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Degradation of Textile Dyes by Isolated *Lysinibacillus Sphaericus* Strain RSV-1 and *Stenotrophomonas maltophilia* Strain RSV-2 and Toxicity Assessment of Degraded Product

Rajeswari K[1]*, Subashkumar R[2] and Vijayaraman K[3]

[1]*Research and Development Centre, Bharathiar University, Coimbatore, Tamil Nadu, India*
[2]*PG & Research Department of Biotechnology, Kongunadu Arts and Science College, Coimbatore, Tamil Nadu, India*
[3]*KSG College of Arts and science, Coimbatore, Tamil Nadu, India*

Abstract

An attempt was made to study the toxic nature of dye degraded product(s) which was degraded by previously isolated two potential strains namely *Lysinibacillus sphaericus* RSV-1 and *stenotrophomonas maltophilia* RSV-2. The phytotoxicity and cytotoxicity of degraded product(s) were tested on *Triticum aestivum* (co w) and human embryonic kidney cell line (HEK 293) respectively. Results revealed that the degraded product(s) was nontoxic in nature with respect to phytotoxicity as well as cytotoxicity study. Further the strains were utilized for the treatment of real textile dyewaste effluent and the result found that there was considerable reduction in Biological Oxygen Demand (BOD), Chemical Oxygen Demand (COD) and Total Organic Carbon (TOC) of real textile effluent. Therefore the strains could be effectively utilized for the treatment of real textile dye effluent having high concentration of reactive dyes.

Keywords: Textile dye waste effluent; *Lysinibacillus* sp; *Stenotrophomonas* sp; Physico-chemical parameter; Waste water treatment

Introduction

With the increased demand for textile products, the textile industry and its wastewater have been increasing proportionally, making it one of the main sources of severe pollution problems worldwide. In particular, the release of coloured effluents into the environment is undesirable, not only because of their colour, but also because many dyes from wastewater and their breakdown products are toxic and/or mutagenic to life [1]. In India, an average mill discharges about 1.5 million litres of contaminated effluent per day, which leads to chronic and acute toxicity [2]. Considering the volume generated and the effluent composition, textile industry wastewater is rated as the most polluting among all industrial sectors [3]. The effluents from textile and dyeing industries have high Chemical oxygen demand (COD), Biochemical oxygen demand (BOD), colour, pH, and also it contains metal ions, hence it is very difficult to treat such effluents [4]. Textile industries generate millions of liters of untreated effluents per day which are directly discharged into drinking water resources, such as rivers and lakes. This alters pH, increases BOD and COD and gives intense coloration [5]. Dye wastewaters are usually treated using physicochemical methods such as flocculation, coagulation, adsorption, membrane filtration, precipitation, irradiation, ozonization and Fenton's oxidation [6]. These methods are effective but may generate significant amounts of chemical sludge, whose disposal in secure landfill increases process cost [7]. There are several studies describing decolorization of reactive dyes mediated by pure bacterial culture such as, *Pseudomonas* sp. SUK1 for Reactive Red 2 [8]; *Exiguobacterium* sp. RD3 for Navy Blue HE2R (Reactive Blue 172) [9]; *Rhizobium radiobacter* MTCC 8161 for Reactive Red 141 [10]; *Pseudomonas aeruginosa* NBAR12 for Reactive Blue 172 [11] and isolated bacterium KMK48 for the degradation of various sulfonated reactive azo dyes [12]. It is very important to know whether biodegradation of a dye leads to detoxification of the dye or not. This can be done by performing toxicity test of the original dye and its biodegradation products. Many studies have shown that reactive dyes can cause allergic dermatoses and respiratory diseases [13,14]. In order to assess the effect of xenobiotic compounds on cell, it is important to determine the cytotoxicity concentration of the dye or its degraded compound. Our previously isolated potential bacterial strains [15] from textile dyewaste effluent were utilized in the present study. An attempt was made to determine the toxic nature of the dye metabolites based on phytotoxicity and cytotoxicity test, further the strains were used to treat real textile effluents and physico chemical parameters of treated and untreated effluents were analysed. Two potential strains namely *Lysinibacillus sphaericus* RSV-1 and *stenotrophomonas maltophilia* RSV-2 were isolated from textile dyewaste effluent capable of decolorizing four different mixed reactive dyes namely Blue RR, Black B, Red RR and Yellow RR up to 2700mg-l and 2100mg-l respectively with 50-60 percent decolorization within 48-70 h of incubation [16]. Strain *Lysinibacillus* sp ZB-1, the first one of this genus, was found to possess the ability to metabolize fomesafen. *Lysinibacillus* are able to survive under extremely harsh conditions, which make them ideal candidates for bioremediation of contaminated environments [17]. Originally, this genus was Bacillus sp. and was transferred into this genus as *Lysinibacillus* sp. in 2007 [18]. A bacterial strain AAP56, isolated from a polluted soil (from Kelibia city) and identified as *Stentrophomonas maltophilia*, was particularly interesting for its ability to decolorize recalcitrant dyes of an industrial effluent SITEX Black [19]. This strain can also decolorize some synthetic dyes such as Methylene Blue, Toluidine Blue, Methyl Green, IndigoBlue, Neutral Red, Congo red, Methyl Orange and Reactive Pink [20]. Many studies have shown that reactive dyes can cause allergic dermatoses and respiratory diseases [21-24]. The result of a clinical and immunological

