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Decolorization and Degradation of Textile Dyes by *Stenotrophomonas maltophilia* RSV-2

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Abstract Dyewaste effluent samples were collected from textile dyeing unit as well as CETP located in Tirupur. Strains having decolorizing ability of various textile reactive dyes were obtained from primary and secondary screening methods. *Stenotrophomonas maltophilia* RSV-2 strain could degrade the mixed dyes up to 2100 ppm with in 67 hrs and 58 % decolorization was obtained through acclimatization study. The strain was identified based on biochemical and 16S ribosomal RNA gene sequence study. Optimization of yeast extract composition, pH, temperature, salinity and biomass composition was determined. Decolorization performance of individual dyes using two different substrates was also studied.

Keywords: *decolorization, dyewaste effluent, Stenotrophomonas maltophilia, textile dyes*

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1. Introduction

Environmental pollution is one of the major problems of the modern world. On one hand, industrialization is necessary to satisfy the needs of the world's overgrowing population but on the other hand, it threatens life on earth by polluting the environment. The problem of environmental pollution is increasing day by day due to the release of xenobiotic substances into water, soil and air. These substances include organic compounds (pesticides, dyes, polymers etc.) and heavy metal ions. The damage caused by these pollutants to plants, animals and humans cannot be neglected and hence strategies must be developed to solve the problem of environmental pollution on priority basis [2]. In India, an average mill discharges about 1.5 million liters of contaminated effluent per day, which leads to chronic and acute toxicity [30]. Considering both the volume generated and the effluent composition, textile industry wastewater is rated as the most polluting among all industrial sectors [19]. Approximately 10,000 different dyes and pigments are used industrially, and over 0.7 million tons of synthetic dyes are produced annually, worldwide. In 1991, the world production of dyes was estimated 668,0002 of which azo dyes contributed to 70 %. During dying process, a substantial amount of azo dye is lost in wastewater. Reactive dyes present in exhausted dye baths and rinsing water are not recyclable and scarcely biodegradable due to their various substituents [28]. The routine use of dyes in day-to-day life is increasing because of rapid industrialization, most widely in textile, rubber and enamel, plastic, cosmetic and many other industries [23]. Dye wastewaters are usually treated using physicochemical methods such as flocculation, coagulation, adsorption, membrane filtration, precipitation, irradiation ozonization and Fenton's oxidation [15]. Recently, coupled chemical–biological treatment technologies are used for the mineralization and/or decolorization of dyes, as these technologies are most acceptable for the recalcitrant compounds like dyes [9]. These methods are effective but may generate significant amounts of chemical sludge, whose disposal in secure landfill increases process cost [14]. An attempt was made to isolate the bacterial strains from dyewaste effluent and study their decolorization activity on high concentration of reactive dyes.

2. Materials and Methods

2.1. Sample Collection

Dyewaste effluent samples were collected from textile dying unit and CETP located in Tirupur. In our previous study, the effective decolorizing strains were isolated based on the primary and secondary screening methods [24].

2.2. Dyes

Ten different reactive dyes were utilized for present study namely Yellow ME4GL, Blue RR, Red RR, Yellow RR, Red M5B, Blue MR, Deep Black RR, Yellow MERL, Red ME4BL and Golden Yellow MR. Stock solution of 5000 ppm was prepared by dissolving the dye in distilled

water and was filter sterilized and kept at 4°C. For acclimatization study only four different reactive dyes (Blue RR, Black B, Red RR and Yellow RR) in mixed form were utilized.

2.3. Media Composition

All the experiments were performed in Minimal salt medium (MSM) of pH 7.0 ± 0.05 containing (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). For cultivation of microbes, Nutrient Agar medium with the composition (g L-1) pH 7.0 ± 0.05 Peptone (5.0), Beef extract (3.0), Yeast extract (2.0), NaCl (5.0), Agar (in case of solid medium) (16.0) was used. All the decolorizing experiments were performed in MSM supplemented with 0.5% yeast extract unless otherwise stated.

