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Degradation of textile dye reactive navy – blue Rx (Reactive blue–59) by an isolated Actinomycete *Streptomyces krainskii* SUK – 5

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ABSTRACT

The isolated Actinomycete, *Streptomyces krainskii*, SUK -5 was found to decolorize and degrade textile dye Reactive blue–59.This azo dye was decolorized and degraded completely by *Streptomyces krainskii* SUK–5 at 24 h in shaking condition in the nutrient medium at pH 8. Induction in the activity of Lignin Peroxidase,and NADH-DCIP Reductase and MR reductase represents their role in degradation .The biodegradation was monitored by TLC, UV vis spectroscopy, FTIR. and GCMS analysis. Microbial and phytotoxicity studies of the product were carried out.

Keywords: Navy – blue Rx (Reactive blue–59), *Streptomyces krainskii* SUK – 5, Biodegradation, azo dye, lignin peroxidase

INTRODUCTION

Synthetic dyes have a wide application in the food, pharmaceutical, textile, cosmetic and paper industries (Claus *et al.,* 2002) due to their ease of production, fastness and color variety as compared to natural dyes. Azo dyes are the largest group of dyes used in industry (Ramalho *et al.,* 2002, Mazimo *et al.,* 2003) representing more than half of the annual production (Stolz, 2001). It has been estimated that about 10% of the dye stuff used during this dyeing processes does not bind to the fibers and is therefore released into the sewage treatment systems or environment (Zollinger, 1991). Azo dyes have structural properties that are not easily degradable under natural conditions (Rajguru *et al*.,) and are not removed from water by conventional wastewater treatment systems. Azo dyes are designated to resist chemical and microbial attacks (Ramalho *et al*., 2002) and to be stable in light and during washing.

Actinomycetes now are being recognized for their degradative capacity of highly recalcitrant compounds, hence actinomycete isolated from Krishna river water was used for the study of its degradative capacity.

The objective of this work was to study the aerobic degradation of azo dye Reactive blue -59 by *S. krainskii* SUK -5 and evaluate the performance in vitro with a view to understand their potential; for treating waste water especially considering the potential for toxicity reduction of the dye solution.

MATERIALS AND METHODS

Microorganism and culture conditions

The actinomycetes were isolated from Krishna river samples at Wai (Maharashtra, India) receiving wastes from domestic, industrial sources and temples. Water samples were pour-plated on various nutrient media as glycerol aspargine agar, colloidal chitin agar, starch casein agar and Bennetis agar by serial dilution technique. Out of the 10 *Streptomyces,* a promising isolate showing decolorization and degradation was identified by morphological and biochemical characteristics and 16S rDNA analysis (Table 1, 2 and 3). The bacterium was placed in the genus *Streptomyces* and had 98% matches with *Streptomyces albus* sub *sp. albus Streptomyces sparophyticus* and *Streptomyces kranskii*. The new isolate was named as *Streptomyces kranskii*. (SUK -5) on the basis of biochemical characteristics

The bacterium was grown on glycerol aspargine agar slants at R.T. till sporulated and stored at 4 ℃. Subclturing after every 3 weeks was done. For decolourization studies the bacterium was grown in Bennets broth (Gluc 10 g, Pep One g, Y.E. 19 g, Casein 10 gm, D/w 1000 mL, pH 8.0) at room temperature 30 ± 2 ℃.

Dye stuff, chemicals and cultural media

2, 2 ´-Azinobis (3-ethy1 benzothiazoline-6sulphonic acid) ABTS and amino pyrine were purchased from sigma Aldrich, USA. Tartaric acid, *n*-propanol, catechol, H_2O methyl Red, (Dichlorophenol indophenol, (DCIP), Nicotine adenine diamine reduced salt was obtained from Sisco research laboratories, India. The textile dye and other fine chemicals were obtained from SRL Chemicals, India. Nutrient media for isolation were purchased from Hi-media, India.

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Decolourization experiments

Decolorization at static and shaking conditions

S. krainskii SUK -5 from preserved culture was inoculated and grown for 9 days at static conditions at 30 ^{0}C in 250 mL Erlenmeyer flask containing 100 ml Bennet's broth. Dye was added and kept at static conditions. The other flask containing 100 mL Bennet's broth was kept at shaking conditions 150 rpm at 30 ^{0}C on orbital shaker for 48 h and after addition of dye it was again kept at shaking conditions. The aliquots (3mL) of culture media was withdrawn at different time intervals and centrifuged at 7000-rpm for 20min. Decolorization was monitored by measuring the absorbance of culture supernatant at 588 nm. pH change during decolorization was observed to see whether decolorization was due to degradation or change in pH. The percentage decolourization was calculated as follows:

$$\% \text{ Decolourization} = \frac{\text{Initial absorbance} - \text{observed absorbance}}{\text{Initial absorbance}} \times 100$$

Decolourization at different dye concentrations

To examine the effect of initial dye concentration the nutrient medium was added with 50 mg/L ,70 mg/L and 100 mgL of Reactive blue 59 and percent decolorization was measured at these different dye concentration

Enzyme activities

Enzyme activities in cells before and after decolourization were studied.

