Identification of *Bacillus subtilis* subsp *subtilis* "RA-29", a Congo Red Decolourizer using 16S rDNA Sequencing

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Abstract: A gram positive aerobic bacillus "RA-29", isolated from the garden soil, in the vicinity of an industrial town, was able to decolourize congo red azo dye (88.26%) under static condition of incubation. The optimization studies were performed to determine the optimal pH and temperature for maximal decolourization of the dye by UV-vis analysis. The maximum decolourization percentage was calculated as 95.67% at pH (8.0) and temperature (37^0C) after 60 hours of incubation at 50 ppm congo red concentration. Identification of the isolated strain "RA-29" was performed by 16S rDNA sequence analysis. The RA-29 strain was phylogenetically positioned in the genus *Bacillus*. The nucleotide alignment and distance matrix showed S_ab score of the strain as 0.999 with closest relation to *Bacillus subtilis* subsp. *subtilis* ZH10 (NCBI accession no. HM103330). RA-29 was grouped as *Bacillus subtilis* subsp subtilis (NCBI, accession no. JF 901735). The work highlights an efficient decolourization of congo red by *Bacillus subtilis* RA-29 from aqueous solution. Since most azo dyes are recalcitrant to aerobic degradation by bacterial cells, the organism could be a valuable tool to develop commercially viable bioremediation technology to remove azo dye from dye contaminated aqueous ecosystem under microaerophilic/static incubation conditions. [Arun Kumar, Rajesh Sawhney. **Identification of *Bacillus subtilis* subsp. *subtilis* "RA-29", a Congo Red Decolourizer using 16S rDNA Sequence.** Researcher. 2011;3(12):18-22]. (ISSN: 1553-9865). http://www.sciencepub.net

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1. Introduction

Azo dyes (-N = N- group) are the largest group of synthetic dyes used in textile, paper, plastic and leather manufacturing units (Gurulakshmi et al., 2008, Saharan and Ranga, 2011). These colourants are environmental pollutants. Their discharge into the aqueous ecosystem cause aesthetic problems, poor light penetration and oxygen transfer into water bodies and impart mutagenic activity to the contaminated water or soil (Slokar and Marechal, 1998, Pinherio et al., 2004, Bae and Freeman, 2007, Elisangela et al., 2009, Saharan and Ranga, 2011). Thus, these xenobiotic compounds are detrimental to the health of humans as well as animals and vegetation. Dye house effluents contain large amount of these dyes. Several physico-chemical techniques such as filtration, coagulation, flocculation, adsorption, oxidation and precipitation by Fenton's reagent, bleaching with Chlorine and ozone, and photo degradation have been used for treatment of coloured industrial effluents (Robinson et al., 2001, Gharbani et al., 2008, Mabrouk and Yusef, 2008, Elisangela et al., 2009, Olukanni et al., 2009, Saharan and Ranga, 2011). All these methods are expensive, unsafe and pose secondary disposal problems (Maier et al., 2004). Biological removal of the dyes from contaminated sources is considered safe, economic and eco-friendly approach. A number of bacterial (*Bacillus, E. coli, Klebsiella, Enterobacter, Pseudomonas, Kerstersia, Eubacterium, Clostridium, Butyrvibrio, Bacteriods, Sphingomonas*) and fungal (*Pleurotus, Schizophyllum, Neurospora*, White rot fungi) genera have been employed for dye degradation (Chen et al., 2003, Bumpus, 2004, Jaladoni-Buan et al., 2010). However, an effective biological solution to dye degradation is still a matter of concern. The screening of microbial wealth for strains with efficient dye decolourization ability could provide a bioremediation tool to tackle the after effects of dye pollution.

Congo red (sodium salt of benzidinediazo-bis-1- naphtylamine-4 sulfonic acid) is one of the important azo dye, used for colouring of paper products (Cripps et. al., 1990, Jaladoni-Buan et al., 2010). It is a carcinogenic direct diazo dye that causes serious environmental and health concerns associated with the discharge of dye effluent from industries. Thus, the present work highlights the efficient decolourization of congo red by indigeneous bacterial isolate, RA-29, identified as *Bacillus subtilis* subsp. *subtilis* at optimal conditions of pH and temperature under static incubation. The successful search for an efficient indigeneous organism could be a valuable tool to develop commercially viable bioremediation technology to remove azo dye from dye contaminated aqueous ecosystem.

