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Biodegradation and Decolorization of Azo Dye (Deep Red Glx) by Alkaliphilic Bacillus Cereus Strain BPL Isolated from Textile Effluent Contaminant Soil

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***Abstract*:- The Gram positive, spore forming bacterium was isolated from textile effluent contaminated soil and was examined for its ability to degrade azo dye (deep red glx). On analysis of 16S rRNA gene sequence, strain showed 99% sequence similarity with *Bacillus cereus* ATCC 14579 and belongs to group Firmicutes of domain Bacteria. The strain has been assigned name BPL and the gene sequence was deposited in Genbank with accession number KU510086. The *Bacillus cereus* strain BPL was able to decolorize deep red glx with efficiency of 78% in minimal salt medium (MSM) supplemented with dye at concentration of 0.5gl^{-1}. Decolorization studies have shown that the strain was able to decolorize deep red glx in pH range of 4-9 at 37ºC with optimum pH of 9 and adaptability to decolorize dye in alkaline conditions. Other parameters such as nitrogen sources, carbon sources and osmotic concentration were also examined. When supplemented with nitrogen source (peptone, 5gl^{-1}), carbon source (maltose, 5gl^{-1}) and NaCl (10gl^{-1}), the decolorization obtained were 85%, 87% and 82% respectively. The *Bacillus cereus* strain BPL was found to decolorize azo dye deep red glx and shows good possibilities for biodegradation and decolorization of textile effluents.**

Keywords: Azo dyes; Bacillus cereus strain BPL; decolorization, deep red glx; optimization; 16S rRNA gene

INTRODUCTION

Textile industries contribute nearly 14% of the total industrial production in India and discharge large volume of effluent after dying process **(Ekambaram *et al.*, 2016; Zolinger *et al.*, 1987)**. Effluent from textile industries contain cotton, wool, silk, synthetic fibers, coloring agents (synthetic dyes), bleaching agents (chlorine), fixing agents (formaldehyde, benzidine etc.), stain removing agents (CCl_4) and printing gums (polyphosphates like trisodium phosphate and sodium hexametaphosphate). Textile effluent contain different types of dyes based on their chemical nature, such as, azo, diazo, cationic, basic, anthraquinone base and metal complex dyes. Synthetic dyes (e.g., azo, xanthenes and anthraquinone dyes) are highly toxic or mutagenic for organisms (**Daneshvar *et al.*, 2007**) and also affect natural resources (**Puvaneswari *et al.*, 2006; Mester and Tien 2000**).

Textile effluent is generated through printing, dying, bleaching, sourcing and mercerizing **(Ponraj *et al.*, 2011; Gupta *et al.*, 200**3). Dye concentration in effluent varies between 10-200mg/ml, depending on the dye processing and it is estimated that, around 10-15% of dyes are lost in effluent during the dye process **(Kumar *et al.*, 2006).** Cotton based textile effluent is usually alkaline, whereas, synthetic and woolen fabric processing generates acidic or low pH effluent. When high concentration dye containing water are used for washing and bathing purposes, it may cause several skin disease like ulceration of skin, dermatitis, respiratory tract infections, perforation of nasal sputum and ingestion may cause omitting, pain, hemorrhage and sharp diarrhea **(Iqbal and Nisar 2015 and Mittal *et al.*, 2005)**. Textile effluents are often contaminated with non-biodegradable organics materials, presence of these chemicals results in high chemical oxygen demand (COD) value of the effluent.

Azo dyes are largest group of dyes which consists of 70% of dyes used in textile industries and produces clear and strong colors. Removal of colors from textile effluent is one of major environmental concern. Degradation and decolorization can be achieved through physical, chemical and biological methods. Physical and chemical methods are generally expensive as compared to biological methods and produce large amount of secondary waste **(Guan *et al.*, 2014; Tantak and Chaudhari 2006)**.

The aim of present study is to isolate potential dye degrading bacteria from textile effluent contaminated soil and to optimize various parameters to enhance decolorization. For this, reactive deep red glx dye was used as a standard.