Preparation of cell free extract

Streptomyces krainskii SUK – 5, grown on Bennet's broth at 30 ℃ for 9 days at static conditions and grown in shaking conditions 150 rpm for 48 h was centrifuged at 7000 rpm for 30 min in cold centrifuge. The cells were suspended in 50 mM phosphate buffer (pH 7.4) for sonication (Soncs vibra cell ultrasonic processor) keeping sonifer out put 50 amplitude maintaining temperature at 4 ° C and giving 5 strokes each of 50 s with 5 min interval. This extract without centrifugation was used as enzyme source. Similar procedure was carried out for cells obtained after decolourization. Enzyme activity in medium supernatant was also studied

Enzyme assays

Lignin peroxidase (LiP) laccase, tyrosinase, azoreductase and DCIP reductase activities were assayed in cell homogenate as well as culture supernatant.

LiP activity was determined by monitoring the formation of propanaldehyde at 300 nm in a reaction mixture of 2.5 containing 100 mM n-propanol, 250 M tartaric acid, 10 mM H_2O_2 (Shanmugam *et al*.,1999). Laccase activity was determined in a reaction mixture of 2 ml containing 10% ABTs in 0.1 M acetate buffer (pH 4.9) and measured increase in O.D at 420nm (Hatvani and Mecs, 2001).

Tyrosinase activity was determined in reaction mixture of 2 mL containing 0.01% catechol in 0.1 M phosphate buffer (pH 7.4) at 495 nm (Zhang and Flurkey, 1997).

Azoreductase activity was determined by taking 2 mL 380 nM of MR added to 0.1 mL of 20 µm NaOH and 0.1 ml of enzyme. The decrease in absorbance was recorded at 440 nm.

NADH – DCIP reductase was determined by using a procedure reported by Salokhe and Govindwar (1999) .The assay mixture contained 50µM DCIP, 28.57 mM NADH in 50 mM potassium buffer (pH 7.4)and 0.1 mL enzyme solution (sonicated cell suspension in a total volume of 5.0mL). The DCIP production was calculated as extinction coefficient 19 mM/cm.

Biodecolorization and Biodegradation analysis

Decolorization was monitored by U.V spectroscopic analysis (Hitachi U – 2800) whereas biodegradation was monitored by FTIR spectroscopy. For this 100 ml sample was taken after decolourization. Centrifugation was carried out at 10,000 rpm and the metabolites were extracted from supernatant using equal volume of ethyl acetate. The extract were dried over anhydrous Na_2SO_4 and evaporated to dryness in a rotary. HPLC analysis was carried out (waters model no.2690) on C_{18} column. (symmetry 4.6 × 250mm).The mobile phase was methanol with a flow rate of 0.75 mL/min and UV detector at 316nm.The biodegraded Navy blue Rx –(Reactive blue – 59) was characterized by fourier transform Infrared Spectroscopy (Perkin Elmer by Spectrum one) and compared with control dye. The FTIR analysis was done in the mid IR region of 400-4000 cm $^{-1}$ with 16 scan speed. The samples were mixed with spectroscopically pure KBr in the ratio of 5:95, pellets were fixed in sample holder, and the analyses were carried out. Rotary vacuum evaporated sample (extracted after 24 h decolourization period) was dissolved in methanol and GC-MS analysis of metabolites was carried out using a Hewlett Packard 989 BMS engine, equipped with integrated gas chromatograph with a HP1 column (30 m long, 0.25 mm id, nonpolar).Helium was used as carrier gas at a flow rate of 1.1 mL/min .The injector temperature was maintained at 300 ℃ with oven condition as 100 ℃ kept constant for 2 min – increased up to 250 ℃ with 10 ℃ per min – raised up to 280 ℃ with 30 ℃ per min rate .The compounds were identified on the basis of mass spectra and using the NIST library. TLC analysis was carried out: ethyl acetate: distilled water in proportion of 6:1:1:2 samples were analyzed and the spots were developed using an I_2 chamber.

Microbial toxicity and phytotoxicity studies

Phytotoxicity

The toxicity of the product was studied on *Triticum aestivum* and *Phaseolus mungo.* 100 mL of decolorized broth was centrifuged at 10,000 rpm for 20 min .The supertanant was mixed equal volumes of ethyl acetate. The extract was dried over anhydrous Na_2So_4 and evaporated to dryness to toxicity in a rotary evaporator.