2.4. Acclimatization Study

In Acclimatization study the culture was previously inoculated in Nutrient broth medium, incubated at 30°C for 24 hrs. The culture was centrifuged and pellet was dissolved in MSM. The OD value of the culture was set to 1.0 approximately 4gL-1. The stock of 5000 ppm was prepared by mixing equal proportion of four different dyes (Blue RR, Black B, Red RR and Yellow RR) in distilled water and then filter sterilized. Stock was stored at 4°C. Initially 100 ppm dye was added in the tube containing 1.0 OD culture, and then, after decolorization 200 ppm was added in the same medium. Likewise, the dyes were added in the increasing concentration after decolorization. The time taken for decolorization and percent decolorization was noted. The absorbance was measured at its maximum (598 nm) against a blank. The percent decolorization was calculated as

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

2.5. Identification of The Strain

Strains were identified based on biochemical and 16S ribosomal RNA gene sequence study. 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 this isolate was extracted with the Bacterial Genomic DNA Isolation Kit (RKT09). The rDNA fragment of ~1.4 kb was amplified using high –fidelity PCR polymerase. The PCR product was sequenced bi-directionally using the forward, reverse primer. The Forward and reverse Prokaryotes 16s rRNA specific primers were 16s Forward Primer: 5'-AGAGTRTGATCMTYGCTWAC-3 and 16s Reverse Primer: 5'-CGYTAMCTTWTTACGRCT-3'. The reaction mixture contained 1µl of template DNA, 400 ng of each 16s forward and reverse primers, 4 µl of dNTPs (2.5 mM), 10 µl of 10x DNA polymerase assay buffer, 1.5mM of $MgCl_2$ as final concentration, 1 µl of Taq DNA polymerase enzyme (3U/ µl) and 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. 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 1min 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 was analyzed using the Blast search and was aligned with sequences from other bacteria in the GenBank database of NCBI using CLUSTAL W .The phylogenetic tree was constructed using Mega 4.0 software.

2.6. Optimization of Physico Chemical Parameters

Effect of yeast extract (0.05, 0.1, 0.2, 0.4, 0.8 % (w/v), pH (3, 5, 7, 9 and 11), Temperature (28, 30, 37, 45, and 50°C), Salinity (1, 3, 5, 7, 9 %) and biomass concentration (4, 8, 12, 16, and 20 %) on decolorization was studied. The culture was inoculated in Nutrient broth medium incubated at 30°C (temperature found at the time of sample collection) for 12 hours .After incubation, cells was 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 Mineral salt medium and triplicates were maintained for all the experiments.

2.7. Effect of Individual Dyes on Decolorization Ability by the Strain

The decolorization ability of the strain was tested against individual dyes with the concentration of 1000 mgL^{-1}. Two different substrates were utilized namely yeast extract and soya chunk powder. The absorbance was measured at its maximum wavelength against a blank.

3. Results and Discussion

Dyes of different structures are often used in the textile processing industry, and, therefore, the effluents from the industry are markedly variable in composition. A non-specific biological process may be vital for the treatment of textile effluents, containing a mixture of dyes [5]. According to the reports [13,25], decolorization of dyes by bacteria can be due to adsorption to microbial cells or biodegradation. The Adaptive study was conducted by adding dyes in increasing concentration in the decolorized medium and the nature of the strain in high concentration of mixed dyes reavealed that the strain could decolorize the reactive mixed dyes up to 2100 ppm concentration with in 67 h with 58 percent decolorization ability. The decolorization of 100 ppm concentration was achieved within 5 h and 20 min with 94 percent decolorization. As the dye concentration increased, the decolorization performance decreased. The results are elaborated in Table 1. The decolorization rate was found to be 18.07 mg h^{-1}. Decline in decolorization rate may be due to the poisonous effect of the dye on bacteria, obstruction of active sites of azoreductase enzyme by complex dye

structure and/or insufficient production of biomass for decolorization of higher concentrations of dye [27]. Adaptation of a microbial community towards toxic or recalcitrant compounds is found to be very useful in improving the rate of decolorization process [6]. The adaptation of microorganisms to higher concentrations of pollutants is called acclimatization and leads to forced or directed evolution [3]. An adaptation procedure was used to acclimatize the microbes [20] to an increasing concentration with methyl red as the carbon source [1]. The single flask procedure of adaptation [32] without transfer of microorganisms was employed. Moreover, the time required for decolorization increased with increasing dye concentrations. This might be attributed to the toxicity of dye to bacterial cells through the inhibition of metabolic activity, saturation of the cells with dye products, inactivation of transport system by the dye or the blockage of active site of azoreductase enzymes by the dye molecules [17].

