz.* pea (*Pisum sativum* L.), lentil (*Lens esculentum* L.) and gram (*Cicer arietinum* L.) because seed germination is an important and vulnerable stage in the life cycle of terrestrial angiosperms.

During the past three decades, several physical, chemical and biological decolourization methods have been accepted by the paper and textile industries (McMullan *et al.*, 2001; Crini, 2006; Kalyani *et al.*, 2008; Saratale *et al.*, 2009). Wide range of microorganisms including bacteria, fungi, yeasts, actinomycetes and algae capable of degrading azo dyes have been reported (McMullan *et al.*, 2001; Bafana and Chakrabarti, 2008; Chen *et al.*, 2009; Saha *et al.*, 2017; Ruhi *et al.*, 2017; Rahman *et al.*, 2019). Hence, the present investigation was also aimed to exploit the decolourization abilities of indigenous bacterial species and their characterization.

Materials and methods

Sample collection

Effluents were collected from different Textile Dyeing Industries in Sathia, Sirajgonj, Gazipur, Madhapdi, Narshingdhi, Bangladesh. Samples were collected from different places, such as drainage canal that carry stagnant textile effluent. Samples were in the form of liquid untreated effluent and untreated sludge. All the samples were collected in sterile plastic bottles and sterilized polythene bags and preserved at 4°C in refrigerator within 24 hours.

Dyes and Media

Azo dye Red 3B was procured from Dysin-Chem limited, Dhaka. All media components and chemicals used in the studies were of analytical grade.

Enrichment and isolation of dye degrading bacteria

All samples (untreated textile effluents) were used for isolation of dye decolourizing bacterial cultures by enrichment culture techniques using Luria-Bertani (LB) medium amended with 20ppm of the test dye Red 3B for adaptation of the microorganisms. For isolation of azo dye decolourizing bacteria, serial dilutions (10^{-1} - 10^{-6}) of enriched samples were inoculated into LB agar plates containing 20ppm respective dye. LB agar plates were prepared by dissolving 1 g NaCl, 1 g tryptone and 0.5 g yeast extract in 100/ml distilled water, pH adjusted at 7 and then 1.5 g agar was added in the 250 ml flasks.

The medium was autoclaved at 121°C and 15 lbs/inch² pressure for 15 min. Bacterial colonies that showed a clear decolourization zone around them on LB agar medium were picked and cultured for 24 hours in MS medium amended with 1 ml/1 TE solution. The growth of the bacterial colonies was observed after 24 hours of incubation at 35°C. Effect of dyes on the growth of bacterial isolates was determined in Minimal Salt Medium (MSM) which contained (g/l): K₂HPO₄, 2; (NH₄)₂SO₄, 0.5; KH₂PO₄, 0.2; MgSO₄·7H₂O, 0.05 amended with 1 ml/1 Trace Element (TE) solution containing (g/l): FeSO₄·7H₂O, 0.4; ZnSO₄·7H₂O, 0.2; MnSO₄, 0.4; CuSO₄, 0.04; KI, 0.3; Na₂MoO₄, 0.05; CoCl₂, 0.04; (pH 7) and supplemented with 20ppm azo dye Red 3B. After certain period of incubation from the decolourized test tubes the dye degrading bacterial strains were stored on LB agar plate at 4°C for further test.

Screening of decolorizing bacteria

The bacterial isolates capable of decolourizing textile azo dye Red 3B were used to inoculate into test tubes, each containing 10 ml MS medium supplemented with 1 ml/L TE solution and amended with 20ppm test dye as the sole source of C and N.

The inoculated tubes were incubated at 35°C for 24 hrs under static condition and centrifuged at 10,000 rpm for 10 minutes. The cell free supernatant from each test tube were plated onto MS agar medium and incubated at 35°C for 48 hrs. Morphologically distinct colonies were selected and purified by streaking twice on LB agar medium. Single colony were transferred on to the agar slant and stored at 4°C for further studies.

Antibiotic sensitivity test

Sensitivity of antibiotic to the isolated bacteria was performed as described by Saha (Saha *et al.*, 2009).

Decolourization activity test

Decolourization activity was expressed in terms of percentage decolorization and was determined by monitoring the decrease in absorbance at absorption maxima (λ_{max}) using UV-Visible spectrophotometer.

The un-inoculated MS medium supplemented with respective dye was used as reference. At different time intervals, the 2ml samples were collected from reaction mixture and centrifuged at 10000 rpm for 10 minute to separate biomass.

The concentration of dye in the supernatant was determined by monitoring the absorbance at the maximum absorption wavelength (λ_{max}) at 660 nm. The decolourization assay was calculated according to the following formula-

$$\text{Dye Decolorization (\%)} = \frac{\text{Initial absorbance} - \text{Final absorbance}}{\text{Initial absorbance}} \times 100$$

Influence of different parameters on process of dye decolourization

The effect of initial dye concentration and co-substrate (yeast extract) concentration on decolourization of Red 3B dye by isolated bacteria was examined after 96 hours of incubation as described previously (Saha *et al.*, 2017). Briefly, to test the effect of different dye concentrations on their decolourization, MS media supplemented with 100 and 200 ppm Red 3B dye were adjusted to pH 7. Then, the media inoculated with bacterial isolates were incubated at 35 °C for 192 hours. To investigate the effect of yeast extract on decolourization, MS media supplemented with 0 %, 0.1 %, 0.5 %, 1 % or 1.5% of yeast-extract, and 100 ppm dye were adjusted to pH 7. Then, the media inoculated with bacterial isolates were incubated at 35 °C for 192 hours.

