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BIODEGRADATION OF REACTIVE DYES BY AN ISOLATED BACTERIUM LYSINIBACILLUS SPHAE RICUS RSV-1

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

[1]*Research and Development Centre, Bharathiar University, Coimbatore-641 046, INDIA*
[2]*PG & Research Department of Biotechnology, Kongunadu Arts and Science College, Coimbatore 641 029, INDIA*
[3]*KSG College of Arts and Science, Coimbatore-641015, INDIA*

ABSTRACT

A potential bacterial strain isolated from dyewaste effluent was characterized and identified as Lysinibacillus sphaericus RSV-1 based on biochemical and 16S ribosomal RNA gene sequence study. The strain was utilized for decolorization and degradation of various reactive dyes. Optimization of yeast extract concentration, pH, temperature, salinity and biomass was determined to be 0.5%, 7.0, 30˚C and 20% respectively. Effects of various nitrogen and carbon sources on decolorization of 100 ppm concentration of ten different mixed reactive dyes revealed, yeast extract, soya chunk powder, rice husk extract showed best nutritional supplement with 95±0.47, 89±0.94 and 92±1.63 percent decolorization within 2-48 hrs of incubation. Our isolated strain could be effectively utilized for the treatment real textile effluent containing high concentration of reactive dyes.

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KEY WORDS

Decolorization ; dyewaste effluent ; Lysinibacillus sphaericus; reactive dyes

***Corresponding author:** Email: rajimicro_grd@yahoo.co.in

[I] INTRODUCTION

Textile effluent discharge is becoming a major environmental pollution in India particularly in Tirupur. During reactive dyeing processes, up to 50% of the dye that present in the original dye bath is lost to the wastewater that let in to nearby river without any treatment [1]. The reactive dyes are not degraded by the conventional wastewater treatment processes [2] are characterized by the presence of a nitrogen–nitrogen double bond (–N=N–), namely the azo group bound to aromatic groups [3]. Azo dyes are as recalcitrant in nature are not readily degraded by conventional methods [4]. More than 10–15% dye does not bind to fiber during color processing and release into the wastewater causing serious environmental pollution [5, 6]. There are several physical and chemical methods available but these have disadvantages of being very expensive, the formation of large amount of sludge and the emission of toxic substances [7]. In addition, the accumulation of concentrated sludge creates a disposal problem [8]. It is estimated that at least 100 tonnes of sludge will have to be disposed of every year. Many microorganisms has been using as bioremediation agents in the treatment of wastewater-containing textile dyes [9]. An advantage of using pure culture system ensures that the data are reproducible and that the interpretation of experimental observations is easier [10]. Many pure culture bacteria has been utilized for the treatment of reactive dyes such as, Pseudomonas sp. SUK1 for Reactive Red 2 [11], *Exiguobacterium* sp. RD3 for Reactive Blue 172 [12], *Rhizobium radiobacter* MTCC 8161 for Reactive Red 141 [13], *Pseudomonas aeruginosa* NBAR12 for Reactive Blue 172 [14] and bacterium KMK48 for the degradation of various sulfonated reactive azo dyes [15]. Bioremediation is a process in which the natural capacity of microbes is enhanced to degrade toxic chemicals and waste [16]. Several reports are available indicating that a variety of microbes have been involved in the bioremediation and biodegradation of dyes, which includes some bacteria such as, *Aeromonas hydrophila* [17], *Pseudomonas* sp SUK 1 [18, 19], *Exiguobacterium sp* RD 3 [12], *Pseudomonas* KF46 and *Kurthia* sp. [20], *Aeromonas hydrophila* [21] *Pseudomonas luteola* [22], bacterial consortium *(Aeromonas caviae, Proteus mirabilis* and *Rhodococcus globerulus)* [23]. Moreover, bacterial degradation is much faster than fungal degradation of textile dyestuffs [24]. Attempts have been made to decolorize the reactive mixed dyes using our previously isolated *Lysinibacillus sphaericus* strain [25].

[II] MATERIALS AND METHODS

2.1. Isolation of bacteria

Dyewaste effluent samples were collected from textile dyeing unit and CETP located in Tirupur. In our previous study, effective decolorizing strains were isolated based on primary, secondary screening and acclimatization study [25].

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2.2. Media preparation

Previously isolated strain was maintained on nutrient agar medium with the composition (g L-1) of pH;7.0 ± 0.02 Peptone(5.0); Yeast extract(2.0); Beef extract(3.0); NaCl (5.0); Agar (16.0). All the decolorization experiments were performed in Minimal Salt 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). In the present study all the experiments were performed with 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. About 1000 ppm stock was prepared by adding equal amount of each dye.

2.3. Identification of the strain

A pure colony of the bacterium was grown in LB medium until log phase growth was obtained. The resulting growing bacteria were obtained with centrifugation. Genomic DNA from the isolate was extracted with the bacterial genomic DNA Isolation Kit (RKT09). Amplification was done using prokaryotes 16S rRNA specific forward Primer: 5'-AGAGTRTGATCMTYGCTWAC-3 and reverse Primer: 5'-CGYTAMCTTWTTACGRCT-3' (Sreekumar and Krishnan 2010). The reaction mixture contained 1µl of template DNA, 400 ng of each forward and reverse primers, 4 µl of dNTPs (2.5 mM), 10 µl of 10x DNA polymerase assay buffer, 1.5mM of MgCl2 as final concentration, 1 µl of Taq DNA polymerase enzyme (3U/ µl), water to make the final volume as 100 µl. The PCR conditions were initial denaturation of 94˚C for 5 min, denaturation of 94˚C for 30 sec, annealing of 55˚C for 30 sec, extension of 72˚C for 2 min and final extension of 72˚C for 15 min. Number of cycles were 35. The reaction was carried out in Thermal Cylcer ABI2720 (Chromous biotech). The sequencing mixture contained 1 µl of Template (100ng/ µl), 2 µl of Primer (10pmol/ µl), 3 µl of Milli Q water and the conditions were initial denaturation of 96˚C for 1 min followed by denaturation of 96˚C for 10 sec followed by hybridization 50˚C for 5 sec finally elongation of 60˚C for 4 min for 25 cycles. The nucleotide sequence was determined automatically using Big Dye Terminator v3.1 Cycle sequencing Kit in the ABI PRISM 3130- Genetic Analyzer with universal primers. DNA sequence analyses were performed using the BLAST search and was aligned with sequences from other bacterial sequences retrieved from GenBank database of NCBI. An alignment of sequences was done by CLUSTAL W. The phylogenetic tree was constructed using Mega 4.0 software.

