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ORIGINAL PAPER

/+//Biodecolorization of Reactive Black5 and Reactive Red120 azo dyes using bacterial strains isolated from dairy effluents

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Abstract

In a biological treatment of an industrial effluent, the indigenous bacteria have already been used. In this study, the three bacteria strains which are potent in the decolorization of azo dyes were isolated from dairy effluent and used for decolorization of Reactive Black5 and Reactive Red120 azo dyes and decolorization test of each dye was conducted at five concentration levels (10, 50, 100, 150, and 200 mg/l). The pH of which was adjusted to 7 and incubated at 37 °C for 3 days. The strains were identified as *Staphylococcus* sp. and *Micrococcus luteus* strains by 16S rRNA gene sequences analysis. The strains were deposited in GenBank with accession numbers of KX180131, KX180132, and KX180133 and submitted to laboratory identifier named *Staphylococcus* sp. MEH038S, *Micrococcus luteus* strain SEH038S, and *Micrococcus luteus* strain FEH038S. Three days into incubation, the lowest efficiency was at a concentration level of 200 mg/l for each dye. Decolorization efficiencies for *Staphylococcus* sp. MEH038S, *Micrococcus luteus* strain SEH038S, and *Micrococcus luteus* strain FEH038S at the concentration level of 200 mg/l for Reactive Red120 were 89.7, 87.1, and 89.3%, and for Reactive Black5 were 90.8, 90.0, and 89.9%, respectively. Based upon this study, dairy effluents can be used as a suitable alternative to the decolorization of textile wastewater. This study demonstrates a report on grounds of elucidation for the use of non-indigenous bacteria in the treatment of industrial wastewater.

Keywords Azo dyes · *Biodecolorization* · *Micrococcus luteus* strain FEH038S · *Micrococcus luteus* strain SEH038S · *Staphylococcus* sp. MEH038S

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Introduction

The primitive man simply extracted the pigments from the natural environment around himself and used them for dyeing clothes and textiles. The use of natural dyes has continued for centuries, until 1856 when the first synthetic dye was accidentally discovered by William Henry Prkyn (Saratale et al. 2011). Today, over 10,000 commercially available synthetic dyes exist and are used extensively in many fields of industries such as textile, leather, paper, food. (Crini 2006; Lade et al. 2015). Over 0.7 million tons of such dyes are produced annually worldwide (Saharan and Ranga 2011; Tripathi and Srivastava 2011; Saroj et al. 2014). Low price, facilitating the production, high stability to the sunshine and temperature, detergents sustainability of synthetic dyes in contrast to natural dyes have caused an increased industrial attraction to the use of such dyes (Lade et al. 2015; Kumari and Naraian 2016; Nouren et al. 2017); 70% of all dyestuffs used in textile

are azo dyes (Jirasripongpun et al. 2007; Ogugbue et al. 2012; Tripathi and Srivastava 2011). The azo dyes containing aromatic rings are joined by one or more azo bond (–N = N–) (Gupta 2009; Hussain et al. 2013).

The textile industries consume large quantities of water. Between 60 and 400 L (on average, 200 L) of water is used to produce 1 kg of textile (Ali et al. 2009). As a result, a large volume of colored wastewater is discharged into the environment (Buthelezi et al. 2012; Daâssi et al. 2013; Hema and Suresha 2014). Moreover, some amounts of these dyes are usually directly lost in wastewater in textile industries, depending on the type of dyes and fibers. In the case of reactive dyes, more than 50% of the initial loading dyes are released in the vicinity of regional dyeing facilities into a waste reservoir (Pandey et al. 2007). Less than 1 mg/l of dyes dissolved in water is highly visible and undesirable, while the amounts of dye concentration in the textile effluents are in the range of 10-200 mg/l (Pandey et al. 2007; Lade et al. 2015). It is reported that around 280,000 tons of dyes discharge into nature annually worldwide. Discharging dyes into receiving water is undesirable and causes damage to the water bodies, such as spoiling water appearance or aesthetic problems, obstructing light penetration, reducing the generation of oxygen by photosynthesis process, and creating anoxic conditions that are lethal to aquatic life. (Kurade et al. 2012; Rana and Sharma 2013). Also, azo dyes in soil have different effects on microbial activity and caused changes in the microbial community composition (Imran et al. 2015). Hence, the effluent from textile industry is one of the most problematic wastewater and cannot be discharged directly into the environment without thorough treatment of such water (Miao 2005; Ali et al. 2009).