Identification and characterization of Dye decolourizing bacterial strain

Genomic DNA was extracted from dye decolourizing bacteria using CTAB method (Smith *et al.*, 1989). The PCR primers used to amplify 16S rDNA fragments were the bacteria-specific primers a forward primer F27 (5' – AGAGTTTGATCCTGGCTCAG – 3'; Tm: 61°C); and a reverse primer R1391 (5' – GACGGGCGGTGTGTRCA – 3'; Tm: 67.4°C). A total of 25 µl of reaction mixture consisted of – water 15µl, MgCl₂ 2.5µl, buffer 2.5, dNTPs 0.5µl, template 1µl, primer (forward 2 µl and reverse 2 µl). The PCR amplification was performed by Swift™ Minipro

Thermal Cycler (Model: SWT-MIP-0.2-2, Singapore) using the following program: Denaturing at 95°C for 5 minutes, followed by 40 cycles of 40 seconds of denaturing at 95°C, 60 seconds of annealing at 65°C and 2 minutes of elongation at 72°C with a final extension at 72°C for 10 minutes. Then, the PCR products were subjected to 1% agarose gel electrophoresis, stained with ethidium bromide and visualized on a UV transilluminator for the presence of about 1500 bp PCR products.

The amplified PCR product was cleaned by using a AccuPrep® Gel Purification Kit (Bioneer corporation, Korea) in accordance to the manufacturer's protocol. PCR amplified 16S rDNA of the screened isolates was sent for automated sequencing (Applied Biosystem 3130) to the Centre for Advanced Research in Science (CARS) under Dhaka University, Bangladesh. The sequence generated from automated sequencing of PCR amplified DNA was analyzed through NCBI BLAST (<http://www.ncbi.nlm.nih.gov>) program to find out possible similar organism through alignment of homologous sequences. Finally, the isolates were identified based on alignment of partial sequence of 16S rDNA with the existing sequences available in the database.

Seed germination test

In this experiment, the effect of four different concentrations of Red 3B dye was evaluated on

germination of seeds of three leguminous crops: pea (*Pisum sativum* L.), lentil (*Lens esculentum* L.) and gram (*Cicer arietinum* L.). Seeds were soaked in solution with different concentrations of dye (50, 100, 200 and 400 ppm) for 12 hours. Then, 10 seeds were placed on a Whatman filter paper in sterilized petri plate. The filter papers were wetted with 5 ml of distilled water (control) or with various concentration of dye in distilled water at every 12 hours. All dishes were kept in room temperature (28±2 °C) for the period of 7 days. At the end of the germination experiment, the root and shoot length were measured in cm/plant. In another independent experiment, 200 ppm dye was treated with different bacterial isolates for 96 hours. After 96 hours, the treated dye was used for seed germination experiment as described above.

Results

In this study potential textile dye decolourizing bacteria were isolated from effluents of the textile industries. Four morphologically distinct bacteria (Isolate A, B, C and D) were isolated from effluent which were able to decolourize Red 3B dye. Among them, 3 isolates (Isolate A, B and C) were selected for 16S rDNA based identification as well as characterization. Result of NCBI blast showed the highest similarity for isolate A (99% similarity) to *Enterobacter* sp. S20112, isolate B (90% similarity) to *Bacterium* MJ20 and isolate C (90% similarity) to *Enterobacter aerogenes* strain HK 20-1.

Table 1. Antibiotic sensitivity pattern of the isolated strains. Here, (5-10mm) = Resistance to antibiotic (R), (15-20mm) = Sensitive to antibiotic (S), (10-15mm) = intermediate resistance (I).

Name of antibiotics	<i>Enterobacter</i> SP. S20112	<i>Bacterium</i> MJ20	<i>E. aerogenes</i> strain HK 20-1
Kanamycin (30 µg)	S	S	S
Doxycycline (30 µg)	S	I	S
Cefradine (25 µg)	S	R	R
Tetracycline (30 µg)	S	S	S
Erythromycin (15 µg)	S	R	R
Ampicillin (25 µg)	R	R	R
Rifampicin (5 µg)	I	R	R
Streptomycin (10 µg)	I	I	S
Azithromycin (30 µg)	S	R	R
Gentamycin (10 µg)	S	S	I
Ciprofloxacin (5 µg)	S	R	S
Amoxicillin (30 µg)	R	R	R
Cefalexin (30 µg)	R	R	R
Neomycin (30 µg)	I	S	S

All three isolates grow optimally at 28°C and pH 7

Optimum temperature for growth of these 3 bacterial isolates was determined at pH7 in nutrient broth medium.

For *Enterobacter* SP. S20112, the maximum growth rate (OD 0.66) was observed at 28° C while the minimum growth rate (OD 0.22) at 37° C (Fig. 1A).

For *Bacterium* MJ20 the maximum growth rate (OD 0.67) was observed at 28°C and minimum growth rate (OD 0.32) at 20°C (Fig. 1B). Similarly, for *E. aerogenes* strain HK 20-1 the maximum growth rate (OD 0.72) was observed at 28°C while the minimum growth rate (OD 0.07) was observed at 20°C (Fig. 1C). Altogether, the optimum temperature for growth of the studied strains was found to be 28°C.

Table 2. Effect of initial concentration of dye on decolourization activity by bacteria.

Name of the Isolate	Time (hours)	Decolourization activity (%)	
		100 ppm	200 ppm
<i>Enterbacter</i> SP	48	4.329	3.09
	96	16.81	10.76
	192	31.85	21.52
<i>Bacterium</i> MJ20	48	8.383	4.53
	96	22.961	11.52
	192	36.58	23.72
<i>Enterobacter aerogenes</i> ___ strain HK 20-1	48	17.10	8.383
	96	42.76	22.96
	192	56.58	36.58

Optimum P^H for growth of these 3 strains was determined at 28°C temperature in liquid broth medium. For *Enterobacter* SP. S20112 the maximum growth (OD 0.89) was observed at pH 7 and minimum growth (OD 0.28) at pH 6 (Fig. 2A). *Bacterium* MJ20 exhibited maximum growth (OD 0.86) at pH 7 and minimum growth (OD 0.28) at pH 6 (Fig. 2B). Similarly, the maximum growth rate (OD 0.87) at pH 7 and minimum growth rate (OD 0.28) at pH 6 was observed for *E. aerogenes* strain HK 20-1 (Fig. 2C). Altogether, the optimum pH for growth of the studied strains was found to be pH 7.