2.4. Optimization of physico chemical parameters

Effect of yeast extract concentration (0.05, 0.1, 0.2, 0.4, 0.8 % (w/v), pH (3, 5, 7, 9 and 11), Temperature (28 ºC, 30 ºC, 37 ºC, 45 ºC, and 50ºC), Salinity (1, 3, 5, 7, 9 %) and biomass concentration (4, 8, 12, 16, and 20 %) on decolorization was studied. The final concentration of mixed dye was 100ppm. The culture was inoculated previously in nutrient broth medium incubated at 30ºC for 12 hours. After incubation, cells were pelleted (6000×g for 20 min) and the growth was monitored spectrophotometrically at 600nm. About 1.0 OD at 600 nm was utilized for optimization parameter study. The medium to inoculums was maintained at 50:1 ratio. All the experiments were performed in MSM and triplicates were maintained for all the experiments.

2.5. Effect of supplemental carbon and nitrogen sources as co-substrates

To study the effect of carbon and nitrogen sources on decolorization, MSM was prepared by adding different carbon and nitrogen sources such as (mannitol (1%), inositol (1%), Corboxy methyl cellulose (CMC) (1%), lactose (1%), sucrose (1%), starch (1%), dextrose (1%) as a carbon source and yeast extract (0.5%), ammonium nitrate (1%), ammonium sulphate (1%), ammonium chloride (1%) and urea (1%) as nitrogen source. In addition, to make the process economically feasible, appropriate concentration of rice husk extract (10%), wheat husk extract (10%), straw waste (1%), sugarcane juice (1%) and Soya chunk power (1%) were mixed with 100 ml distilled water individually and autoclaved at 121˚C for 20 min, MSM was made up with appropriate concentration of each substrate.

2.6. Effect of yeast extract and soya chunk powder on decolorization of individual as well as mixed reactive dye by RSV-1

To study the effect of yeast extract and soya powder (selected based on previous study result) on decolorization, MSM was prepared by adding 1000 ppm concentration of each Yellow ME4GL, Blue RR, Red RR, Yellow RR, Red M5B, Golden Yellow MR, Deep Black RR, Yellow MERL, Red ME4BL, and Blue MR and maintained in appropriate optimised conditions. Spectrophotometric reading was done for the analysis of percent decolorization of individual dyes where as for the mixed dyes, colour of the samples was determined using HACH DR 2800 spectrophotometer and the results were expressed as (pt/co).The percentage of decolorization was calculated as follows:

% Decolorization = Initial absorbance - Observed absorbance/Initial absorbance x 100

[III] RESULTS

3.1. Isolation and Identification of bacteria

Dye waste effluent samples were collected from textile dyeing unit and CETP located in and around Tirupur, Tamilnadu, India. About 112 isolates were obtained from Primary screening and 50 isolates from secondary screening. The effective strain having decolorization ability of four mixed reactive dyes up to 2700 ppm concentration with 52% decolorization within 49 hrs of incubation was selected from acclimatization study was utilized in the present study(data not shown)25. Biochemical and 16S ribosomal RNA gene sequence was performed for identification of the strain. The strain was identified on the basis of cell shape, cell arrangement, nutritional characteristics, physiological and biochemical characteristics. The morphological, physiological, and biochemical characteristics of the isolated strain RSV-1 was listed in **[Table– 1]**. Gram Staining result showed that the strain RSV-1 was Gram positive and showed negative results for indole, methyl Red, V-P Test, citrate utilization test, TSI Test, glucose, lactose, was sucrose, xylose, mannitol, arabinose, hydrolysis of starch, catalase and nitrate reduction test, where as positive for oxidase and gelatin liquefaction test. Presence of growth was observed in temperature (10–50°C) and NaCl (2.0–7.0%). The strain was motile when observed under inverted microscope. The cell morphology was spherical rods, smooth coherent cluster, convex and Opaque. The culture was light grey in colour. 16S rRNA sequence was analyzed using BLASTn and multiple sequence alignment was performed using CLUSTAL W. Phylogenetic tree was constructed using MEGA 4.0 **[Figure–1]**. Result revealed that the strain RSV-1 identified as *Lysinibacillus sphaericus* which was found to be 99% identical to *L. sphaericus* (JF343178) among selected taxonomic group

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of other bacillus species. The strain was deposited in the GenBank database under accession number JF502569. A bootstrap value of 500 indicates that a branching pattern is confirmed in all the resampling, whereas a bootstrap value of 60 indicates that the branching pattern is reproduced only in 60% of the resamplings.