100 ppm-1000 ppm of dye concentration were used to determine the toxicity of the dye to wheat and mung 1000-ppm conc. was found to be toxic. The same conc. of product and control i.e. distilled water was used for observing the effect of product.

The seeds were grown for even days in the above said product, dye and distilled water by daily watering the plant with fixed volume of dye product and distilled water. On 7th day difference in root stem length was measured in all the sets.

Microbial toxicity

Azotobacter vinelandii and *Pseudomonas aeruginosa* obtained from NCIM was cultivated on nutrient agar plates for 24 h by spread plate technique Two wells were bored in which the same concentrations of degraded product and undegraded dye were placed. The plates were incubated to see the zone of clearance.

RESULTS

The actinomycetes showing decolorization and degradation of textile dye Navy blue Rx –Reactive blue-59 was sent for 16S rDNA analysis to GeneOm bio laboratories, Pune.

Table 1: Hit list - 16SrDNA analysis of *Streptomyces isolate from* NCBI data base library

Sequence Name	% Match	No. Of bases searched	Total mismatches
Streptomyces spp.	98	735	11
Streptomyces albus subsp.	98	735	11
*Streptomyces spp.*3196	98	735	11
Streptomyces saprophyticus	98	735	11
Streptomyces krainskii.	98	735	11

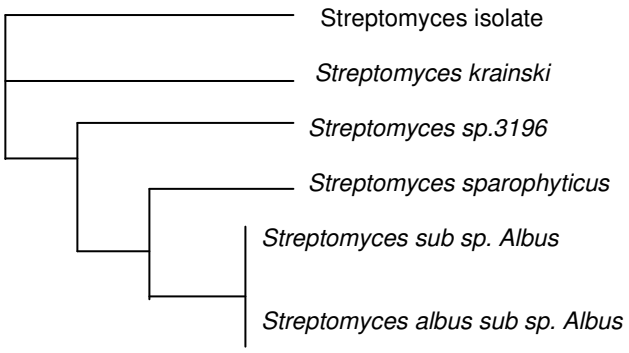
Table 2: Concise alignment of bases:

Base Position →

74	56	180	305	411	543	619	626	681	715	724
	T	G	T	C	T	T	T	T	G	A
C	A	A	A	G	-	G	G	-	-	-

Ai =Actinomycete isolate

Table 3: Phylogenetic tree



The actinomycete was found to have 98 % similarities with 4 *Streptomyces*. It was placed close to *S .krainskii* in phylogenic tree hence was named as *S. krainskii* SUK – 5.

Effect of static and shaking condition

S. krainskii SUK –5 did not decolorize Navy-blue Rx at static conditions well as in plain sterile distilled water. When organism was grown and kept for decolourization at shaking conditions 95.33% decolourization was possible at 24 h. The dye was seen to be adsorbed by the cell in the first 12 h, which suggest adsorption of dye and then degradation. To confirm whether this decolourization was due to microbial action or change in pH, the change in pH was recorded, which was in the range of 7.4 to 8 at shaking conditions .UV–Vis spectra of Navy blue Rx did not show any change at this pH range.

Effect of initial dye concentration on decolourization

Percent decolourization of Navy blue Rx by *S. krainskii* SUK –5 varied at initial dye concentration (30- 100 mg/L). At 30-50 mg/L, 95 and 87 % dye removal was observed. The time required for complete decolourization of dye was 24 and 48 h for 30 & 50 mg/L respectively. 70 and 100 mg/L concentration of the dye was seen to be toxic to the organism.

Enzymes activities while decolorization in shaking condition

The time course of Lignin Peroxidase, laccase, tyrosinase, NADH- DCIP reductase azo reductase was observed for 96 h. Induction of oxidative enzymes (LiP), up to complete decolorization (24h) was observed. DCIP and MR reductase activity was found be increased at 48h as compared to their activities at 24 h, after complete decolorization of the dye. These enzymes were later on decreased at 96 h.

From this study it can be deduced that there was direct involvement of lignin peroxidase, DCIP reductase

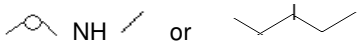
and azoreductase and formation of simple metabolites from rather complex structure of Navy blue Rx as taken place. There were no activities of laccase and tyrosinase in the culture and supernatant.

Biodecolorization & Biodegradation analysis

TLC analysis of undegraded dye and the product showed RF values 5.6 and 4.2 indicating biodegradation of the dye. U V. Vis scan (400 – 800 nm) of supernatant at 0 h and after decolorization of the dye at 24 h showed decolorization and decrease in dye concentration from the medium. Peak observed at 588 nm (0 h) was decreased showing disappearance of the original peak with complete decolorization at 24 h incubation time. HPLC analysis of the dye sample extracted at 0h incubation showed a peak at retention time 2.5 min and sample extracted at 24 h showed to major metabolites at retention time 2.6 and 3.9 min. Detection of two peaks in HPLC of sample as compared to a single peak in control clearly indicates the degradation of the dye. The FTIR spectra of undecolorized Navy blue Rx has shown the presence of different peaks at 2924 cm^{-1} ,1635 cm^{-1} ,1478 cm^{-1},1221 cm^{-1} and 1020 cm^{-1}.In the FTIR spectrum of 24 h,extracted sample, peak at 3393 cm^{-1} , indicates NH or OH stretching ,2926 cm^{-1}, represents aromatic ring-.Hydrogen stretching, a broad signal at 1650 cm^{-1} represents breaking of N=N and formation of – NHCO- group (data not shown).