Table. 1 Acclimatization study

Step	Total dye added in medium (ppm)	Time (h)	Decolorization (%)
1	100	5.20	94
2	200	2.45	92
3	300	4.30	86
4	400	8.10	71
5	500	15.00	63
6	600	32.00	58
7	600	-	-
Total	2100	67	77.33

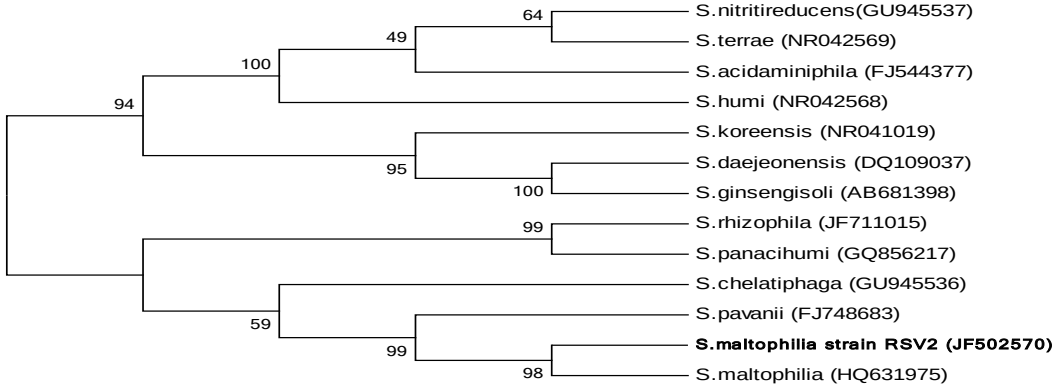

Figure 1. Phylogenetic analysis of 16 s rRNA sequence of *Stenotrophomonas maltophilia* strain RSV-2 using MEGA-4.0 software. The percent numbers at the nodes indicate the levels of bootstrap support based on neighbor-joining analyses of 500 replicates. Brackets represent sequence accession

3.1. Characteristics of *Stenotrophomonas Maltophilia* RSV-1

The strain is Gram Negative in nature. The biochemical test result showed that the strain gave negative for Indole, V-P test, Citrate Utilization Test, Sucrose, Xylose, Mannitol, Arabinose, Hydrolysis of starch, Catalase, Gelatin liquefaction and Phenylalanine deaminase and positive for TSI Test (A/A), Glucose, Lactose, Oxidase and Nitrate reduction test Table 2. Presence of growth at temperature (10–50°C) and NaCl (2.0–7.0%) was observed. 16S rRNA phylogenetic analysis was performed using BLAST and the results reavealed that the strain belongs to *Stenotrophomonas maltophilia*. The sequence was deposited in the GenBank database under accession number JF502570 Figure 1. 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 [10]. This strain can also decolorize some synthetic dyes: Methylene Blue, Toluidine Blue, Methyl Green, Indigo Blue, Neutral Red, Congo red, Methyl Orange and Reactive Pink. A rod-shaped, gram-negative bacterium *Stenotrophomonas* sp. SMSP-1 was isolated from the sludge of a wastewater treating system from a pesticide manufacturing industry. Strain SMSP-1 could hydrolyze methylparathion to p-nitrophenol (PNP) and dimethyl phosphorothioate but could not degrade PNP further. Strain SMSP-1 was able to hydrolyze other organophosphate pesticides, including fenitrothion, ethyl parathion, fenthion, and phoxim, but not chlorpyrifos [29].