During the last few decades, several physicochemical techniques were used for the treatment of colored textile effluents. Despite these methods to have a good efficiency in decolorization, such problems like large amounts of toxic sludge, formation of hazardous by-products may arise. Furthermore, in a method like adsorption, the dyes only transfer from one phase to another and it leaves the problem unsolved (Aksu and Dönmez 2003; Crini 2006; Saratale et al. 2011; Vijayanand and Hemapriya 2013; Govindwar et al. 2014; Saroj et al. 2014). Despite other methods, biodecolorization is considered as an environmental friendly and viable alternative that can lead to complete mineralization of xenobiotic compounds at low cost and produces less toxic compounds or sludge with less water consumption (Saratale et al. 2011; Hema and Suresha 2014; Pathak et al. 2014).

Recently, numerous microorganisms, including bacteria, fungi, yeasts, and algae, were investigated for decolorization of azo dyes (Liao et al. 2013; Rana and Sharma 2013). Previous studies had used the indigenous microorganisms in the treatment of wastewater (Ali et al. 2009; Jain et al. 2012; Forss et al. 2017; He et al. 2017). Dairy effluent is one of the resources, which possesses various types of microorganisms such as bacteria and yeasts. The main purpose of this study is the use of bacteria strains isolated from dairy effluent to decolorization of two commonly used azo dyes in the textile industry. In addition, this work investigates the (I) screen and phenotypic identification of azo dye decolorizing bacteria strains from dairy wastewater, (II) determine the potentiality of the isolated strains in the decolorization of Reactive Black5 (RR5) and Reactive Red120 (RR120) azo dyes, and (III) genetic identification of strains based on the 16S rRNA gene. This research was conducted in Shahrekord, the province of Chaharmahal and Bakhtiari in Iran in 2016.

Materials and methods

Dyes, growth mediums, and chemicals

Textile azo dyes used in this study consist of the RB5 and RR120 that were purchased from Sigma–Aldrich (USA). All growth mediums including nutrient broth, nutrient agar, and Luria broth were purchased from Merck, Germany. Ethidium bromide, Taq DNA Polymerase, DNA ladder 100 bp, dNTP, preparation solutions, wash buffer, and 10X PCR buffer were purchased from CinnaGen, Iran. Primers were purchased from Faza Biotech, Iran.

Sample collection and primary isolation

The dairy effluent samples were collected from Zagros Pak Pooyan Co. (Shahrekord, Iran). Three samples of dairy effluent were collected in sterile clean plastic containers every 8 h and transported to the laboratory in a coolbox. In the laboratory, the samples were mixed and a composite sample was prepared.

In the primary isolation of bacteria, the dilutions of dairy effluent samples were prepared through successive dilution techniques to 10^{-5} times using sterile distilled water. Diluted sample was spread on nutrient agar and incubated for 24 h at 37 °C. After 24 h of incubation, growing colonies were picked up. Purification of bacteria was performed using four-quadrant streaking on the nutrient

agar and gram staining. Cultivation and gram staining of purification of bacteria were repeated several times. Initially, isolated colonies were assessed in terms of the ability of growth in the environment contaminated with dyes. For this purpose, single colonies were streaked on nutrient agar medium containing different dye concentrations (10, 50, 100, 150, and 200 mg/l).

Secondary isolation and screening of high-efficiency strains in decolorization

To select the most potent bacterial strains in the decolorization, the pretest was carried out on primarily separated colonies. For this purpose, nutrient broth medium containing 200 mg/l of each dye (RB5 and RR120) was used and 20 ml Pyrex tubes were selected as bioreactors. In each of the tubes, the bacterial strains were inoculated and incubated at 37 °C for three days and decolorization was compared with a control sample through direct observation.

Appropriate wavelength alternative for decolorization assay

To select the maximum absorption wavelength for each dye, nutrient broth mediums supplemented with a concentration of 10 mg/l of dyes were used. They were autoclaved to match decolorization assay and neutralize the effect of temperature on dyes. Then, the absorption was measured with a spectrophotometer (DR6000, Hach, Germany) at different wavelengths. In the end, the maximum absorption wavelength was selected for the decolorization assay.