MATERIAL AND METHODS

Study site

Sanganer, a small town located 16 km from south of Jaipur, state capital of Rajasthan, India was selected for present study (Fig.1).

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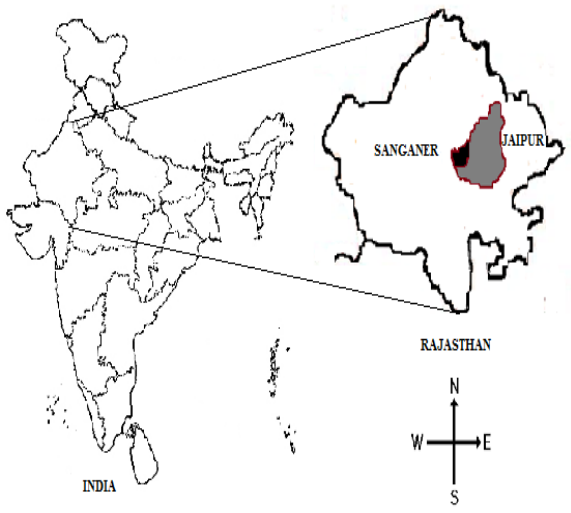

Fig. 1 Map showing location of sampling site. Samples were collected from Sanganer industrial area of Jaipur, Rajasthan.

Sanganer is famous for its textile and hand block printing and had received geographical location tag (GI) in 2010. Dye contaminated industrial effluent disposal sites were subjected for sample collection (Fig.2).

Fig. 2 Textile effluent discharge sites from where soil samples are collected for isolation of dye degrading and decolorizing bacteria.

Sample collection and preservation

Surface soil was removed 30cm from the surroundings of effluent discharge area and 20-50g of soil samples from the depth of 9-10 cm were collected in sterile polyethylene bags. The samples were transported to the laboratory in an icebox and processed within 4 h of collection or stored at 4ºC temperature until processed within 24h.

Characterization of textile effluents

The textile effluents were characterized in triplicates for pH, Chemical Oxygen Demand (COD), Biochemical Oxygen Demand (BOD), Total Solids (TS), Total Suspended Solids (TSS), Total Dissolved Solids (TDS), Total Organic Carbon (TOC) and Chlorides by standard methods **(APHA, 1985)**.

Isolation of azo dye degrading bacteria

The samples collected from effluent contaminated soils were enriched for dye degrading bacteria by incubating 5g of the textile dye effluent contaminated soil in 100ml of minimal salt medium (MSM) with following ingredients (gl^{-1}); Glucose 5.0, Yeast Extract 5.0, $MgSO_4.7H_2O$ 0.5, KH_2PO_4 0.5, KCl 0.5 **(Gahlout *et al.*, 2013)** autoclaved at 121ºC for 15 min and supplemented with deep red glx at concentration of $0.5gl^{-1}$. Incubation was done at 37ºC for 3-4 days at 180 rpm/min, till prominent decolorization was observed. Enriched samples showing decolorization were subjected to sector plating on minimal salt medium with $20gl^{-1}$ agar added for solidification. Bacterial isolates showing decolorization and different morphologies were picked up and purified.

Screening of bacterial isolates for azo dye degradation

The isolated bacterial strains were grown in 5ml nutrient broth and transferred to minimal salt medium supplemented with deep red glx ($0.5gl^{-1}$) and incubated at 37ºC under shaking condition at 180rpm/min for 48 h and were obsrved for decolorization (Fig.3).