Antibiotic sensitivity pattern of the isolated strains

Study of antibiotic sensitivity pattern is vital to maintain pure culture of a bacterial isolate as well as to take a decision for using it safely in any environmental application.

In this study the pattern of antibiotic sensitivity and resistance of isolating bacterial strains to 14 different antibiotics were tested by disk diffusion method using nutrient agar medium. All of these 3 strains showed mixed pattern of antibiotic sensitivity (Table 1).

Effect of textile dye concentration on decolourization

Effect of initial dye concentration on dye decolourization rate by these 3 bacterial isolates was measured. It was found that the percentage of decolourization activity decreased as the initial dye concentration increased (Table 2). It was found that 31.85%, 36.58% and 56.58% decolourization were achieved after 192 hours incubation by *Enterbacter* SP., *Bacterium* MJ20 and *Enterobacter aerogenes* strain HK 20-1 respectively when initial concentration of dye was 100 ppm (Table 2). But, decolourization rate decreased when initial concentration of dye was 200 ppm.

Effect of yeast extract (Co-substrate) on decolourization

It was reported that different concentration of yeast extract plays an important role for decolourization activity (Saha *et al.*, 2017). Here, effect of yeast extract on decolourization of Red 3B dye by one of three isolates (*Enterobacter aerogenes* strain HK 20-1) was studied. It was found that the decolourization activity was increased with the increase of concentration of the co-substrate (Fig. 3).

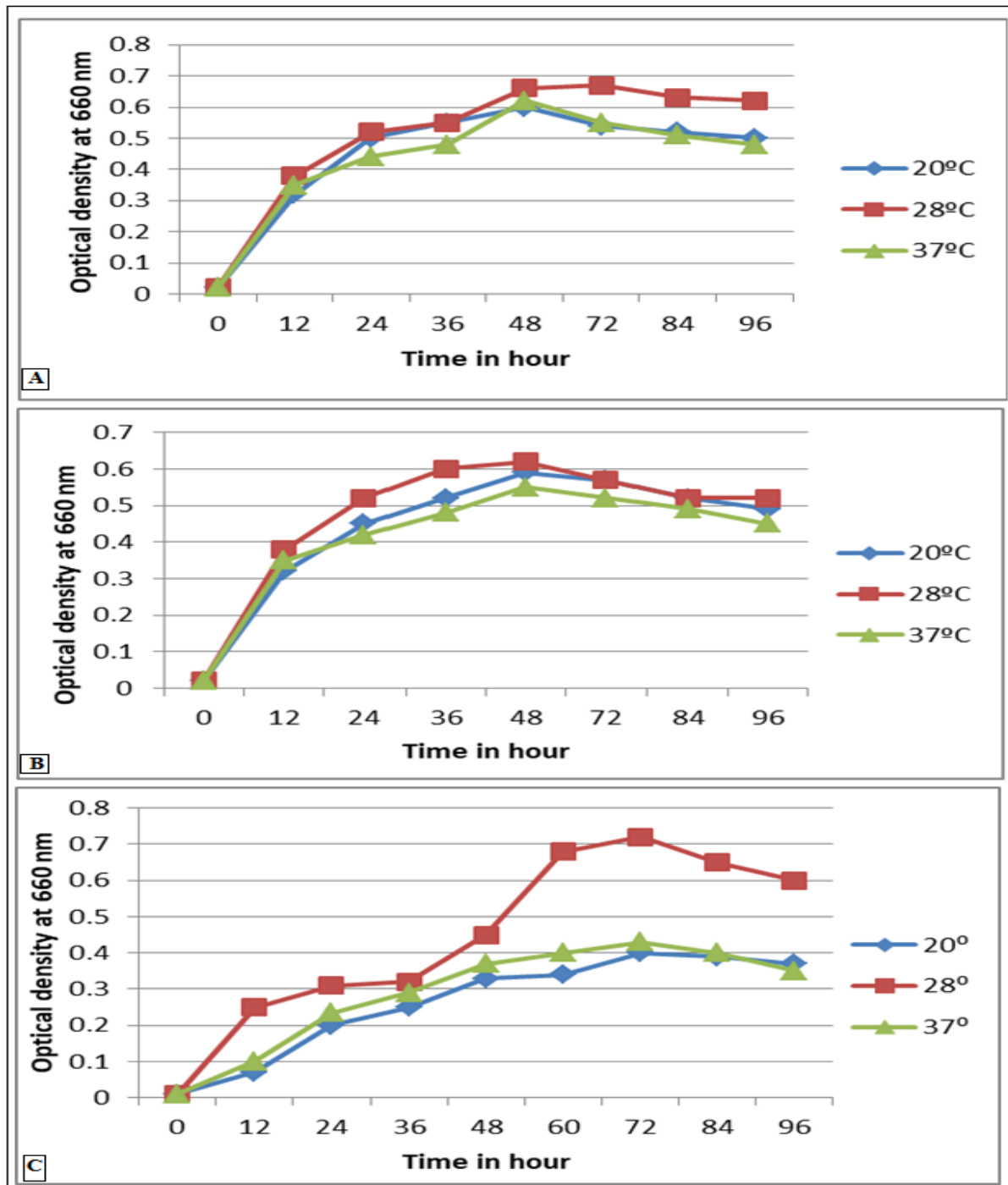


Fig. 1. Optimum temperature for growth of the bacterial strains *Enterobacter* SP. S20112 (A), *Bacterium* MJ20 (B) and *E. aerogenes* strain HK 20-1 (C) at pH 7.0. The optimum temperature of bacterial growth was determined at every 12-hours interval up to 96 hours incubation at 20°C, 28°C and 37°C by measuring optical density at 660 nm.