2. Material and Methods

2.1 Collection of Samples

The garden soil samples were collected at random in duplicate from different locations in Baddi, Distt. Solan (H.P), India under aseptic conditions in sterile plastic bottles. Baddi is an industrial town located in Himachal Pradesh, India ($30^0$57' 31.08"N, 76^0 47' 17.87" E) at 1375 ft. above sea level.

2.2 Chemicals and media

The azo dye congo red was purchased from CDH. The mineral salt media (MSM) at pH 7 used during study contained: K_2HPO_4-6.3, KH_2PO4-1.8, NH_4NO3-1.0, $MgSO_4.7H_2O$-0.006, Yeast Extract-5.0 gL^{-1}. The culture media was autoclaved at 121^0C for 15 min.

2.3 Bacterial isolation and identification

The bacteria were isolated from different garden soils. Serial dilutions (upto 10^{-7}) of samples were inoculated into nutrient agar medium by spread plate technique. The isolated bacteria were inoculated into MSM supplemented with 50 ppm congo red (Cohen-Bazire et al., 1957). The flasks containing the cultures were incubated under static condition in REMI-CIS-24BL at 37^0C for 7 days. The strain that achieved the best decolourization was taken for further study.

Identification of the isolated strain was performed by 16S rDNA sequence analysis. Genomic DNA was obtained from the culture by using Chromus Genomic DNA isolation kit (RKT09). The 16S rRNA was amplified by PCR using the 16S rRNA specific primers, (16S Forward primer 5'-AGAGTRTGATCMTYGCTWAC-3' and 16S Reverse primer 5'-CGYTAMC TTWTTACGRCT-3').

100 µl reaction mixtures were prepared containing 1ul of total DNA, 3U/µl of Taq DNA polymerase, 2.5 mM each of deoxynucleoside triphosphates (4 µl d NTP's -2.5mM each), 400 ng of each primer. The PCR amplifications (total 35 cycles) were done using an initial denaturation step of 5 min at 94^0C, followed by denaturation step of 30s at 94^0C, hybridization step of 30s at 55^0C, elongation step of 2 min at 72^0C followed by final extension for 5 min. at 72^0C, in an Applied Biosystems (ABI2720) thermal cycler.

The sequencing was carried out using the Big Dye Terminator version 3.1" Cycle Sequencing Kit for the ABI 3130 Genetic Analyzer (Applied Biosystems), according to the manufacturer's instructions. The data was analyzed using Seq Scape_v 5.2 software.

Identification was achieved by comparing the contiguous 16S rRNA sequences obtained with the 16S rRNA sequence data from the reference and type strains available in public databases GenBank using the BLAST. The sequences were aligned using Jukes Cantor Model. The phylogenetic reconstruction was done using the neighbour-joining (NJ) algorithm, with bootstrap values (Bruno et al., 2000).

2.4 Congo red decolourization

10 ppm congo red dye solution in distilled water was scanned spectrophotometrically (Systronics 2202) to find out maximum absorbance (λ_{max}) for congo red. Decolourization experiments were done under static incubation condition with 50 ml of the nutrient broth (pH 7) supplemented with 50 ppm. congo red. The dye containing nutrient broth was inoculated with approximately 20 mg dry cell mass (Biomass measured using OD_{600}) and incubated under microaerophilic conditions at 37^0C for 168 h. One set of the flasks with dye and bacterial culture was incubated on rotary shaker at 120 rpm to promote decolourization through oxidative degradation. Dye decolorization was measured in a UV-visible spectrophotometer (Systronics 2202) for both static and shaking conditions at λ_{max} (495.2 nm). The samples were centrifuged at 4000 g for 15 minutes to exclude biomass and the percentage decolourization was calculated as per method documented (Olukanni et al., 2006).

$$\text{Decolourization (\%)} = \frac{A_0 - At}{A_0} \times 100$$

A_0 = Absorbance of the blank (dye solution)
At = Absorbance of the treated dyes solution at specific time.

2.5 Dye decolourization at different pH and Temperature:

Optimization of pH and temperature was done to achieve maximum congo red decolourization by the bacterial strain. The effect of pH was determined with in the pH range of 5.0 and 10.0. under static incubation condition with 50 ml of the nutrient broth (pH 7) mixed with 50 ppm. congo red and 20 mg dry cell mass (OD_{600}) and incubated at 37^0C for 168 h.