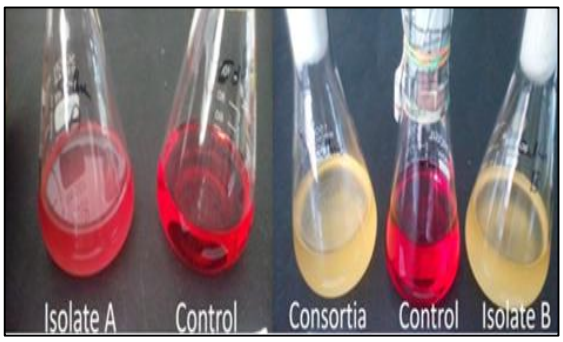

Fig. 3 Dye decolorization by isolated strain A, strain BPL and consortia of strain A and strain BPL with reference to control

After 48h, aliquots (5ml) were withdrawn, centrifuged at 10,000 rpm for 5 minutes at room temperature to separate the bacterial cell mass. The supernatant was used for analysis of decolorization and all the experiments were repeated in triplicates. Absorbance of the supernatant withdrawn at different time intervals were measured at the absorbance maximum wavelength for the dye deep red glx (λ max =570 nm) in the visible region on a shimadzu double beam spectrophotometer (UV 1601). The percentage of decolorization was calculated from the difference between initial and final values using the following formula:

$$\% \text{ decolorization} = \frac{\text{initial absorbance value} - \text{final absorbance value}}{\text{initial absorbance value}} \times 100$$

The bacterial strains showing maximum decolorization values were selected and used further for decolorization experiments.

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Morphological and biochemical characterization

Morphological and biochemical characterization of bacterial isolates were carried out by the routine bacteriological methods *i.e.*, by the colony morphology, preliminary tests like Gram's staining, motility, catalase and oxidase.

Identification of bacterial isolate using 16S rRNA

Genomic DNA of isolate was extracted using method describe by **Sambrook and Russell (2001)**. The 16S rRNA gene amplification was performed using 27f (*Escherichia coli* position 8-27, 5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (*E.coli* position 1510-1492 5'-GGCTACCTT GTT ACG ACT T-3') primers **(Ikenaga *et al.*, 2002; Lane 1991)**. The PCR amplification conditions involved an initial denaturation step at 94ºC for 5 min, 30 extension cycle of 94ºC, 1 min; 53ºC, 1 min; 72ºC, 1.5 min; and a final extension step at 72ºC for 10 min. Purified PCR product were sequenced using an automated sequencer by Perkin Elmer ABI Prism 377 DNA sequencer. The phylogenic relationship of the isolate was determined by comparing the sequencing data with sequences available through the GenBank database of the National Center for Biotechnology Information (NCBI). The obtained 16S rRNA gene sequence was deposited in the NCBI gene bank database **(http://www.ncbi.nlm.nih.gov/)** and accession number, KU510086 was assigned.

Optimization of dye decolorization

Effect of carbon and nitrogen sources

To observe effect of carbon, decolorization medium was supplemented with different carbon sources (*viz;* maltose, glucose, sucrose and fructose) at concentration of $5gl^{-1}$. Similarly, to observe effect of nitrogen, organic nitrogen sources (peptone and yeast extract) and inorganic nitrogen sources (ammonium sulfate, ammonium nitrate and ammonium chloride) were added at concentration of $5gl^{-1}$. Incubation conditions were 37ºC for 24 to 48h.

Effect of physiochemical parameters

To observe effect of different temperatures and pH, dye decolorization was studied at pH 4-9 and temperature 25-45ºC. The decolorizing medium (100ml) was inoculated with 1ml of the 24h old culture and incubated at 37ºC for 24 to 48h under shaking condition at 180 rpm/min. Aliquots (5ml) were collected and examined for percentage decolorization.

RESULTS

Characterization of textile effluent

Observed values of effluent discharged into the environmentwere compared with that of Bureau of Indian standards (BIS) (Table 1).

Table 1 Qualitative parameters of textile effluent of Sanganer industrial area

Parameters	**BIS (Bureau of Indian Standards) (mg/L)**		**Observed values**
	Public sewer	**Irrigation**	
pH	6 - 8.5	6 – 8.5	10* ± 1
Biological oxygen demand (BOD)	350	100	120 ± 4
Chemical oxygen demand (COD)	1000	30	1112 ± 6
Total suspended solids (TSS)	600	200	1800 ± 200
Total dissolved solids (TDS)	2000	1200	2866 ± 411
Total solids (TS)	2600	1400	4666 ± 611
Total organic carbon (TOC)	-	-	24 ± 2
Chlorides	250	250	190 ± 3.2
* = no unit for pH			

The values observed were critically higher as compare to standard values.