Table: 1. Morphology, Cultural and Biochemical Characterization of *Lysinibacillus sphaericus* RSV-2 strain

Biochemical and culture conditions	*Lysinibacillus sphaericus* RSV-2
Gram staining	Gram-positive
Indole	-
Methyl red	-
V-P test	-
Citrate Utilization Test	-
TSI Test	-
Glucose	-
Lactose	-
Sucrose	-
Xylose	-
Mannitol	-
Arabinose	-
Hydrolysis of starch	-
Catalase	-
Oxidase	+
Gelatin liquefaction	+
Nitrate reduction	-
Phenylalanine deaminase	+
Growth at temperature (10–50°C)	+
Growth on NaCl (2.0–7.0%)	+
Cell type (shape)	Spherical rods
Color	light grey
Surface	Smooth
Arrangement	Coherent cluster
Density	Opaque
Elevation	Convex
Motility	Positive

3.2. Optimization of Yeast extracts concentration

Effects of yeast extract concentration on maximum decolorization was studied by adding the medium with 0.05, 0.1, 0.2, 0.4, 0.8 % of yeast extract along with 100 ppm of mixed dye in 100 ml containing MSM. The maximum decolorization was obtained in medium containing 0.8% of yeast extract **[Figure– 2A]**. The percentage decolorization of RSV-1 was found to be in the range of 50.77-98.75 in increasing concentration of yeast extract respectively. The highest biomass was found to be 1.409 OD at 0.8% yeast extract concentration. Considering the high cost, further experiments were performed by using 0.4% yeast extract.

3.3. Optimization of pH

The effect of pH on decolorization was investigated in the pH ranges of 3, 5, 7, 9, and 11. It was found that change in pH significantly affect the decolorization rate. The isolates showed good growth at pH 7 and 9, while at pH 3 and 5 were found to be inhibitory for the growth. Bacterial cultures generally exhibit maximum decolorization at pH values near 7.0 **[Figure– 2B]**. The percentage decolorization of RSV-1 was found to be 94.37 and 91.99 at pH 7 and 9 respectively at 36th hour of incubation. The biomass OD 1.128 and 0.681 obtained for pH 7 and 9 respectively.

3.4. Optimization of temperature

Temperature plays an important role in microbial growth and enzyme activity; it is one of the most important parameter taken into consideration for the development of biodecolorization processes. The influence of temperature on decolorization was performed in the temperature range of 26ºC, 30ºC, 37ºC, 45ºC, and 50ºC. The maximum percentage of decolorization was found to be 99.61, 98.64 and 91.01 in the temperature range of 30ºC, 37 ºC, and 45ºC respectively by RSV-1. The biomass OD was found to be 1.00 -1.5 in that temperature [**Figure–2C**]. The temperature conditions studied in the present investigation were selected based on the average high and low temperatures of the concerned zone. Optimal temperature to decolorize mixed azo dye for RSV-1 strain was found to be 30°C.

3.5. Optimization of NaCl concentration

Effect of NaCl concentration on decolorization was studied by adding the medium with the range of 1, 3, 5, 7, 9 % concentration of NaCl. The optimized pH, and Temperature of 7.0 and 30ºC was kept as constant. When the concentration of NaCl increase there was decrease in decolorization activity. Our isolate RSV-1 achieved maximum decolorization and the biomass OD of 82.72, 82.95 and 0.826, 0.760 at 1 % and 3 % NaCl concentrations, respectively [**Figure–2D**].

3.6. Optimization of biomass concentration

Effect of biomass concentration on decolorization was performed by adding the medium with 1.0 OD at 600nm culture of 4, 8, 12, 16, and 20 % (v/v) concentration inoculum. When the concentration of biomass increased, the growth and percentage decolorization also increased. At 36th hours, the percentage removal of dye by the isolate RSV-1 was found to be 50.04, 67.44, 91.20, 94.57 and 99.07% at inoculum concentrations 4, 8, 12, 16 and 20% respectively and the OD was 0.9 and 1.2 at 16 and 20%, respectively [**Figure- 2E**)].

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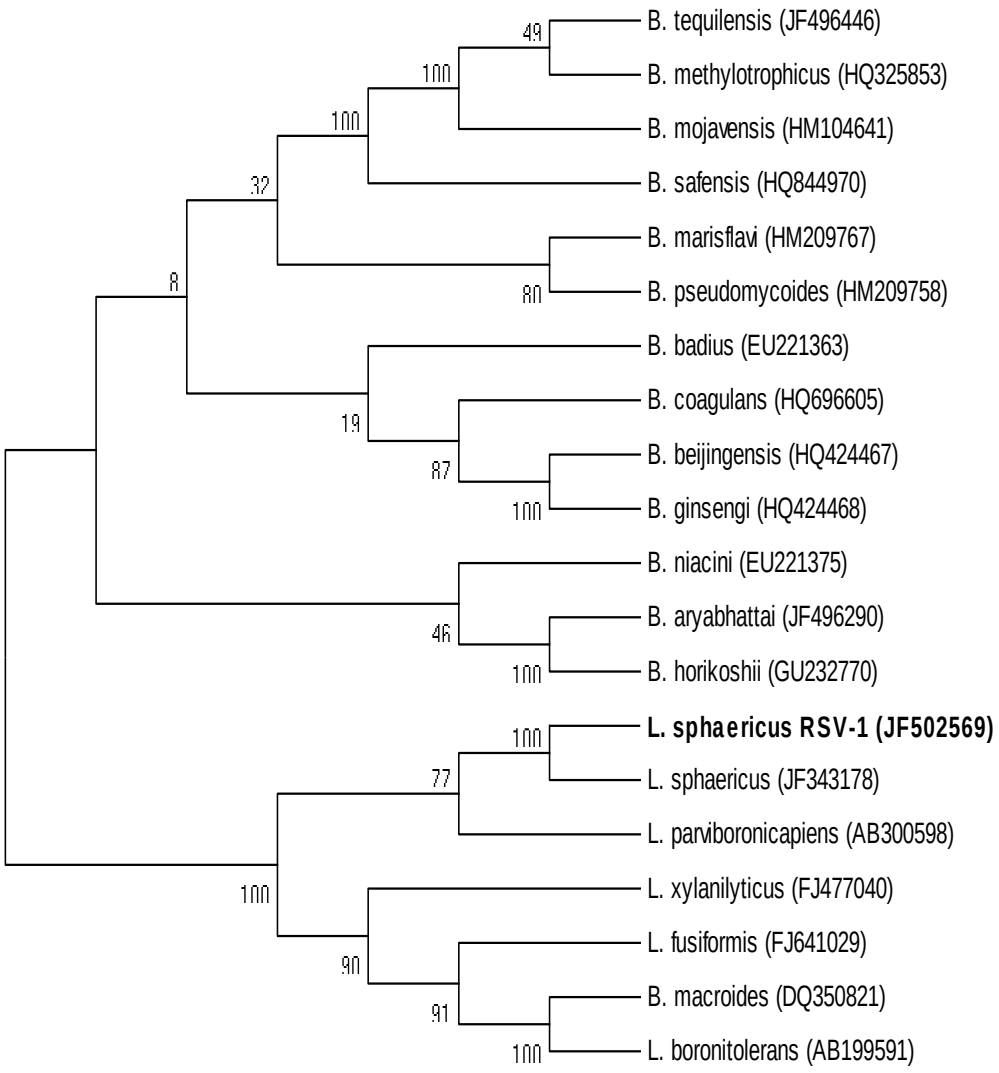