The optimum temperature for dye decolourization was also determined at different temperatures (20^0C, 25^0C, 30^0C, 37^0C, 40^0C, 45^0C and 50^0C) at optimal pH value. Percentage decolourization was calculated as described earlier. All experiments were carried out in triplicate.

3. Results and Discussion

A congo red decolourizing, gram positive bacillus "RA-29" was obtained after series of isolations from soil samples. RA-29 showed maximum (88.26%) decolourization of congo red (50 ppm concentration) measured at λ_{max} 495.2 nm (data not shown). Congo red decolourization has been achieved earlier to the extent of 85% by *Bacillus* sp. (Sawhney and Kumar, 2011).

To know the exact identity of organism, the 1.5 kb genomic DNA fragment was obtained on agarose gel electrophoris (Figure 1).

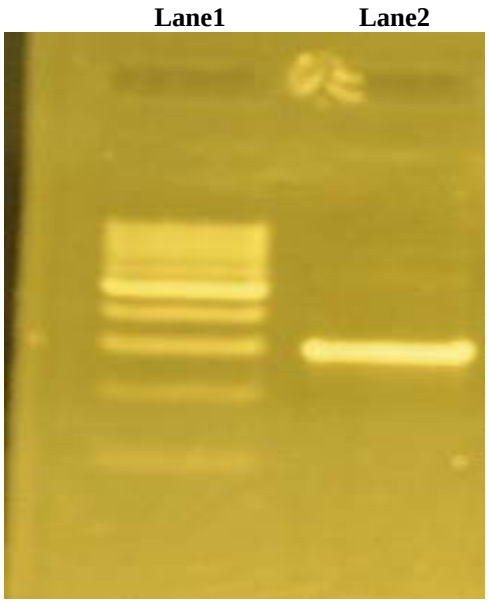


Figure 1. Agarose gel electrophoresis of PCR product for the estimation of molecular weight of genomic DNA. Lane 1 contain 500bp DNA ladder and Lane 2 contain 1.5kb 16srDNA amplified fragment.

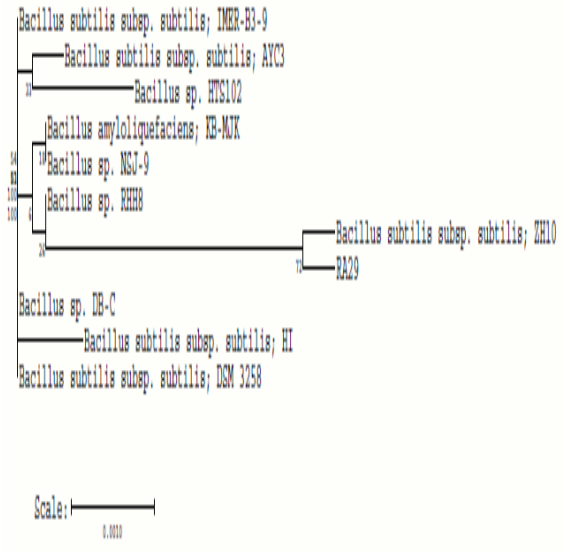
The 16S rRNA gene sequence of RA-29 strain was determined and compared with available 16S rRNA gene sequences from organisms in the GenBank databases. The RA-29 strain was phylogenetically positioned in genus *Bacillus* (Figure 2). The nucleotide alignment and distance matrix showed S_ab score of the strain as 0.999 with closest relation with *Bacillus subtilis* subsp. *subtilis* ZH10 (NCBI accession no. HM103330) (Table 1).

Earlier research reported the decolourization of Acid Blue 113, Vaxent Red HE7B by *Bacillus subtilis* (Gurulakshmi et al., 2008, Saharan and Ranga, 2011). It is established that the decolourization of dye pH and temperature dependent activity and that the behaviour of each strain varied for dye decolourization with variation in pH and temperature (Maier et al., 2004, Olukanni et al., 2009, Nosheen et al., 2010, Kumar et al., 2011, Sawhney and Kumar, 2011).