Preparing the inoculums concentrations

To prepare the inoculation density, one loop of isolated bacteria was added in 100 ml of Luria broth medium and incubated for 24 h at 37 °C and was shaken (150 rpm). Then, the density of inoculation was adjusted to McFarland 0.5 turbidity standard (0.1 at 625 nm).

Decolorization assay

Decolorization test was carried out at concentrations of 10, 50, 100, 150, and 200 mg/l for each dye, and then, 200 ml nutrient broth medium containing different dye concentrations was poured in 250 ml Erlenmeyer flasks. The pH of mediums was adjusted to 7 and autoclaved. Aliquots of 10% (v/v) of inoculums were added to each flask. The flasks were closed with sterilized cotton and aluminum foil, incubated at 37 °C, and shaken at 150 rpm. Every 24 h interval, aliquots of 45 ml were regularly withdrawn from each flask in sterile conditions and poured in 50 ml Falcon tubes, and cells were pelleted by centrifugation for 15 min at 4500 rpm. The obtained clear supernatant was transferred to the Cuvette and tested with a spectrophotometer (DR6000, Hach, Germany) at maximum absorption wavelength. Percentage of decolorization was calculated using Eq. (1):

$$\text{Decolorization } (\%) = \left[\frac{C_0 - C_1}{C_0}\right] \times 100, \tag{1}$$

where C_0 is the initial and C_1 is the executed decolorized dye concentration. All the experiments were performed in triplicates, and the mean values were considered.

Assessing the biological resistance of strains, against RB5 and RR120 azo dyes

In order to determine the ability and tolerance of isolated strains, resiliency test was done in different dye concentrations. To do that, different dye concentrations were added to nutrient agar medium and autoclaved. One loop of each colony was streaked in each plate and incubated for 48 h at 37 °C. The test was continued to obtain a minimum inhibitory dye concentration.

Differential identification of strains

The initial identification of most potent bacterial strains in decolorization was conducted based on morphological characters on nutrient agar media and differential identification including gram staining, biochemical activity (motility, indole, catalase, fermentation of glucose, and mannitol) and physiological properties were implemented using the protocol of the Bergey's Manual of Determinative Bacteriology (Buchaman and Gibbons 1974).

The final identification of bacteria using 16S rRNA Gene Sequencing

The genomic DNA was extracted from the pure culture pellets via a Cinnagen DNA extraction kit according to the manufacturer protocol. The 16S rRNA gene was amplified by PCR using the bacteria universal primers 27F (5′-CAG CGGTACCAGAGTTTGATCCTGGCTCAG-3′) and 1492R

(5′-CTCTCTGCAGTACGGCTACCTTGTTACGACTT-3′), and 1500 bp products were amplified. PCR steps including an initial denaturation were performed for 5 min at 94 °C followed by 32 cycles of 1 min at 94 °C, 1 min at 58 °C, and 2 min at 72 °C. The amplicons were purified using Cinnagen several reactive gel extraction kit and sequenced. The phylogenetic tree was constructed by the neighbor-joining method using Kimura-2-parameter distances in Mega 6 package. The support of monophyletic groups was assessed by the bootstrap method with 1000 replicates. The stock was prepared for an extended time preservation of strains.

Fig. 1 (Left): Single colonies isolated from dairy effluents and (right): Grown colonies on nutrient agar medium containing 200 mg/l of dye for each

Fig. 2 Visual comparison of decolorization by eight colonies: (left) decolorization of RR120 and (right) decolorization of RR5

Table 1 Percentage decolorization efficiencies of isolated strains (mean ± SD)