Screening of dye degrading isolates

The percentage dye degradation by strainA, strain BPL and consortia of strain A and strain BPL are shown in Fig. 4.

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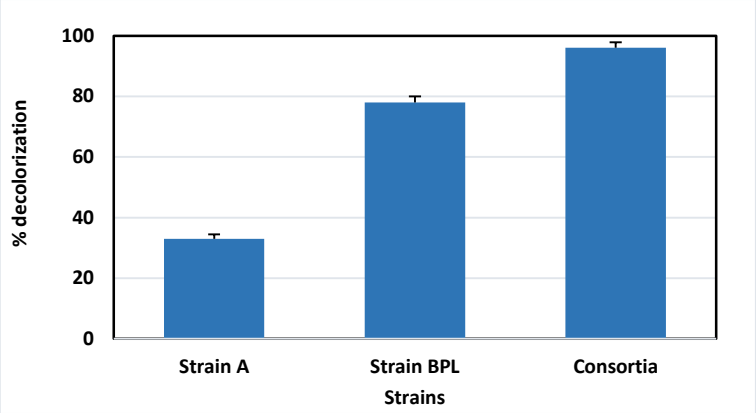

Fig. 4 Percentage decolorization by strain A, strain BPL and consortia of strain A and strain BPL. Error bar indictaes standard deviation of duplicate observations.

The strain BPL and consortia of strain A and strainBPL shows maximum dye decolorizationof 78% and 96% respectively as compared to strain A with 33% rate of decolorization. Isolated strain BPLshowing maximum decolorizationwas further selected for identification by 16S rRNA gene sequencing and for optimization of various parameters for dye degradation.

Morphological and biochemical characterization of isolates

The colonies of strain A and strain BPL were irregular, flat and opaque. The strains were found to be Gram's positive, spore forming rods. The biochemical tests for strain A and strain BPL are depicted in Table 2.

Table 2 Biochemical characterization of strain A and strain BPL

Biochemical characteristics	**Strain A**	**StrainBPL**
Gram's Staining	Gram positive rods	Gram positive rods
Catalase	-	+
Oxidase	-	+
Motility	-	+
Urease	+	-
Starch Hydrolysis	-	+

Strain A showed positive result for urease, whereas, strain BPL showed positive result for catalase, oxidase, motility and starch hydrolysis. Strain A was found to be capable of hydrolyzing urea to produce ammonia and carbon dioxide,whereas,strain BPL showed presence of catalase and extracellularamylases. Catalase confirms presence cytochrome oxidases an important enzymes system required for degradation of colored products. Results of morphological and biochemical studies shows that isolated strain A and strain BPL belongs to genus *Bacillus*. In order to characterize strains at species level 16S rRNA gene analysis was performed.

Identification of Bacillus St. BPL by 16S rRNA gene sequencing

16S rRNA gene sequences of *Bacillus*strain BPLwere compared with those in the NCBI and RDP databases using FASTA **(Pearson & Lipman 1988)**. Multiple alignments of sequences were executed using CLUSTAL W (**Thompson *et al.*, 1994**) and evolutionary distances were calculated by pair-wise comparison of the aligned sequences using Phylogeny.fr **(Dereeper *et al.*, 2010 & 2008)**. Results of phylogenetic relationships revels that the strain BPL showed 99% sequence similarity with *Bacillus cereus* ATCC 14579 (Fig.5)