Effect of synergism activity on decolourization of Red 3B dye

In this experiment the decolourization activity increased when mixed microbial populations (*Bacterium* MJ20 and *Enterobacter aerogenes* HK 20-1) were used to achieve rapid decolourization rate

(Fig. 4). The highest decolourization activity (85.52%) was achieved when consortium of these two bacteria were used. The lower decolourization activity (36.58% and 56.58%) was achieved when these two bacterial strains used separately (Fig.4).

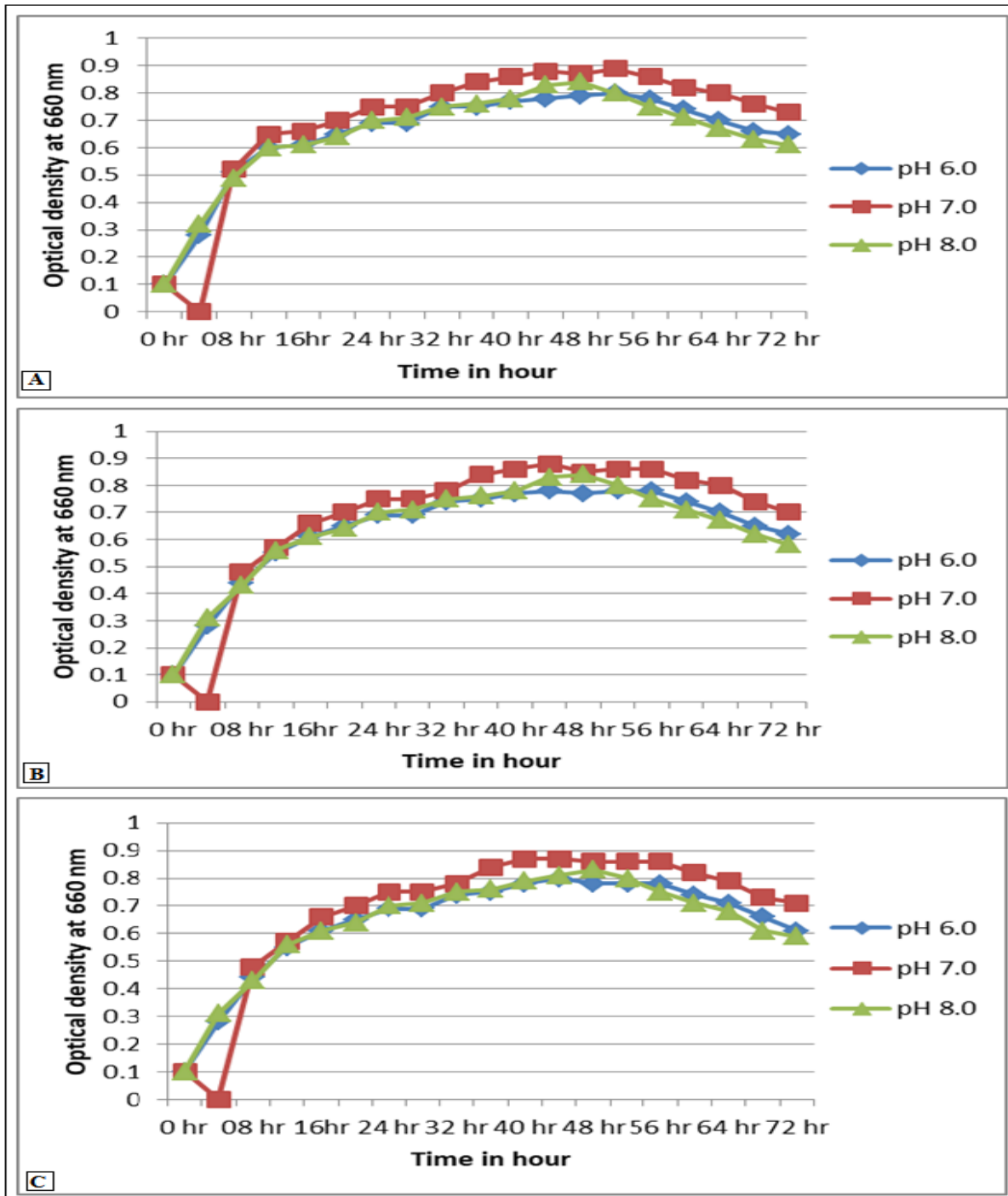


Fig. 2. Optimum pH for growth of the bacterial isolates *Enterobacter* SP. S20112 (A), *Bacterium* MJ20 (B) and *E. aerogenes* strain HK 20-1 (C) at 28°C. The optimum pH of bacterial growth was determined at every 8-hours interval up to 72 hours incubation at pH 6.0, 7.0 and 8.0 by measuring optical density at 660 nm.

Effect of textile dye on seed germination of 3 leguminous crops

The effect of textile dye Red 3B on seed germination of three leguminous crops pea (*Pisum sativum* L.) lentil (*Lens esculentum* L.) and gram (*Cicer arietinum* L.) were studied. It was found that percentage of seed germination decreased with

increase of concentration of dye (Fig. 5, 6 and 7) indicating that the germination percentage is concentration dependent. The highest rate of seed germination was found in control (seed irrigated with water) while the lowest rate of germination was observed in seed irrigated with 400 ppm dye (Fig. 5, 6 and 7).