Fig: 1. Phylogenetic analysis of 16S rRNA sequence of *Lysinibacillus sphaericus* strain RSV-1 using MEGA-4.0 software. The percent numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analyses of 500 replicates. Brackets represent sequence accession

3.7. Effect of supplemental carbon and nitrogen sources as co-substrates

In order to check the growth and decolorization efficiency of the strain, the MSM was supplemented with various carbon and nitrogen sources. The decolorization and growth was absent in MSM, since it contains no added C or N sources. About 3-5% decolorization was observed when dextrose and sucrose was used and 12% was observed when starch was used as carbon source. There was no decolorization in lactose and CMC supplemented medium. The strain couldn't able to grow and decolorize the dye incorporated in inorganic nitrogen supplements such as ammonium nitrate, urea, ammonium chloride and ammonium sulphate. The strain effectively utilized yeast extract and decolorized the dyes up to 95% within 2 hr. Other than yeast extract, one more substrate served better nutrient supplement with 89% decolorization with in 3 hrs of incubation was soya chunk powder. Agricultural waste also served better decolorization rate, when rice husk extract was utilized and the percentage was found to be 92% in 48 hrs **[Table– 2]**. The effective decolorization may due to the strain be able to utilize the husk particles as a supportive material and also utilized the nutrients present in it for growth. The wheat husk served only 36% of decolorization while straw waste and sugar cane juice served only 15 and 12% decolorization.

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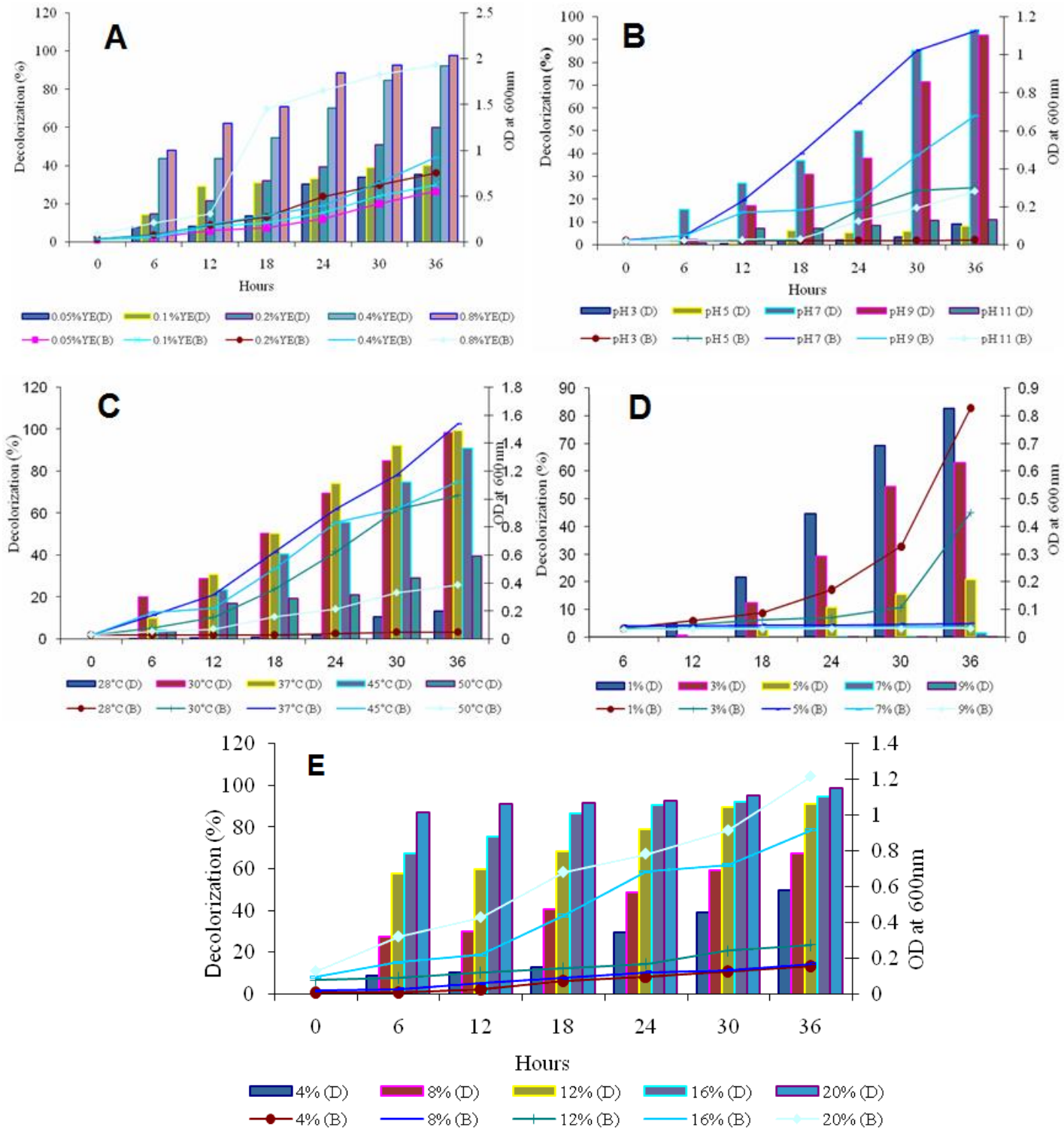