***Corresponding author:** Rajeswari K, Research and Development Centre, Bharathiar University, Coimbatore, Tamil Nadu, India, Tel: 09944933846; E-mail: rajimicro_grd@yahoo.co.in

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investigation of respiratory disease indicated that about 15% of 400 workers handling reactive dyes experienced work-related respiratory and nasal symptoms [25]. Recent toxicological studies with the azo dye Red HE3B (Reactive Red 120), before and after bacterial treatments, showed that the dye was able to induce oxidative stress and a high frequency of chromosome aberrations and micronuclei in root cells of *A. cepa*, when compared to the effects caused by its metabolites [26]. The textile industries are to satisfy the ever growing demands in terms of quality, variety, fastness and other technical requirements, but the use of dye stuffs has become increasingly a subject of environmental concern. Therefore, it is essential to evolve regulations designated to improve the health and safety and the human and natural environment [27].

Materials and Methods

Microorganisms and growth media

Previously isolated bacterial strains *Lysinibacillus sphaericus* strain RSV-1 and *stenotrophomonas maltophilia* strain RSV-2 were cultured and maintained on Nutrient agar medium with the composition (g L^{-1}) of Peptone (5.0); Yeast extract (2.0); Beef extract (3.0); NaCl (5.0); agar (16.0) pH 7.0 ± 0.02. All the decolorization experiments were performed in Mineral Salts Medium (MSM) of pH 7.0 contained (g L^{-1}) the following composition NaCl (1.0), $CaCl_2.2H_2O$ (0.1), $MgSO_4.7H_2O$ (0.5), KH_2PO_4 (1.0) and Na_2HPO_4 (1.0). Decolorization experiment was performed in mixture of ten reactive dyes namely Yellow ME4GL, Blue RR, Red RR, Yellow RR, Red M5B, Blue MR, Deep Black RR, Yellow MERL, Red ME4BL and Golden Yellow MR. These strains could able to decolourise more than four mixed dye hence further experiments were performed with ten mixed dyes. Dyes were procured from textile dyeing unit in Tirupur. About 1000 ppm of stock was maintained by adding equal amount of each dye.