Table 2. Morphology, Cultural and Biochemical Characterization of isolated Strains

Biochemical and culture conditions	RSV-2
Gram staining	Gram-negative
Indole	-
Methyl red	+
V-P test	-
Citrate Utilization Test	-
TSI Test	A/A
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%)	+
Morphology under Microscope	Short Rods
Cell type (shape)	Yellowish
Color	Smooth/mucoid
Surface	cluster
Arrangement	Opaque
Density	Convex
Elevation	Positive
Motility	

+ Positive reaction, - negative reaction

3.2. Effects of Yeast Extract Concentration on Decolorization

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. The percentage decolorization was ranged between 35.35 and 98.04. The highest biomass was found to be 1.935 OD at 0.8% medium. The increase in concentration of yeast extract resulted in increase of growth rate. Even though our isolate performed maximum decolorization at 0.8% yeast extract Figure 2a, considering the expensive nature of yeast extract, we performed our further experiments at 0.4% of yeast extract to minimize the cost. 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 increase in yeast extract concentrations (from 0 to 10 g l^{-1}) resulted in higher decolorization rates and the decolorization rates reached a plateau as yeast extract was higher than 8 g l^{-1} [5]. *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 the best medium for faster decolorization [7]. The use of yeast extract for increased decolorization of dye has also been reported [4,12].