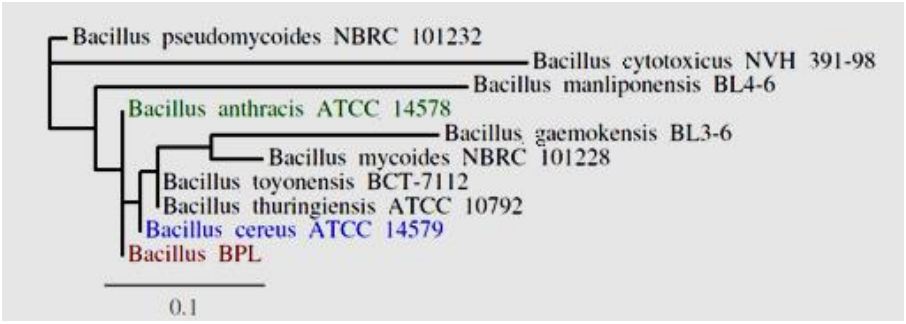

Fig. 5 Phylogenetic relationships of strain *Bacillus* BPL based on partial 16s rRNA gene sequence. Strain *Bacillus* BPL shows 99% similarity with *Bacillus cereus* ATCC 1457 and belongs to group Firmicutes of domain Bacteria. The nearest homolog was found to be *Bacillus anthracis* ATCC 14579.

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Optimizations of dye decolorization

To scrutinize effect of various parameters (carbon and nitrogen sources, pH, salinity and temperature) experiments were performed by keeping test parameter variable and other parameters constant. On observing effect of carbon sources (maltose, glucose, sucrose and fructose) maximum dye degradation up to 87% was observed for maltose (Fig.6).

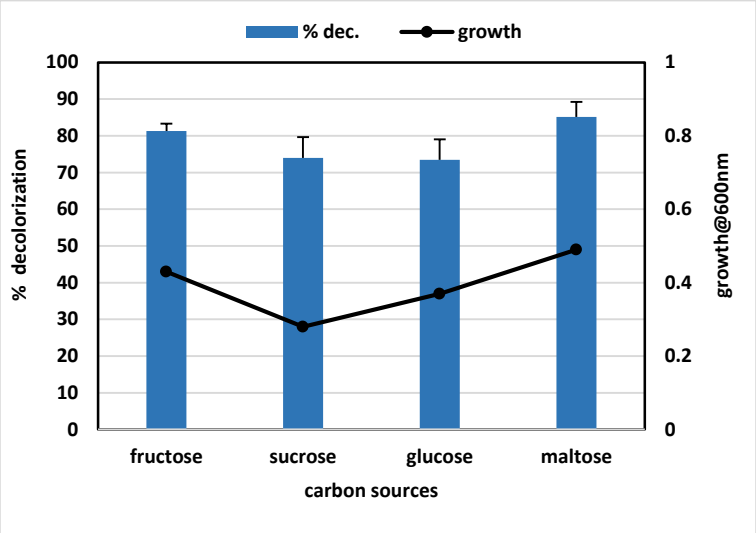

Fig. 6 Percentage decolorization and growth of *Bacillus cereus*strainBPL under influence of different carbon sources.Error bar indictaes standard deviation of duplicate observations

With different nitrogen sources (ammonium sulphate, ammonium nitrate, ammonium chloride, peptone and yeast extract) maximum dye degradation up to 85% was observed for (Fig.7).