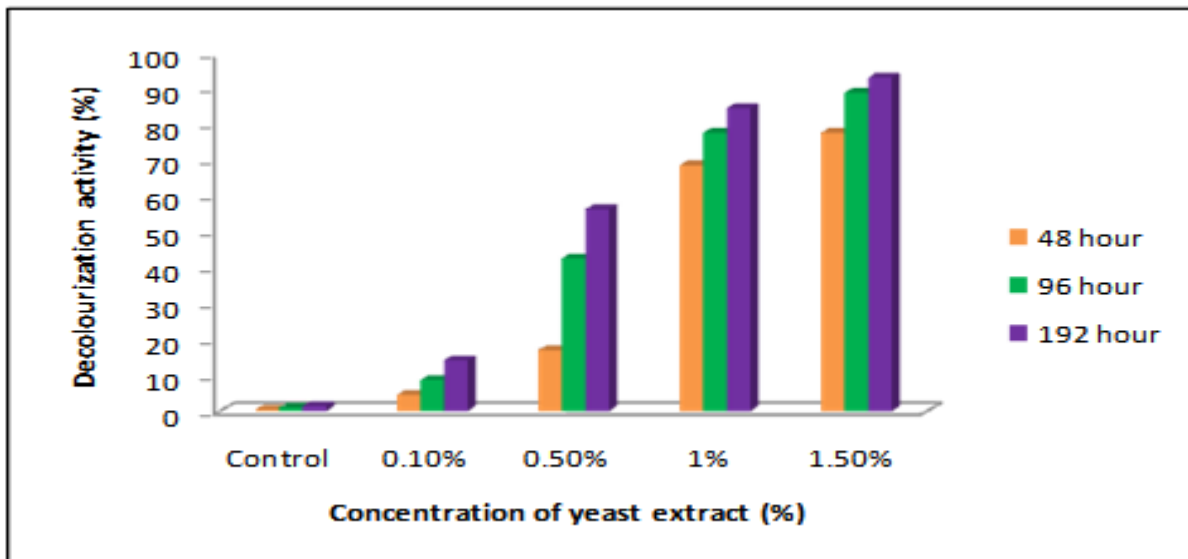


Fig. 3. Effect of yeast extract (co-substrate) on decolourization of Red 3B dye.

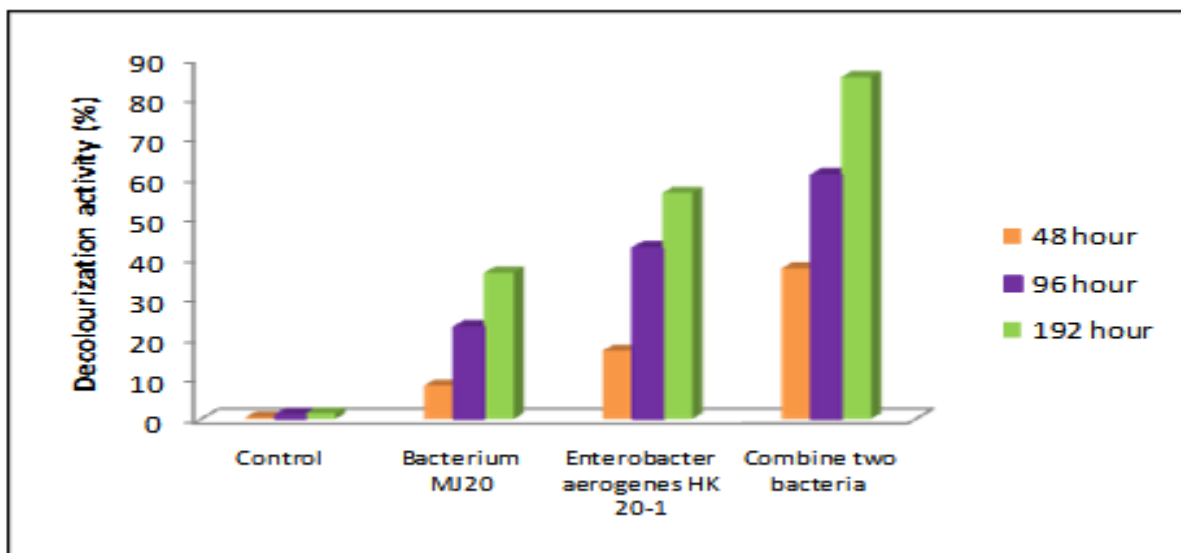


Fig. 4. Synergistic effects on decolourization of Red 3B dye. The Synergistic effects of bacterial isolates on decolourization of Red 3B dye was determined at 48, 96 and 192 hours incubation at pH 7.0 and 28°C.

In this experiment, seed germination effect of 200 ppm Red 3 dye which was either treated (decolourized) with consortium of 3 isolated bacteria or untreated was studied to know the feasibility of isolated bacteria for release in crop field.

It was found that effect of treated dye on seed germination was comparable to that of control (water without dye) (Fig. 8). But, germination rates of seed soaked with untreated dye was remarkable low as compared with germination rate of seed soaked with treated dye and distilled water (Control) (Fig. 8).

Effect of textile dye on root and shoot length of 3 leguminous crops

The inhibition of plant growth by toxic pollutants is a global agricultural problem. In this study, it was found that root and shoot length of three studied legumes decreased gradually with the increase of dye concentration indicating the toxic effect of Red 3B dye on growth of legumes (Fig. 9, 10 and 11). The highest and lowest growth of legumes was observed at control and 400 ppm dye concentration respectively. However, root and shoot length of studied legumes were not significantly different between control and treatment with lower concentration of dye (50 ppm).