Fig: 2. A) Effect of yeast extract concentration on decolorization by RSV-1, B) Effect of various pH ranges on decolorization by RSV-1. C) Effect of various temparatures on decolorization by RSV-1. D) Effect of NaCl concentrations on decolorization by RSV-1. E) Effect of biomass on decolorization by RSV-1

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Table: 2. Effect of supplemental carbon and nitrogen sources on decolorization of textile dyes by RSV-1 strain

Substrate	RSV-1	Time (h)
MSM	ND	48
MSM + Dextrose (1%)	03±0.81	48
MSM + Lactose (1%)	ND	48
MSM + Sucrose (1%)	05±0.47	48
MSM + Mannitol (1%)	ND	48
MSM + Inositol (1%)	ND	48
MSM + CMC (1%)	ND	48
MSM + Starch (1%)	12±0.81	48
MSM + Ammonium nitrate (1%)	ND	48
MSM + Ammonium sulphate (1%)	ND	48
MSM + Ammonium chloride (1%)	ND	48
MSM + Urea (1%)	ND	48
MSM + Yeast extract (0.5%)	95±0.47	2
MSM + Soya chunk power (1%)	89±0.94	3
MSM + Wheat husk[a] (10%)	36±0.94	30
MSM + Rice husk[a] (10%)	92±1.63	48
MSM + Straw waste[b] (1%)	15±0.47	48
MSM + Sugar cane juice (1%)	12±2.49	48

ND=no decolorization also absence of growth; CMC=Carboxy methyl cellulose; MSM=Minimal salt medium. [a]The extracts were prepared by mixing 20g of each rice and wheat husk with 100ml of boiled distilled water(stock) and 10% of each substrate was made in MSM taken from the stock.these were autoclaved at 121˚ C for 20 min as mentioned by (Jadhav et al., 2008) with little modification

3.8. Effect of yeast extract and soya chunk powder on decolorization of dyes by RSV-1

The strain was inoculated in MSM prepared with optimized concentration of yeast extract, pH, temperature, dye concentration and inoculum concentration of 0.4%, 7.0, 30˚C, 1000 ppm and 20% of 1.0 OD (v/v) respectively. Static condition was maintained throughout the experiment. Strain RSV-1 could effectively decolorized the follwing dyes namely Deep Black RR, Yellow MERL, Red ME4BL, and Golden Yellow MR in the range of 90-99% decolorization. The decolorization percentage was within the range of 80-90% when Red M5B and Blue RR were used. Red RR achieved 76% of decolorization. Moderate decolorization was obtained in the range 40-65% when Yellow RR and Blue MR were utilized by the strain. The strain could achieve only 21% of decolorization when Yellow ME4GL used, this may be due to complex structure of the dye. No significant difference was observed when soya chunk powder was used as a co-substrate [**Table– 3**]. Structure of some of the study dyes were given in [**Table– 4**].

Table: 3. Effect of yeast extract and soya chunk powder on decolorization of dyes by RSV-1

Name of reactive dyes	λ_{max} (nm)	% Decolorization	
		Yeast extract medium	Soya chunk medium
Yellow ME4GL	418.8	21±0.94	10±0.47
Blue RR	576.9	83±2.62	81±0.94
Red RR	503.2	76±1.69	74±0.47
Yellow RR	442.8	65±0.00	79±0.47
Red M5B	508.5	88±1.69	82±1.69
Blue MR	564.5	45±1.63	52±1.69
Deep Black RR	566.3	92±1.14	86±0.00
Yellow MERL	404.5	98±1.14	87±1.69
Red ME4BL	418.8	98±0.47	94±1.69
Golden Yellow MR	414.6	98±1.14	87±0.47
Mixed dye	514.9	67±0.47	63±1.69

Values are mean of three experiments ± SEM. There is no significant difference were observed when yeast extract and soya chunk powder were used as a substrate since p>0.05 by one way ANOVA with Post Hoc test

[IV]DISCUSSION

Tirupur is an Indian textile town which constitutes many dyeing and bleaching units situated in the upstream. Tirupur serves as one of the major exporters of textiles. The industrial pollution have affected not only the surface water but also the soils and ground water even though industrial units have either constructed or connected to effluent treatment plants, the level of treatment has not been satisfactory at most of the places. Even today, some of the unit operate illegally and let the untreated effluent in to nearby river. It has caused serious impact on agriculture, livestock and fisheries. The effluents of CETP contain the mixture of various dyes, complex substances (from dyeing process), since it is necessary to collect the microbes from their native environment. The dyeing and bleaching units use a variety of toxic chemicals, including hydroxides, hydrochloric acid, sulphuric acid and sodium nitrate. The effluents caused various health problems such as skin allergies and lung infections. The Tirupur municipal hospital reports widespread incidence of skin diseases and pulmonological disorders. Biological methods are simple to use and low cost is involved in operation [26]. The biological treatment has many advantages over physical or chemical methods. Hence the survey was undertaken for the isolation of effective microorganism for the effective treatment of textile dye effluent. Effluent discharge area of textile industry is observed to be rich in biodegradation microbial flora. These can thrive there because of their metabolic adaptability.