Decolorization study

The superior Strain RSV-1 was used in toxicity studies was inoculated separately in nutrient broth medium and incubated at 30°C for 24 hrs. After incubation cells were centrifuged at 6000 rpm for 15 minutes. Pellets were dissolved in MSM containing 0.5% yeast extract. Cell density of 1.0 OD at 600nm was made for decolorization experiment. The medium to inoculum ratio was maintained at 50:1. MSM was prepared by adding ten different reactive dyes with initial concentration of 500 ppm, after complete decolorization, another 500 ppm was added. Complete decolorizarion was achieved within 24-30 hrs of incubation rather 5-6 hrs (data provided in supplementary material) when four mixed dyes were used and then, the cell-free supernatant was collected after decolorization was extracted first with ethyl acetate to extract the biotransformed products and the aqueous phase was further extracted with n-butanol to extract the residual dye. Degraded product(s) was analysed using TLC, the developing solvent systems used were ethyl acetate:hexane (2:3, v/v) for biotransformed intermediates/products and ethyl acetate:methanol (7:3, v/v) for residual dye. The bands of aromatic components were observed under UV light (365 nm) and other bands were observed by exposing the plates to iodine vapor in an iodine chamber. Ethyl acetate is rarely selected as a reaction solvent because it is prone to hydrolysis and transesterification. The aqueous phase, thus obtained, was subsequently extracted twice with n-butanol to extract the residual dye (extract-2), as dye was more soluble in n-butanol [28].

Phytotoxicity study

The degraded metabolites of mixed dye, extracted in ethyl acetate were dried and dissolved in water to form the final concentration of 1,000 ppm were used for phytotoxicity studies. The phytotoxicity study was carried out at room temp (30 ± 2 °C) in relation to *Triticum aestivum* (co w) seeds (ten seeds) by watering separately. The seeds were procured from the Department of Millets, TNAU, Coimbatore. About 5 ml samples of mixed dye and its degradation product (1,000 ppm) was supplied per day. Control set was carried out using plain water at the same time. Length of plumule (shoot), radical (root) and germination (%) was recorded after 7 days.

Cytotoxicity study (In vitro cell proliferation assay)

Cell line: The human embryonic kidney cell line (HEK 293) was obtained from National Centre for Cell Science (NCCS), Pune, and grown in Eagles Minimum Essential Medium (EMEM) containing 10% fetal bovine serum (FBS). All cells were maintained at 37°C, 5% CO_2, 95% air and 100% relative humidity. Maintenance cultures were passaged weekly, and the culture medium was changed twice a week.

Cell treatment: The monolayer cells were detached with trypsin-ethylene diamine tetra acetic acid (EDTA) to make single cell suspensions and viable cells were counted using a hemocytometer and diluted with medium with 5% FBS to give final density of $1x10^5$ cells/ml. one hundred microlitres per well of cell suspension were seeded into 96-well plates at plating density of 10,000 cells/well and incubated to allow for cell attachment at 37°C, 5% CO_2, 95% air and 100% relative humidity. After 24 hrs, the cells were treated with at least six concentrations of degraded samples. They were initially dissolved in dimethylsulfoxide (DMSO) and stored frozen prior to use. The obtained product was dissolved in DMSO to prepare a final concentration 1mg/ml. The samples were further diluted in serum free medium and 100 µl per well was added to plates to obtain final concentrations of 500, 250, 125, 62.5, and 31.25 µg/ml. The final volume in each well was 200 µl and the plates were incubated at 37°C, 5% CO_2, 95% air and 100% relative humidity for 48 hrs. The medium containing without samples were served as control. Triplicate was maintained for all concentrations.