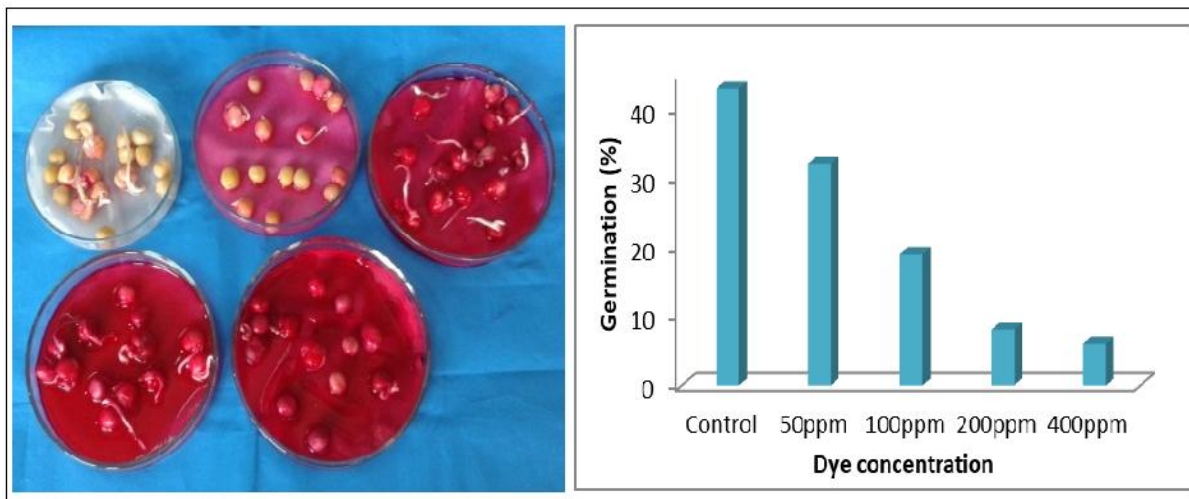


Fig. 5. Effect of textile dye on germination rate of pea (*Pisum sativum* L.).

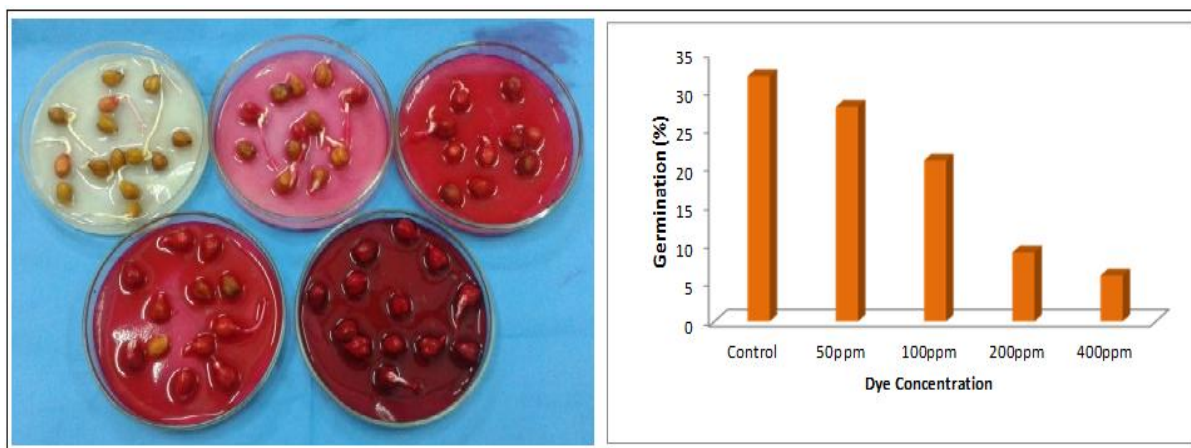


Fig. 6. Effect of textile dye on germination rate of gram (*Cicer arietinum* L.).

Discussion

In this study, three strains of azo dye decolourizing bacteria viz. *Enterobacter* sp S20112, *Bacterium* MJ20 and *Enterobacter aerogenes* strain HK 20-1 were isolated and characterized. These bacterial strains were selected after being grown in an enrichment medium supplemented with dye as the sole carbon source as well as in mineral salt medium which confirm the ability of the isolated bacterial species to survive in the presence of the dye and utilize the dye as the sole carbon source. Biodegradation without any extra carbon sources is very difficult. So, optimization experiments were initiated by supplementing the mineral salt medium containing dyes with different percentage of yeast extract. The color removal percentage of dye increased sharply after the addition of yeast extract and this is in accordance with other reports (Chen *et*

al., 2003; Saha *et al.*, 2017). Metabolism of yeast extract is considered essential for the regeneration of NADH, which is the electron donor for the azo bond reduction (Asad *et al.*, 2007). Azo reductase is reported to be the key enzyme for azo dye degradation.

Bacterium MJ20 and *Enterobacter aerogenes* strain HK 20-1 showed comparatively lower decolourization activity after 48, 96 and 192 hours incubation separately, but the decolourization rate increased sharply to reach up to 85.52% when they are used together indicating the effective synergistic activity. *Bacterium* MJ20 and *Enterobacter aerogenes* strain HK 20-1 are mesophilic bacteria because they all showed better decolourization in the temperature range of 25 to 28°C, similar results were also reported by (Guo *et al.*, 2008).