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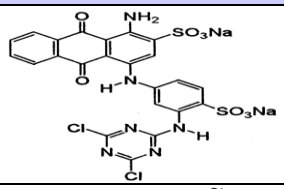
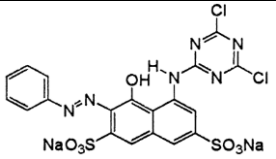
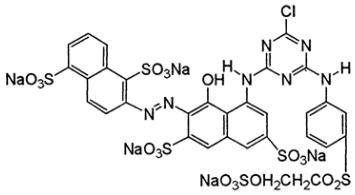
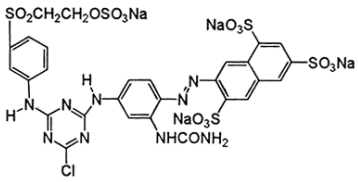
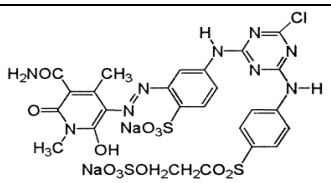
Biodegradation of textile dyes by living forms occurs naturally in the natural habitats [27]. Considering the volume generated and the effluent composition, textile industry wastewater is rated as the most polluting among all industrial sectors [28]. Hundreds of dyeing industries are in Tirupur, an Indian town in Tamil Nadu. About 610 dyeing units located on the bank of the river Noyyal release untreated effluents into the river. The high bacterial counts reflect that the textile dyeing effluents are good sources of nutrients to facilitate the growth of certain bacteria [29]. Azo dyes are selected from the list of dyes mostly used by dye industries at Tirupur and Karur districts in Tamilnadu, India. These dyes are regularly used in textile industries and untreated wastewater is always let out into adjacent channels. The isolation of efficient dye decolourisation bacteria from the samples collected from dye contaminated soil and wastewater indicates the natural adaptation of these microorganisms to survive in the presence of the toxic dyes [30]. In Mathew and Madamwar studies, among the various organic and inorganic nitrogen sources used, yeast extract was the best nitrogen source for efficient decolorization of RFB by the SV5 bacterial consortium. This may be owing to the metabolism of yeast extract, which is considered essential for the regeneration of NADH [31]. The strain *Bacillus endophyticus* VITABR13 with strong decolorizing ability was isolated from textile effluent to decolorize the textile Azo dye Acid Red 128 (100mg/l) [32]. Dawkar et al. studied Decolorization of RHE7B was 100% under static conditions and 0% under shaking conditions. But the growth of Bacillus sp. VUS was more in shaking conditions (44 mg/l) as compared to static conditions (33 mg/l) [12].

Table: 4. Name of the decolorized dyes with structure

Name of Dye	Structure	Class of dye	Molecular weight
Reactive Blue MR		anthraquinones	681.39
Reactive Red M5B		Single azo class	615.34
Red ME4BL		Single azo class	1136.32
Reactive Yellow MERL		Single azo class	1026.25
Yellow ME4GL		Single azo class	818.13

Originally, *Lysinibacillus sphaericus* genus was *Bacillus* sp. and was transferred into this genus as *Lysinibacillus* sp. in 2007 [33]. *Lysinibacillus* are ubiquitous and numerous in soils possess the ability to catabolism various compounds and xenobiotics. *L. sphaericus* has been reported to degrade p-nitrophenol, caprolactam and dichloromethane [34-36]. Strain

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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 [37]. Wan et al. isolated a new bacterial strain from activated sludge has been identified as *Lysinibacillus sphaericus* based on its morphology, physiochemical properties, and the results of 16S ribosomal RNA (rRNA) gene sequence analysis. This new bacterial strain uses ethanethiol as both carbon source and energy source [38]. *Lysinibacillus* sp. could degrade fomesafen (one of the diphenyl ethers, is specifically used for early post-emergent control of broad leaf weeds in soybean and bean) was isolated from contaminated agricultural soil, and identified as based on the comparative analysis of 16S rRNA gene. The optimum temperature for fomesafen degradation by strain was 30 ˚C [37]. *B. sphaericus* is having mosquito larvicidal activity and it is active against Culex and Anopheles mosquito larvae. An advantage of *B. sphaericus* over other larvicidal bacterial strains like *Bacillus thuringiensis* var *israelensis* can grow even in polluted water [39]. Russell et al. studied the carbohydrate metabolism in mosquito pathogenic strain *B. sphaericus* 2362. This bacterium was found to be unable to transport glucose or sucrose into the cell and it lacked glucokinase and hexokinase activities [40]. *Lysinibacillus sphaericus* produced light grayish colour colonies and effective growth was observed in pH 7.