MTT assay: MTT is a yellow water soluble tetrazolium salt. A mitochondrial enzyme in living cells, succinate-dehydrogenase, cleaves the tetrazolium ring, converting the MTT to an insoluble purple formazan. Therefore, the amount of formazan produced is directly proportional to the number of viable cells. After 48 hrs of incubation, 15 µl of MTT (5 mg/ml) in phosphate buffered saline (PBS) was added to each well and incubated at 37°C for 4 hrs. The medium with MTT was then flicked off and the formed formazan crystals were solubilized in 100 µl of DMSO and then measured the absorbance at 570 nm using micro plate reader. The percentage cell viability was then calculated with respect to control as follows:

% Cell viability=[A] Test/[A]controlx100

Treatment of real dye waste effluent by RSV1 and RSV2

The decolorization and biodegradation capacity of RSV-1 and RSV-2 was performed on real textile effluent. For the experiment, raw effluent was collected from one of the CETP unit located in Tirupur and the strains were inoculated individually as well as in consortium form. The set up was maintained as per optimized physicochemical condition. Two controls were maintained, control A (effluent without any nutrition supplement); control B (effluent with 0.5 % of soya powder); RSV-1(effluent inoculated with RSV-1 strain along with 0.5% of soya powder); RSV-2 (effluent inoculated with RSV-2 strain with 0.5% of soya powder); CRM (effluent contains strain RSV-1 and RSV-2

along with 0.5% of soya powder). All the physico-chemical parameters were analysed as per the method of APHA [29].

Results and Discussion

In our previous study about 112 bacterial strains were isolated from dyewaste effluent. Based on various screening methods, the two most competent strains were selected for further studies [15]. In acclimatization study, these two bacterial strains RSV-1 and RSV-2 decolorized four different mixed reactive dyes namely Blue RR, Black B, Red RR and Yellow RR up to 2700 mg^{-L} and 2100 mg^{-L} respectively with 50-60 percent decolorization within 48-70 hrs of incubation (Tables S1 and S2). The strains were identified as *Lysinibacillus sphaericus* RSV-1 and *Stenotrophomonas maltophilia* RSV-2 1 based on morphology, physiochemical properties, and the results of 16S ribosomal RNA (rRNA) gene sequence analysis [16]. The sequences of RSV-1 and RSV-2 were deposited in Gen Bank database under accession number JF502569 and JF502570 respectively. The identification of microorganisms capable of assimilation or removal of textile dyes is one approach to reduce environmental pollution by textile dyes. Bioremediation of the textile effluents is an attractive method of color removal due to its environmental friendly technology. Hence, it is of outstanding interest to find an effective microflora and biochemical approach for the decolorization and detoxification of textile wastewater [30].

Phytotoxicity analysis of degraded product

Most common methods employed to study phytotoxicity are monitoring of seed germination and plant growth. As dyewaste from industries are most commonly discharged to the nearby agricultural area and water bodies, it was important to reveal the toxicity of Reactive dyes and its degraded metabolites on plant systems. Germination (%) of *Triticum aestivum* seeds was found to be less with mixed reactive dyes as compared with its degraded metabolites. The phytotoxicity study showed that length of plumule and radical was affected in case of the dyes whereas with degraded metabolites it showed significant growth, compared to control (Table 1), which indicate that, the isolated RSV-1 was not only able to decolorize mixed dyes but was also able to detoxify it. The mean of plumule length and radical length of *T. aestivum* was 13.30 ± 1.161cm and 9.25 ± 0.75 cm, respectively of 10 seeds in distilled water as a control with 100% germination. When seeds were treated with 500 ppm concentration of mixed reactive dyes, the plumule length and radical length was found to be 3.57 ± 1.59 and 2.05 ± 0.83 cm, respectively with 30% germination. Whereas when treated with the degraded metabolites the plumule length and radical length was found to be 12.34 ± 0.73 and 8.71 ± 0.50 cm, respectively with 80% germination when treated with 1000 ppm degradation product . This indicates the less toxic nature of the degradation product to the plants. We found that the degraded metabolites were non-toxic to the test plant; hence the strains could be utilized for the treatment of real textile effluent. The relative sensitivity towards the dye DR5B and its degradation products in relation to *T.aestivum* was studied by Jadhav et al., [31]. Parshetti et al. also showed germination of *T. aestivum* was less with malachite green treatment as compared to its degradation product and distilled water [32].