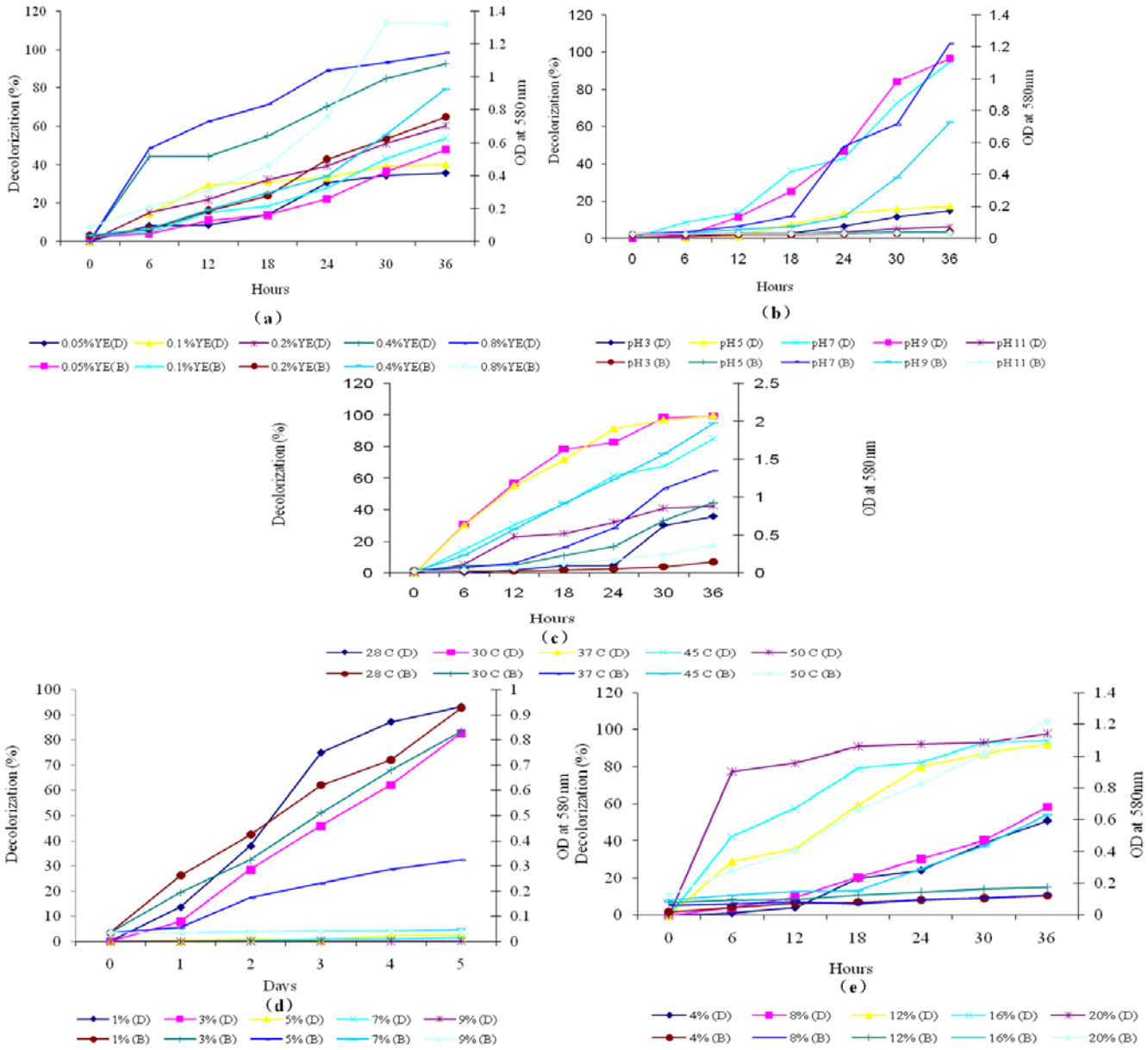

Figure 2. (a): Effect of Yeast extract concentration on Decolorization by RSV-2. (b): Effect of Various pH Ranges on Decolorization by RSV-2. (c): Effect of Various Temperature on Decolorization by RSV-2. (d): Effect of NaCl Concentration on Decolorization by RSV-2. (e): Effect of Biomass on Decolorization by RSV-2

3.3. Effect of pH on Decolorization

The effect of pH on decolorization was investigated for the pH values 3, 5, 7, 9, and 11. It was found that change in pH significantly affected the decolorization rate. The isolates showed good growth at pH 7 and 9, while, at 3 and 5 were found to be inhibitory for the growth. Bacterial cultures generally exhibit maximum decolorization at pH values near 7.0. The percentage of decolorization and biomass OD was found to be 94.83, 96.44 and 1.219,

0.725 at pH 7 and 9 respectively Figure 2b. Our isolates could decolorize the dye even in the pH of 11, but the incubation time required for the process was 3-4 days to achieve maximum decolorizaion of 70-80% (data not shown). So this indigenous isolate can be effectively utilized for real textile dyes having the pH of neutral to alkaline condition. A significant increase in decolorization was observed as the pH increased from 7 to 9. However, relatively, a rapid decrease in decolorization was found when the pH was increased from 9 to 11. 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 decolorization [16].

3.4. Effect of Temperature on Decolorization .

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 at temperatures 26°C, 30°C, 37°C, 45°C, and 50°C. The maximum decolorization was found to be 99.42, 99.9 and 85.21 at 30°C, 37°C and 45°C respectively. The biomass OD was found to be in the range of 0.9-1.000 Figure 2c. The temperature conditions were selected based on the average high and low temperatures of the concerned zone. Optimal temperature to decolorize mixed azo dye by RSV-2 strain was between 30°C and 37°C. However, the decolorization rate dropped sharply as the temperature increased from 45 to 50°C. Most textile and other dye effluents are produced at relatively high temperatures and hence temperature will be an important factor [31].

3.5. Effect of Salinity on Decolorization

Wastewaters from textile processing and dyestuff manufacture industries contain substantial amounts of salts in addition to azo dye residues [11]. Salt concentrations up to 15–20% have been measured in wastewaters from dyestuff industries [8]. Hyper-salinity chemical industry wastewaters often contain a range of chemicals, which are recalcitrant to biodegradation. For example, reactive dyes are very soluble by design and as a result, not all are used up by textile fibers during the dyeing process and therefore end up in the discharge from dye houses [22]. Our strain achieved maximum decolorization of 93.03 and 82.72 and the biomass OD of 0.927 and 0.835 at 1 and 3 NaCl concentrations respectively Figure 2d. The maximum decolorization was achieved only after the 6th day of incubation. Wastewaters from textile processing and dye stuff manufacture industries contain substantial amount of salts in addition to azo dye residues [11]. Salt concentrations up to 15–20% have been measured in wastewaters from dyestuff industries [8]. Thus, microbial species capable of tolerating salt stress will be beneficial for treating such wastewaters.