The metabolism of yeast extract is considered essential to the regeneration of NADH that acts as the electron donor for the reduction of azo bonds. It had also been found that increasing yeast extract concentrations (from 0 to 10 g/l) resulted in higher decolorization rates, and the decolorization rates reached a plateau as yeast extract was higher than 8 g/l [17]. When compare to beef extract and nutrient broth medium, yeast extract medium was more appropriate for the decolorization of dye Navy blue 2GL by Bacillus sp. VUS [42]. Bacterial consortium consists of Bacillus cereus (BN-7), *Pseudomonas putida* (BN-4), *Pseudomonas fluorescence* (BN-5) and *Stenotrophomonas acidaminiphila* (BN-3) capable of completely decolorizing C.I. Acid Red 88 (AR-88) (20 mg/l) in 24 h, whereas individual cultures took more than 60 h to achieve complete decolorization of the added dye. It was able to decolorize 78% of C.I. Acid Red 88, 99% of C.I. Acid Red 119, 94% of C.I. Acid Red 97, 99% of C.I. Acid Blue 113 and 82% of C.I. Reactive Red 120 dyes at an initial concentration of 60 mg/l of mineral salts medium (MSM) in 24 h [43]. Effect of different concentrations of yeast extract in the range of 0-0.15% (w/v) in MSM broth on the decolorization efficiency of HM-4 was evaluated. In the absence of any yeast extract supplement in MSM, only 34% of colour removal was observed whereas complete decolorization of the dye was achieved at 0.1% (w/v) of yeast extract in medium in 12 h [17].

It is thought that the pH effect may be more likely related to the transport of dye molecules across the cell membrane, which was considered as the rate limiting step for the decolorization [44]. Repeated addition of dye aliquots in decolorized medium by bacterial consortium RVM 11.1 was studied, which is significant for its commercial application also the rate of decolourization of individual dyes depends on structural difference of the dyes. [45]. It has been indicated that neutral pH would be more favorable for decolorization of the azo dye. In the case of live bacterial cells, the optimum pH for color removal is often at a neutral pH value or a slightly alkaline pH value. About 91% decolourization of 300 ppm Reactive Red BS dye within 5.5 h was decolourized by *Pseudomonas aeruginosa* [46]. Reactive Red 180 was decolorized by *Citrobacter sp*. CK3 with the concentration of (200 mg/l), resulting in approximately 95% decolorization within 36 h, and could tolerate up to 1000 mg/l of dye [47]. The pH tolerance is quite important because reactive azo dyes bind to cotton fibers by addition or substitution mechanisms under alkaline conditions and high temperatures [48]. The optimal pH and temperature for the decolorization of RED RBN (1000ppm concentration) by the strain Proteus mirabilis were 6.5-7.5 and 30-35˚C. The strain could decolorize the dye within 20 h of incubation. [49]. Most textile and other dye effluents are produced at relatively high temperatures and hence temperature is an important factor [50]. At 37°C the bacterial consortium showed maximum decolorization of Ranocid Fast Blue Dye RFB, followed by at 30°C. This may be owing to a greater production of enzymes and optimal growth conditions of the consortium SV5 for its dye-decolorizing ability. The decolorization at this optimal temperature may be owing to higher respiration and substrate metabolism. This also demonstrates that decolorization of the dye was through the microbial reaction, which relies on optimal temperature, and not by adsorption, where the temperature effect was not great [6]. The optimal temperature and pH for the decolorization of azo-dye reactive red 22 by *Pseudomonas luteola* was found to be 37˚C and 7-9 respectively. Yeast extract and tryptone were strongly enhanced the decolorization while glucose concentration retarded decolorization of reactive red 22 [51]. A novel bacterial species identified as *Exiguobacterium* sp. RD3 degraded the diazo dye reactive yellow 84A (50 mg/l) within 48 h at static condition, at 30˚C and pH 7 [27]. Wastewaters from textile processing and dye stuff manufacture industries contain substantial amounts of salts in addition to azo dye residues [52]. Salt concentrations up to 15–20% have been measured in wastewaters from dyestuff industries [53]. An inoculum size, beyond 10% was not very significant in decolorization was observed [31]. Decolorization activity of *Bacillus* sp. has high (86.72%) in 4% of inoculums [54].

The decolorization rate increased with increase in the inoculum size, reaching maximum (2.53 mg l-1) at 20% (v/v) inoculum size. However, beyond 20% (v/v) inoculum size, rate of decolorization did not vary significantly [55]. Triphenylmethane group of dyes was decolorized intracellularly by a strain *Kurthia* sp under aerobic conditions with 98% color removal [56]. The reduction of azo dyes depends on the presence and availability of cosubstrates, because it acts, as an electron donor for the azo dye reduction [57]. Strain *Pseudomonas aeruginosa*

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strain BCH capable of decolorizing Direct Orange 39 (50 mg/l) within 45 ± 5 min, with 93.06% decolorization ability was isolated from sediments contaminated with dyestuff [58]. It is thought that in case of complex substrates such as extract of rice husk and rice straw, the strains could convert and degrade them, producing some volatile organic acids or alcohols (such as acetic acid and ethanol), which acts as electron donors and apparently induces the reductive cleavage of azo bonds [59,60]. Decolorization was favoured by strictly anaerobic conditions and highly proteinaceous media. [61]. Sugarcane extract is eco-friendly as compared to others reduction method likes electrochemical and ultrasonic energy [62]. Azo dye decolorization by mixed, as well as pure, cultures generally required complex organic sources, such as yeast extract, peptone, or a combination of complex organic source and carbohydrate [17, 43]. The supplementation of cheap co-substrates (e.g., extracts of agricultural wastes) could enhance the decolorization performance of consortium GR consisting of *Proteus vulgaris* NCIM-2027 and *Micrococcus glutamicus* NCIM-2168 could rapidly decolorize and degrade commonly-used sulfonated reactive dye Green HE4BD and many other reactive dyes [63]. In contrast, the bacterial reduction of the azo bond is usually nonspecific and bacterial decolourization is normally faster. Microbial decolourization and degradation has appeared as an environmentally friendly and cost-competitive alternative to chemical decomposition processes although this bacterium has shown greater dye degradation ability as compared to other bacteria. The chemical structures of the dyes greatly influence their decolourization rates, and the decolourization efficiency is limited to several azo dye structures [64].