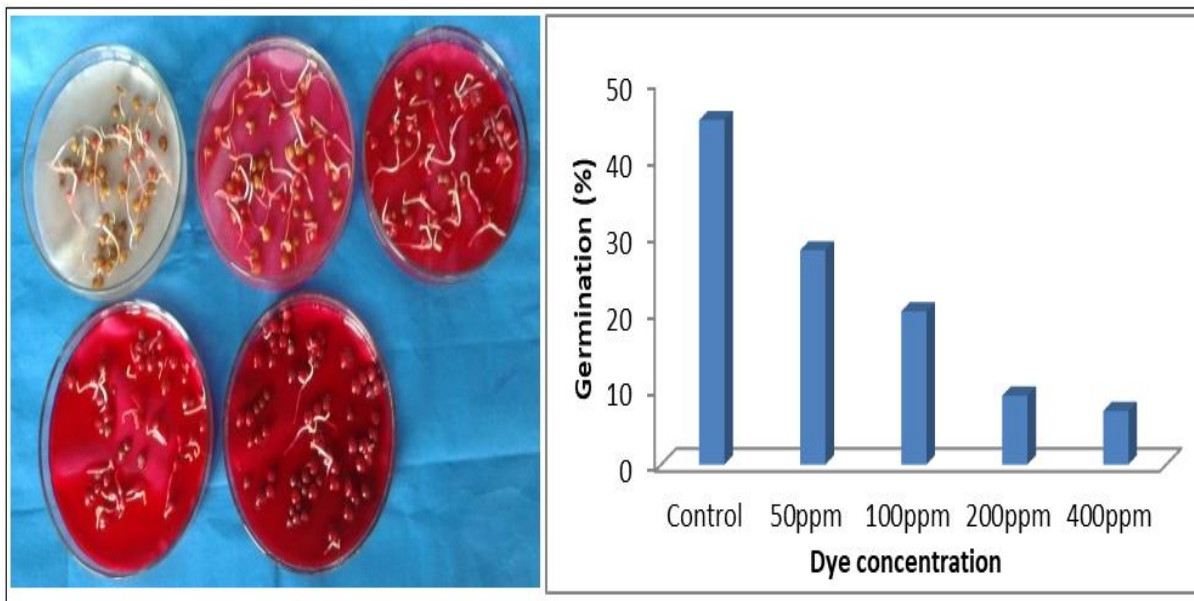


Fig. 7. Effect of textile dye on germination rate of lentil (*Lens esculentum* L.).

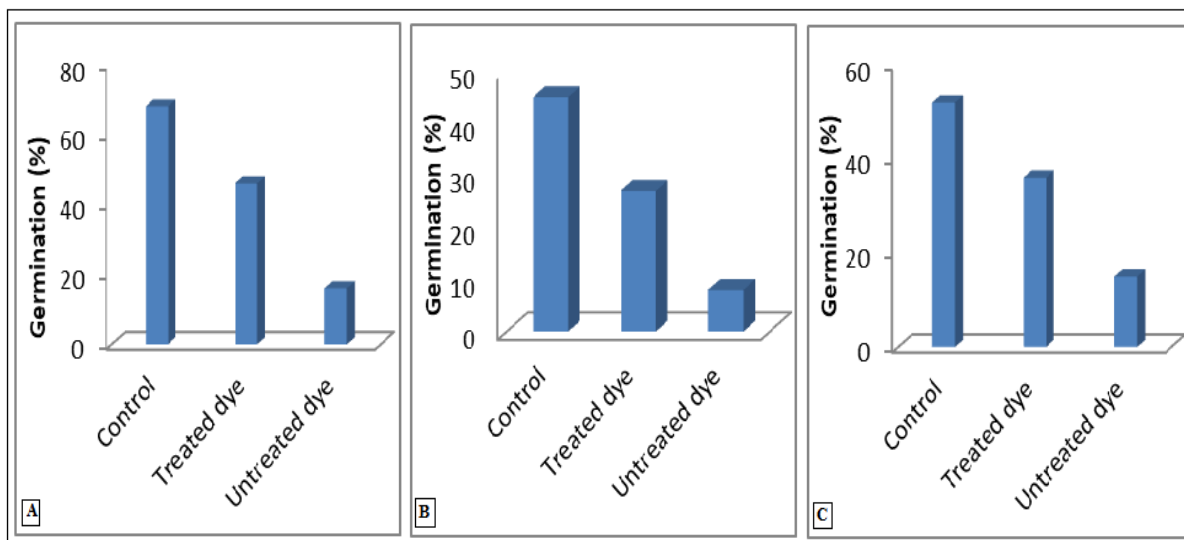


Fig. 8. Effect of treated (decolourized) textile dye on germination of legume seeds. Here, pea (A), gram (B) and lentil(C) seeds were wetted with water (Control), 200 ppm dye treated with bacteria and 200 ppm untreated dye.

At lower dye concentration, bacteria were showed maximum decolourization activity. Decrease in decolourization ability at high dye concentration might be due to the toxicity of the dye (Chen *et al.*, 2003). Azo dyes generally contain one or more sulphonic-acid groups on aromatic rings, which might act as detergents to inhibit the growth of microorganisms (Chen *et al.*, 2003). Another reason of the toxicity at higher concentration may be due to the presence of heavy metals (metal-complex dyes) and/or the presence of non-hydrolyzed reactive groups which may retard the bacterial growth

(reactive dyes) (Sponza and Isik, 2005). It is evident from our results that plants exhibited a stimulation in germination %, biomass, and various attributes of root development at lower concentrations. In contrary, a substantial decrease was observed in these parameters at higher concentrations of textile dyes. Our results are in agreement with some earlier reports which have also demonstrated a same response of plants when irrigated with effluent (Mohammad and Khan, 1985; Srivastava and Sahai, 1987; Kaushik *et al.*, 2005; Nawaz *et al.*, 2006; Garg and Kaushik, 2008).