	Germination (%)	Plumule (cm)	Radial (cm)
Distilled water	100	13.30 ± 1.161	9.25 ± 0.75
Mixed dye	30	3.57 ± 1.59	2.05 ± 0.83
Metabolites	80	12.34 ± 0.73	8.71 ± 0.50

Values of mean of ten germinated seeds of three sets SEM (±)

Table 1: Phytotoxicity study of reactive mixed dye and metabolites on Triticum aestivum CO(W).

Concentration	31.25 µg/ml	62.5 µg/ml	125 µg/ml	250 µg/ml	500 µg/ml	Control1
Absorbance at 570 nm	0.274	0.255	0.263	0.248	0.223	0.284
	0.264	0.264	0.248	0.255	0.218	0.271
	0.275	0.27	0.255	0.249	0.215	0.271
	0.271	0.263	0.255333	0.250667	0.218667	0.275333
Average	0.271	0.263	0.255333	0.250667	0.218667	0.275333

1- Contains no added substances but only the cells

Table 2: Different concentrations of metabolites and their absorbance.

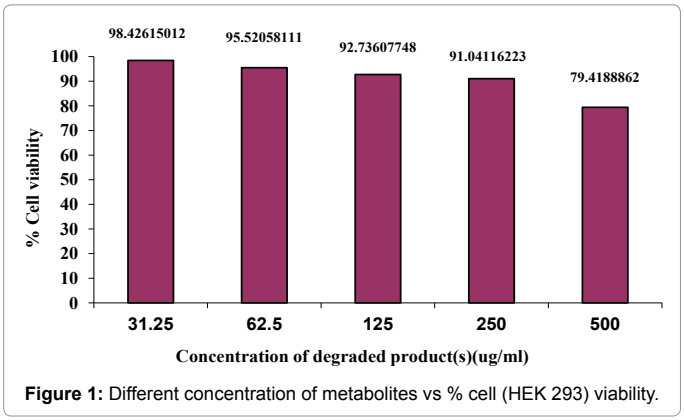

Figure 1: Different concentration of metabolites vs % cell (HEK 293) viability.

Cytotoxicity analysis of degraded product

The cytotoxicity study was conducted to check the toxic nature of degraded metabolites on human embryonic kidney cell line (HEK 293) using MTT assay. The assay was performed with varying concentration of degraded product ranged from 31.25-500 µg/ml (Table 2) and percentage cell viability was observed in the range of 79.41-98.42 (Figure 1) since the degraded product at high concentration because only a mild interference in cell viability, hence it's said to be non-toxic in nature. We performed MTT for mixed dye, but we could not interpret the result since it interefere with the colour, also the cells were not clearly detected when observed under inverted microscope (S3). Cytotoxicity of DR28 dyes at various stages of biodegradation by the strain *Bacillus velezensis* AB was studied by Bafana et al. [33]. Cytotoxicity was tested on HL-60 cell line using MTT assay. Toxicity towards HL-60 cells increased on the 3rd day, followed by gradual reduction up to 15th day ($p<0.01$). The mouse fibroblast cell line from subcutaneous connective tissue (L929 cell line) was utilized or testing the parent dye Reactive Red 141 and Reactive Red 2 dye and their metabolites produced by the strain *Bacillus lentus* [34]. Eczema, contact dermatitis, asthma, chronic bronchitis, tuberculosis, haematoma, bladder cancer and irritation to eyes, have been reported amongst the workers of textile industries in Sanganer [35].