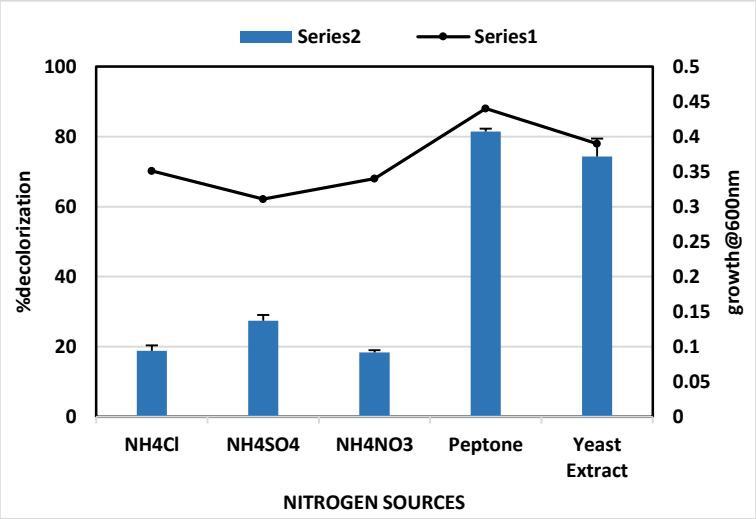

Fig. 7 Percentage decolorization and growth of *Bacillus cereus* strain BPL under influence of different nitrogen sources. Error bar indictaes standard deviation of duplicate observations

Similar observations were recorded for pH and salinity. For pH, maximum degradation (84%) was observed at pH 9, may be due to adaptation of strain at high pH conditions (Fig.8).

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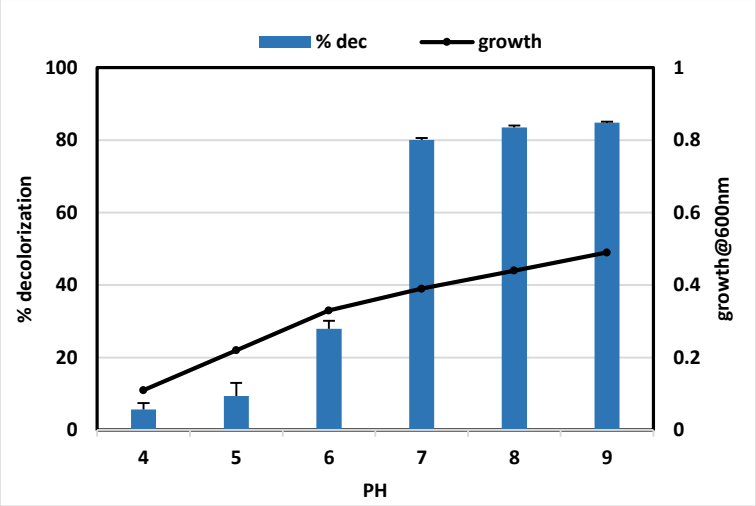

Fig. 8 Percentage decolorization and growth of *Bacillus cereus* strain BPL under different pH conditions. Error bar indictaes standard deviation of duplicate observations

With increasing osmotic concentration, increase in dye degradation (82%) was observed up to $10gl^{-1}$ (1%) of NaCl, after that rate of degradation decreases with further increase in salt concentration (Fig.9).

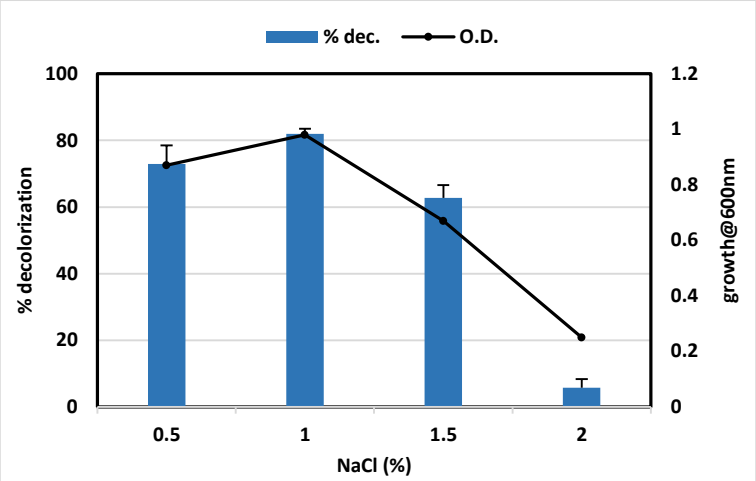

Fig. 9 Percentage decolorization and growth of *Bacillus cereus* strain BPL under saline conditions. Error bar indictaes standard deviation of duplicate observations

The optimum and sub optimal temperature recorded for dye degradation were 37ºC and 30ºC and 40ºC (Fig.10).