3.6. Effect of Biomass Concentration on Decolorization

Decolorization was performed by adding the medium with 1.0 OD culture of 4, 8, 12, 16, and 20 % concentration inoculum. When the concentration of yeast extract increased, the growth and percentage decolorization also increased. RSV-2 achieved 51.11, 58.37, 92.16, 94.15 and 98.14 % decolorization at inoculum concentrations of 4, 8, 12, 16 and 20% respectively and the OD was 1.13 and 1.77 at 16 and 20% respectively. There was no significant difference in percentage removal at 12, 16 and 20% inoculum concentrations and hence 20 % inoculum concentration was selected for further experiments Figure 2e. A trend of increase in decolorization was noticed with increase in inoculum concentration. However, beyond 10% (v/v) inoculum size, rate of increasing decolorization was not very significant [18].

3.7. Effect of Yeast Extract and Soya Chunk Powder on Decolorization of Individual Dye by *Stentrophomonas Maltophilia* RSV-1

The strain inoculated in optimized MSM contains yeast extract, pH, temperature, dye concentration and inoculum concentration of 0.4%, 7.0, 37°C, 1000 mg l^{-1}, and 1.0 OD respectively. Static condition was maintained throughout the experiment. Ten structurally different reactive dyes with the concentration (1000 mg l^{-1}) were used in this study. Most of the dyes were efficiently decolorized by RSV-2 in 24 h. A maximum decolorization of 90% was recorded in Reactive Yellow MERL, Red ME4BL and Golden Yellow MR, and for Blue RR, Red M5B and Deep Black RR dyes, the value varied from 80-90%. Yellow RR and Yellow ME4GL achieved 52% and 14% respectively Table 3. No significant different was observed when soya powder was used. But the decolorization percentage of blue MR was slightly increased to 37%.The time required for decolorization and total percentage decolorization was different for different dyes. Variation in decolorization efficiency and time required for decolorization may be due to structural differences of dyes [21]. *M. glutamicus* strain was applied to decolorize a mixture of ten reactive dyes (Reactive Green 19A, Reactive Yellow 17, ReactiveRed 2, Reactive Orange 4, Reactive Blue 171, Reactive Orange 94, Reactive Blue 172, Reactive Red 141, Reactive Red 120, and ReactiveBlue 59) at a concentration of 50 mg l^{-1} at 37°C under static condition. It showed 63% decolorization within 72 h [26].

Table 3. Decolorization of individual dyes by *Stenotrophomonas maltophilia* RSV-2

Name of reactive dyes	Percent Decolorization	
	Yeast	Soya
Yellow ME4GL	14 ± 0.47	02 ± 1.69
Blue RR	88 ± 1.14	86 ± 0.47
Red RR	62 ± 0.47	58 ± 3.55
Yellow RR	52 ± 0.47	68 ± 0.47
Red M5B	82 ± 1.24	79 ± 0.94
Blue MR	23 ± 1.69	37 ± 0.94
Deep Black RR	88 ± 2.62	85 ± 1.69
Yellow MERL	96 ± 0.94	77 ± 1.63
Red ME4BL	93 ± 1.69	94 ± 0.94
Golden Yellow MR	96 ± 0.94	92 ± 0.47

4. Conclusion

The effective strain having a potential decolorizing ability was isolated from dyewaste effluent. The strain *Stentrophomonas maltophilia* RSV-2 could adapt to high concentration of reactive mixed dyes so it could be used for treating real textile dyes containing high concentration of mixed reactive dyes. The effects of yeast extract concentration, pH, temperatures, NaCl concentration and biomass concentration on decolorization of mixed reactive dyes were investigated. In addition to yeast extract, Soya chunk powder also served as a better nutritional supplement for growth and decolorization performance.

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