Treatment of real dye effluent by the strains RSV1 and RSV2

The decolorization and degradation ability of RSV-1, RSV-2 and consortium (CRM) was determined on real textile effluent. The study effluent was collected from CETP plant and the strains were inoculated individually as well as in consortium form (Table 3). There was an average reduction in BOD (76%), COD (62.48%) and TOC (47.57%) in the effluent containing RSV-1 strain was found. The percent reduction of BOD, COD and TOC for RSV-2 and consortium was found to be 36.86%, 60.71%, 44.48% and 56.56%, 67.10% and 48.96% respectively,

Parameters	Control A	Control B	RSV-1	RSV-2	CRM	Treatment *Time(hrs)*	PCB *Standard*
Color	Reddish Black	Light black	Colourless	Light black	Colourless	12	-
Color(pt/co)	1245	397	103	412	87	12	-
EC(mohs cm)-1	12410	13786	13367	13229	13787	24	-
pH	8.3	7.5	8.0	8.2	7.8	24	5.5-9.0
% Decolorization	16.09	56.89	87.05	53.88	89.56	30	-
Total Suspended Solids (mg/l)	165	188	176	185	194	30	50-150 mg/l
Total Dissolved Solids(mg/l)	8054	8215	8764	8973	8765	30	Not more than 3,000 mg/l
Total hardness as CaCo3(mg/l)	345	367	398	365	415	30	-
BOD(mg/l)	198	69	47	125	86	48	20-60 mg/l
COD(mg/l)	845	320	317	332	278	48	120-400 mg/l
Total Alkalinity(mg/l)	1435	1539	1545	1533	1503	48	-
Chlorides(mg/l)	759	722	673	747	681	48	-
Sulphats (mg/l)	1466	1410	1449	1424	1398	48	-
TOC(mg/l)	1650	987	865	916	842	48	-

Control A= effluent without any nutritional supplement; control B =effluent with 0.5% of soya powder; RSV-1(effluent inoculated with RSV-1 strain along with 0.5% of soya powder); RSV-2 (effluent inoculated with RSV-2 strain with 0.5% of soya powder); CRM (effluent contains strain RSV-1 and RSV-2 along with 0.5% of soya powder)

Table 3: Physico chemical parameters of treated and untreated real textile effluent using effective strains.

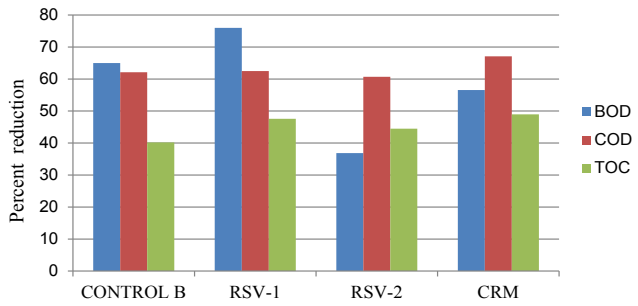

Figure 2: Comparative study of percent reduction of BOD, COD and TOC.

whereas in control B, the percent reduction of BOD, COD and TOC was found to be 65%, 62.13% and 40.18% respectively within 48 hrs (Figure 2). Considerable differences were not observed in other parameters. Based on the experiment, we could conclude that comparatively our strain RSV-1 was a good candidate for effective decolorization of real textile effluent. The ability of the strain to tolerate, decolorize and degrade azo dyes at high concentration gives it an advantage for treatment of textile industry wastewaters. However, potential of culture needs to be demonstrated for its application in treatment of real dye bearing wastewaters using appropriate bioreactors. Consortium-AP of *Aspergillus ochraceus* NCIM-1146 fungi and *Pseudomonas* sp. SUK1 bacterium achieved a significant reduction in COD (96%), BOD (82%) and TOC (48%) of textile effluent [36].

Conclusion

This is the first report to date, and to our knowledge, that investigates the use of *Lysinibacillus sphaericus* RSV-1 and *Stenotrophomonas maltophilia* RSV-2 culture in treating industrial wastewaters containing various types of dyes. While treating the real textile effluent, the reduction of BOD, COD and TOC shows that the strains could mineralize the dyes. Thus our microbes can be a suitable candidate for the treatment of real effluent containing high concentration of textile dyes.

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