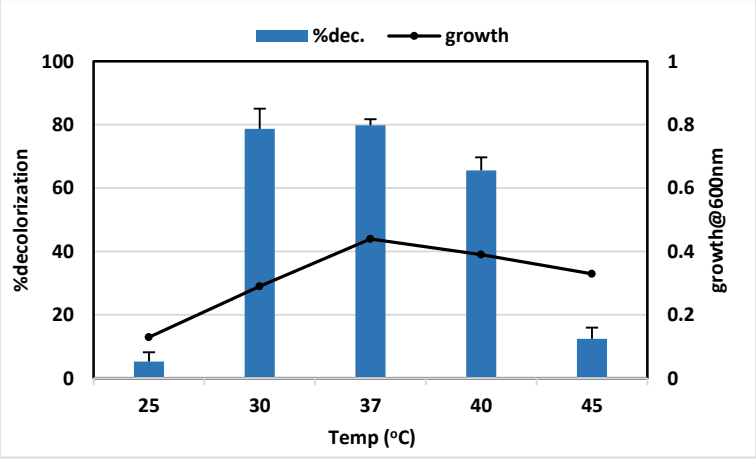

Fig. 10 Percentage decolorization and growth of *Bacillus cereus* strain BPL under different temperature conditions. Error bar indictaes standard deviation of duplicate observations

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DISCUSSION

Occurrence of bacterial population and diversity in the textile effluent has intensified research in the field of bioremediation **(Jadhav *et al.*, 2016; Dawkar *et al.*, 2009)**. It was found that bacteria as compare to fungi and actinomycetes degrade and even can completely mineralize dye under certain conditions **(Asad *et al.*, 2007; Kapdan and Erten 2007; Chen *et al.*, 2003)**. In the present study, *Bacillus cereus* strain BPL with potential azo dye (deep red glx) degrading capacity was isolated from effluent contaminated soil of textile industries located in Sanganer industrial area of Jaipur, state capital of Rajasthan, India. On molecular identification, *Bacillus cereus* strain BPL was found to have 99% similarity with Bacillus *cereus* ATCC 14579 and belongs to group Firmicutes of domain Bacteria.

Decolorization studies have shown that, strain *Bacillus cereus* BPL was able to decolorize deep red glx at a decolorization rate of 78% within 4 days under shaking conditions. No residual dye was found on the biomass, suggesting that the decolorization was due to extracellular enzymes produce by the strain **(Gahlout *et al.*., 2013)**. For optimizing condition for maximum decolorization, maltose ($5gl^{-1}$) and peptone ($5gl^{-1}$) were found to be more suitable as compare to basal media with no modifications. This suggest that dye itself does not serve as a sole carbon source and degradation rate can be enhanced by adding supplementary carbon and nitrogen sources **(Gahlout *et al.*, 2013)**. The optimum pH conditions for decolorization was observed at pH 9, this may be attributed to the acclimatization of strain in high alkaline condition of effluent. pH tolerance in dye degrading bacteria is quite common because reactive azo dyes bind to cotton fibers by addition or substitution mechanism under alkaline conditions **(Aksu *et al.*, 2003)**.

In the present study *Bacillus cereus* strain BPL was found to be potential degrader of azo dye deep red glx and shows good possibilities in treatment of textile effluents.

CONCLUSION

Discharge of textile effluent into open environment is serious environmental problem and a major environmental concern. Physical and chemical methods are effective for color removal but need energy and may cause pollution. Hence, economical, natural and eco-friendly techniques using microorganisms can be an alternative method to treat textile industrial effluent. Microbial degradation of dyes offers an easy, cheaper and effective method for decolorizing textile dyes. In present study, we have isolated bacteria that have potential to degrade and decolorize azo dye deep red glx used in textile industries. Our finding reveals that the rate of decolorization can be enhanced by optimization of condition for dye degradation. In the present case, maltose, peptone, pH 9 and 37°C temperature were found to be optimum parameters to increase rate of degradation.

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