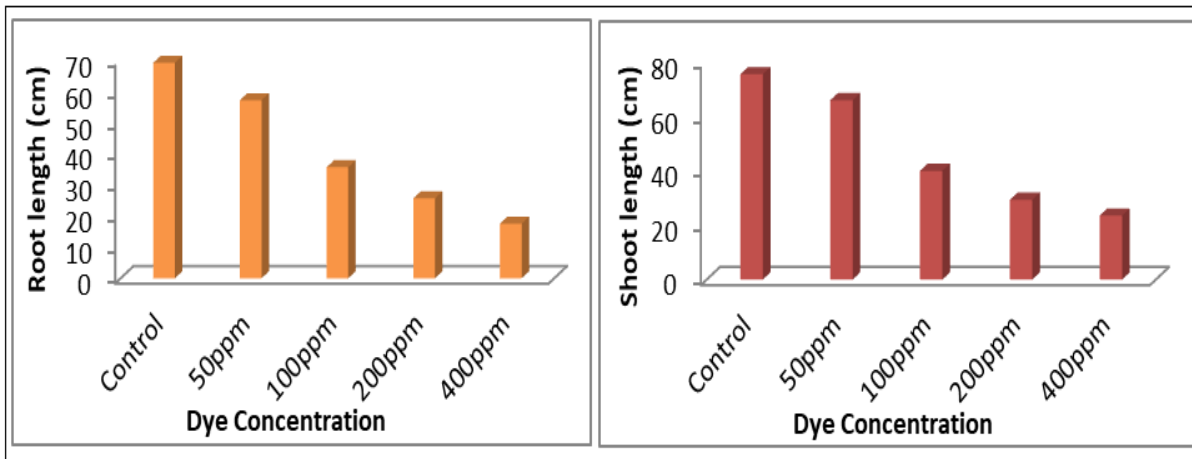


Fig. 9. Effect of textile dye on root and shoot length of pea (*Pisum sativum* L.)

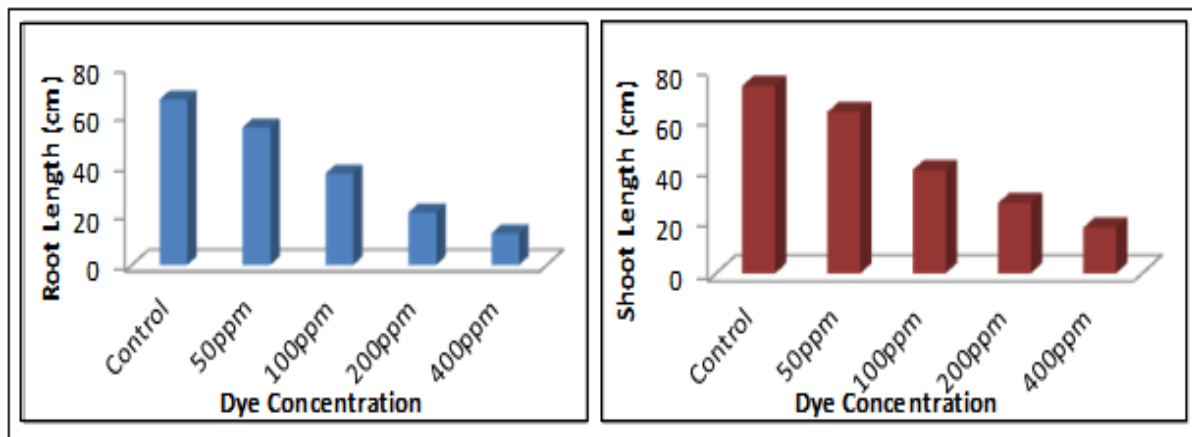


Fig. 10. Effect of textile dye on root and shoot length of gram (*Cicer arietinum* L.).

It has been established by various analysis that effluents from industrial establishments and sewage contain heavy metals and also nutrients (Rodrigues *et al.*, 1996; Dhevagi and Oblisami, 2002; Akbar *et al.*, 2007; Amin *et al.*, 2009) which affect plants and soils

in a variety of ways. Our results indicate that at lower concentration, the effect of effluent was negligible which is indicative of minimum action of heavy metals in lower concentration.

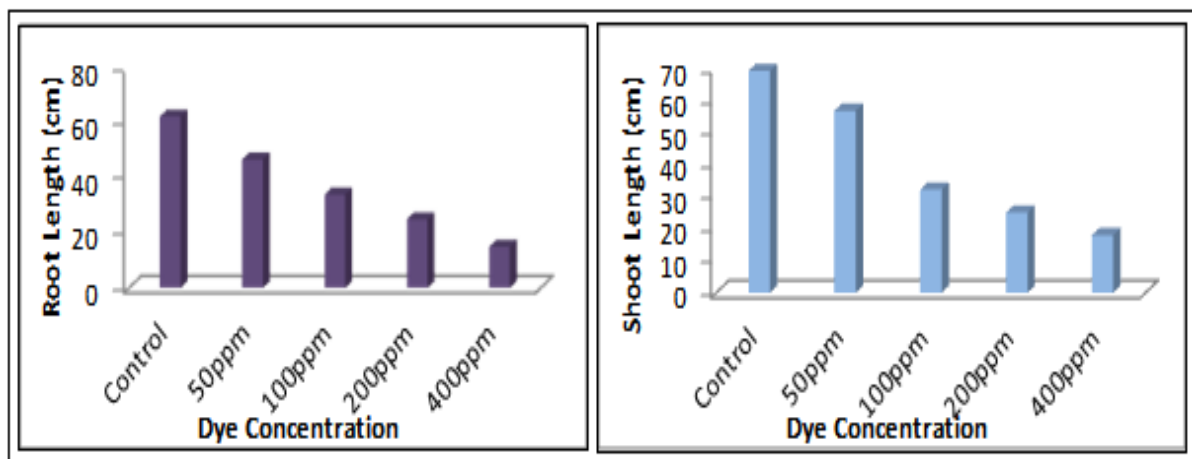


Fig. 11. Effect of textile dye on root and shoot length of lentil (*Lens esculentum* L.).

In this study, it was found that the seeds germination of pea, gram and lentil were affected by the textile dyes. But after treatment of the dyes with bacteria, the germination of treated seeds was comparable to that of control seeds indicating that the used bacteria were able to detoxify the toxic dye.

Although the decolourization efficacy of *Enterobacter* sp S20112, *Bacterium* MJ20 and *Enterobacter aerogenes* strain HK 20-1 was prompting there were few limitations of this study. Decolourization efficacy of the isolated bacteria was studied only for one type of azo dyes but there efficacy to decolourize many other types of azo dyes remained obscured. Likewise, decolourization efficacy of the isolated bacteria was tested in small-scale in lab which does not ensure their similar decolourization capacity in large scale in industrial bioreactor.

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