Metabolism of the yeast extract is considered essential for regeneration of NADH, which is the electron donor for azo bond reduction [65]. Azo dyes generally contain one, or more sulphonic-acid groups on the aromatic rings, which might act as detergents, thereby inhibiting the growth of the microorganisms. Such dyes may affect DNA synthesis since it has also been reported that dyes are inhibitors of the nucleic acid syntheses, or cell growth [66, 17]. The effect of carbon (1 g%) and nitrogen (0.5 g%) sources on the decolorization ability of the bacterial isolates M1 (Bacillus cereus) and M6 was studied by Modi et al. [67] Decolorization of azo dye Red 3BN by two bacterial species Bacillus cereus and *B. megaterium* was studied by Praveen Kumar et al. Optimal condition for B. cereus was found to be 1% sucrose 0.25% peptone, pH 7, 37°C and 8% inoculum and that for *B. megaterium* was found to be glucose 1%, 0.25% yeast extract, pH 6, 37°C and 10% inoculum. Extent of decolorization recorded by B. cereus under ideal conditions was 93.64% and that by *B. megaterium* was 96.88% [69]. *Pseudomonas pseudomallei* 13NA and *Citrobacter* sp. Decolorized both triphenylmethane and azo dyes by a single species of bacterium [70]. *Bacillus* sp. VUS decolorized azo dye Navy blue 2GL in 48 h at static anoxic condition in yeast extract medium. The time required for 94% decolorization of 50 mg/l dye was 48 h. Yeast extract was best medium for faster decolorization than other media [42, 68]. B. cereus performed well because they are nutritionally versatile and carries an efficient enzymatic system for the cleavage of azo bonds, which cause rapid decolourization of different azo dyes and thus they are able to biodegrade many natural and synthetic organic compounds. This could be a consequence of natural adaptation of the organism as the sample from which the bacterial isolate was obtained was highly contaminated with dyes [71].

A strain *M. glutamicus* was applied to decolorize a mixture of ten reactive dyes (Reactive Green 19A, Reactive Yellow 17, Reactive Red 2, Reactive Orange 4, Reactive Blue 171, Reactive Orange 94, Reactive Blue 172, Reactive Red 141, Reactive Red 120, and Reactive Blue 59) at a concentration of 50 mg/l and 37˚C under static condition. Decolorization was found to be 63% within 72 h [72]. There are also several studies describing decolorization of reactive dyes mediated by pure bacterial culture such as, *Pseudomonas sp*. SUK1 for Reactive Red 2 [11], *Exiguobacterium* sp. RD3 for Navy Blue HE2R (Reactive Blue 172) [12], *Rhizobium radiobacter* MTCC 8161 for Reactive Red141 [13], *Pseudomonas aeruginosa* NBAR12 for Reactive Blue 172 [14] and isolated bacterium KMK48 for the degradation of various sulfonated reactive azo dyes [15]. Decolorization of Reactive Red 2, Reactive Red 141, Reactive Orange 4, Reactive Orange 7 and Reactive Violet 5 was observed by the unidentified bacterium, KMK 48. Complete decolorization (100%) of Reactive Red 2, at the concentration of 200 and 1000 mg/l was observed after 30 h. However, 200 mg/l of Reactive Red 141 was decolorized within 24 h, whereas decolorization of 1000 mg/l was achieved after 30 h. Moreover, 200 mg/l of Reactive Orange 4 was decolorized completely after 30 h whereas; with 1000 mg/l decolorization was observed at 36 h. The time taken for total decolorization of Reactive Orange 7, having the concentration of 200 mg/l was 18 h whereas that of 1000 mg/l was found to be 24 h [15].

The difference in decolorization rate between Acid Red 27 and Reactive Red 2 is possibly caused by the presence of the triazine group, as the report of Van der Zee showed that dyes containing triazine group were among the dyes that reduced at slowest rates [73]. The growth characteristics and capacity of *E. faecalis* to decolorize Acid Red 27 was better and higher than that on Reactive Red 2 because this dye has a triazine group in its chemical structure. The more complicated structure of Reactive Red 2 was a factor that could decrease growth quality and capacity of *E. faecalis* to decolorize azo dye [74]. The bacterial consortium NBNJ6 showed rapid decolorization of all dyes within 24–35 h at 37 ˚C. Culture could decolorize upto 250 mg/l of Reactive Black B, Reactive Blue 172, Reactive Violet 5R, Reactive Red 5B and Reactive Black RL, within 30–35 h at 37 ˚C in the range of 85–90%. Food colors like Ponceau 4R (500 mg/l) Raspberry Red (1000 mg/l) Tartrazine (300 mg/l) and Sunset Yellow FCF (300 mg/l) were decolorized within 24 h in the range of 84–95%. The rate of decolorization of individual dyes varied [75].

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[IV] CONCLUTION

The use of a pure culture system ensures reproducible data, and thus interpretation of experimental observations becomes easier. The present study reveals that the isolated strain could effectively utilized for the treatment of real textile effluent containing high concentration of reactive dyes. The optimum pH and temperature for maximum decolorization ability was found to be 7-9 and 30°C. Moreover, 0.4 % of yeast extract or soya chunk powder was served best nutritional supplements

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CONFLICT OF INTEREST

Author declares no conflict of interest.

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ABOUT AUTHORS

Mrs. Rajeswari K. *is currently a Research Scholar in the field of Microbiology.* ***Dr. Subashkumar*** *is Assistant Professor and HoD in PG & Research Department of Biotechnology, Kongunadu Arts and Science College ,Coimbatore 641 029 and* ***Dr. Vijayaraman*** *is a Principal of KSG College of Arts and Science, Coimbatore also Principal investigator of this project.*