Therefore it can be said that primary and secondary amines may be preset in degraded form of the dye. GC-MS analysis of 24 h metabolites was carried out to investigate the metabolites formed during the biodegradation process. As shown in Table 4, GC-MS has revealed one major compound at retention time 7.940. The mass spectrum has revealed base peak at 61 and mass peak at 379.Mass peak of the degraded product is closer is diamino stilbene disulphonic acid Base peak indicates that the framework of the degraded product is of

OH OH OH



NH or Type

Table 4: GC-MS spectral data of biodegraded product of Navy Blue Rx

Peak#	R.Time	I.time	F.Time	Area	Area %	Name
1	7.94	7.742	8.075	446904	100	Compound closer to diamino stilbene disulphonic acid
				446904	100	

Phytotoxic and Microbial toxicity studies

Phytotoxic studies on *Triticum aestivum* and *Phaseolus mungo* were carried out at a concentration of 1000 ppm of untreated and degraded dye .The germination of seed and length of root and shoot of both the plants were recorded. The result pointed towards maximum detoxification of Navy blue Rx, its metabolites showing induction in root and shoot length of both the plants. In contrast even though germination of seeds of both plants was observed in the untreated dye there was reduction in shoot and root growth as compared to product.

Similarly microbial toxicity of the undegraded and degraded dye was studied on agriculturally important microorganisms *Azotobacter vinelandii* and *Pseudomonas aeruginosa.* The dye Navy blue Rx was found to be toxic whereas the dye metabolies were found to be stimulatory to the microorganisms.

DISCUSSION

In the study the main objective was to observe microbial decolorization and biodegradation of Navy blue Rx in shaking conditions. The aerobic-anaerobic decolorization and degradation of Red HE -7 B in textile effluent was reported by *P. desmolyticum* by Carliell *et al*. (1995) O' Neill *et al.* (2000). It was observed that the *S. krainskii* SUK– 5 could not utilize Navy blue Rx as sole carbon source, as it could not decolorize the dye added in sterile distilled water. In our study the decolourization of Navy blue Rx took place only under shaking condition and in nutrient medium. This suggests that the precondition for reduction of azo dyes is the presence and availability of a co-substrate (Nigam *et al*., 1996), because it acts as an electron donor for the azo dye reduction.

Oxidative biodegradation takes place upon action of enzymes such as peroxidases and laccases. The involvement of fungal peroxidases and laccases for the oxidation of sulfonated azo dyes has been reported earlier (Kandelbauer *et al*., 2004a) bacterial extra cellular azo dye oxidizing peroxidases have been characterized in *Streptomyces chromofocus* (Pasti-Grigsby *et al*., 1996). In this study, induction in oxidative enzymes (LiP), up to complete decolorization period (24 h) was probably responsible for decolorization of Navy blue Rx. Induction in reductase for DCIP and MR was also recorded.

The progress of decolorization biodegradation was assessed by UV–Vis spectroscopy, FTIR spectroscopy and HPLC.Identification of biodegradation product was carried out by GC–MS .Identified product was close to diamino stilbene sulphonic acid. Degraded product had much lower mass than the parent compound.

Pourbabaee *et al.,* (2006) had observed 100 % germination inhibition of *Triticum aestivum* at 150 ppm of untreated effluent and 10% inhibition by treated effluent. We observed that at 1000 ppm concentration of untreated dye there was no seed germination inhibition in *Triticum aestivum* and *phaseolus mungo*. At this concentration of dye metabolites, 100 % germination of the seeds of both

the plants was observed, instead growth stimulation took place .This suggests that the product metobolites fromed after dye degradation contained some growth in enhancing compounds. Dye Navy blue Rx was toxic for the growth of agriculturally important microorganisms viz. *Azotobacter vinelandii* and *Pseudomonas aeruginosa*. The dye metabolites were found to be stimulatory to the microorganisms. This made a conclusion that the dye metabolites are not only non-toxic but stimulatory to agricultural crops and organisms.Thus biodecolorization and biodegradation of textile azodye- Navy blue Rx by an isolated actinomycete *Streptomyces krainskii* SUK-5 from Krishna river water can be said to be an ecofriendly and ecoefficient manner of dye degradation.

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