Table 1. Alignment view and distance matrix table of ten closest neighbor's bacterial strains, RA-29 sequence taken as reference sequence

S_ab score	Organism Name	NCBI accession Number
0.998	Bacillus subtilis subsp. subtilis; DSM 3258	DQ452509
0.997	Bacillus subtilis subsp. subtilis; HI;	DQ452513
0.999	Bacillus sp. DB-C	EU343721
1.000	Bacillus subtilis subsp. subtilis	FJ772081
1.000	Bacillus sp. NSJ-9	FJ941086
0.999	Bacillus subtilis subsp. subtilis; ZH10	HM103330
0.999	Bacillus amyloliquefaciens; KB-MJK	HQ113235
0.997	Bacillus sp. HTS102	HQ698269
0.997	Bacillus sp. RHH8	HQ202545
0.998	Bacillus subtilis subsp. subtilis	HQ263248

Figure 2. Phylogenetic tree of bacterial strain RA-29



Thus, optimization of reaction conditions for efficient congo red removal by *Bacillus subtilis* subsp. *subtilis* was also done. The effect of pH and temperature was studied. "RA-29" achieved maximum decolourization (95.67%) at pH 8.0 after 60 hour incubation at 37^0C under static condition. There was gradual increase in decolorization with

increasing pH from 5.0 to 8.0. Minimum decolourization of congo red was observed at alkaline pH 10.

The percentage decolourization of azo dye was also carried out at different incubation temperatures at optimized pH 8.0. RA-29 exhibited highest (95.67%) colour removal at 37^{0}C. The increase in temperature beyond 37^{0}C led to decline in decolourization activity of the strain. Moreover, the congo red colour removal was directly proportionate to the increase in cell biomass (Figure 3, Table 2).

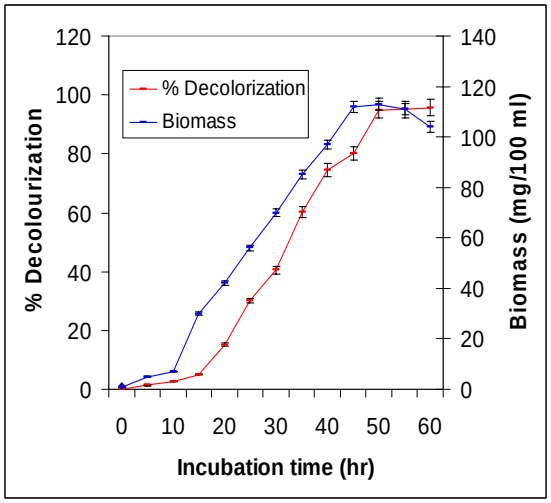


Figure 3. Decolorization of Congo red azo dye Vs. biomass generated.

Table 2. Two way ANOVA analysis for decolorization of Congo red.

Source of Variation	Interaction	Column Factor	Row Factor
% of total variation	1.57	5.04	92.98
Sum-of-squares	2033	6525	120500
Df.	12	1	12
Mean square	169.4	6525	10040
P value	<0.0001	<0.0001	<0.0001
F value	16.51	635.7	978.2
Significant	Yes	Yes	Yes

It was interesting to note that RA-29 could decolourize the dye only under static conditions with formation of a thin surface pellicle with scanty biosorbed congo red. The parallel experiment performed under shaking conditions (120 rpm) yielded no significant decolourization (data not shown). The azo dye decolourization by bacterial species is often initiated by enzymatic reduction of azo bonds. It has been documented that the presence of oxygen normally inhibits the azo bond reduction activity as the aerobic respiration may dominate utilization of NADH; thus impeding the electron transfer from NADH to azo bonds (Chang and Lin, 2001). Our findings are supported by the results of decolourization of azo dyes by *Bacillus subtilis*, *E. coli* and *Pseudomonas luteola* (Hu, 1994, Chang and Kuo, 2000, Gurulakshmi et al., 2008). However, the azo dye reduction being a strain specific mechanism, search for indigenous strains and strain specific studies would be a promising approach.

4. Conclusion

The congo red decolourizing strain, "RA-29", isolated from the soil sample was identified by 16S rDNA sequence, as *Bacillus subtilis* subsp. *subtilis*. The azo dye was maximally (> 95%) decolourized at optimal pH 8 and temperature 37^{0}C under static condition, indicating pH and temperature dependent microaerophilic/anaerobic mechanism of dye decolourization. The "RA-29" could be exploited for its bioremediation ability to treat azo dye contaminated aqueous ecosystem. Moreover, further studies on this isolate could explore new tools and techniques to evolve commercially viable and ecofriendly microbial solutions for treatment of dye industry effluents.

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