Type of dye	Initial concentration (mg/l)	Bacteria code 1			Bacteria code 2			Bacteria code 3		
		24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
R R120	10	96.1 ± 0.20	96.1 ± 0.26	96.6 ± 0.00	84.5 ± 0.17	91.1 ± 0.20	95.5 ± 0.17	93.3 ± 0.00	93.8 ± 0.17	96.1 ± 0.2
	50	94.7 ± 0.10	94.9 ± 0.10	95.1 ± 0.30	84.2 ± 0.10	91.0 ± 0.30	95.3 ± 0.00	92.7 ± 0.10	93.5 ± 0.00	94.9 ± 0.10
	100	94.0 ± 0.30	94.5 ± 0.20	94.5 ± 0.20	82.6 ± 0.00	89.0 ± 0.00	92.7 ± 0.10	91.0 ± 0.20	91.7 ± 0.15	93.4 ± 0.00
	150	92.0 ± 0.20	93.6 ± 0.00	93.7 ± 0.40	81.1 ± 0.30	86.4 ± 0.26	90.3 ± 0.20	85.8 ± 0.34	88.2 ± 0.34	91.8 ± 0.10
	200	88.9 ± 0.17	89.5 ± 0.10	89.7 ± 0.17	79.7 ± 0.20	83.5 ± 0.36	87.1 ± 0.17	83.2 ± 0.26	86.1 ± 0.34	89.3 ± 0.10
RB5	10	85.0 ± 0.20	92.0 ± 0.17	95.0 ± 0.35	80.0 ± 0.00	89.6 ± 0.17	94.4 ± 0.36	83.3 ± 0.10	91.6 ± 0.00	95.0 ± 0.26
	50	84.2 ± 0.00	91.2 ± 0.17	94.8 ± 0.17	79.3 ± 0.17	88.2 ± 0.35	93.9 ± 0.17	82.9 ± 0.17	91.2 ± 0.17	94.3 ± 0.00
	100	82.8 ± 0.20	89.6 ± 0.10	93.1 ± 0.20	76.9 ± 0.20	83.3 ± 0.35	92.4 ± 0.26	79.6 ± 0.30	89.3 ± 0.17	92.6 ± 0.17
	150	81.4 ± 0.26	84.5 ± 0.20	92.6 ± 0.17	75.8 ± 0.26	82.7 ± 0.10	91.3 ± 0.10	76.3 ± 0.10	84.0 ± 0.26	91.5 ± 0.20
	200	79.7 ± 0.10	80.3 ± 0.10	90.8 ± 0.26	75.5 ± 0.20	82.4 ± 0.20	90.0 ± 0.20	76.0 ± 0.00	80.0 ± 0.17	89.9 ± 0.10

Results and discussion

Isolation and screening of azo dye decolorizing bacterial strains

At primary screening, ten bacterial isolates were obtained from cultured dairy effluents on nutrient agar media. Eight of these colonies showed good growth on medium supplemented with a dye concentration of 200 mg/l. Figure 1 shows the samples of isolated single colonies and the colonies grew on nutrient agar medium containing dyes.

The results of the decolorization pretest showed that among the eight selected isolates, only three of which had a desirable decolorization efficiency within three days of incubation. The three most potent colonies in decolorization were coded from 1 to 3. The visual comparison of decolorization by colonies is shown in Fig. 2.

The maximum absorption wavelength for the RB5 and RR120 dyes

The results showed that the maximum absorption wavelength for the RR120 and RB5 dyes were occurring at 538 nm and 597 nm, respectively.

Effect of initial dye concentration

Decolorization efficiency was greatly affected by the initial dye concentration (Singh et al. 2014). As seen in Table 1, decolorization efficiency of RB5 and RR120 by three isolated strain was decreased with an increase in dye concentration. After 24 h of incubation, maximum decolorization efficiency for RR120 was 96.1% at the concentration of 10 mg/l. Also, the minimum decolorization efficiency of RR120 was 79.7% at the concentration of 200 mg/l. Maximum decolorization efficiency for RB5 dye was 85.0% at the concentration of 10 mg/l, and minimum decolorization efficiency of RB5 was 75.5%, at the concentration of 200 mg/l. There is a report in which decolorization was decreased due to increase in initial dye concentration (Jadhav et al. 2008).

Effect of incubation time

It was observed, depending on the initial dye concentration, most decolorization efficiency was occurred within 24 h of incubation. In the first 24 h of incubation, decolorization efficiencies for RR120 and RB5 were 88.9 to 96.1% and 79.7 to 85.0%, respectively. In accordance with Table 1 and Fig. 3, the rise of incubation time only leads from 0.5 to 11% increase in decolorization efficiency. So, dye concentration at high levels required more time to achieve similar efficiencies. Similar results have previously been reported (Kale et al. 2010; Das and Mishra 2016) (Table 2).

Phenotypical and molecular identification of strains

The results of phenotypic characterization and identification of azo dye decolorization strains showed that bacteria code 1 was gram-positive, nonmotile catalase-positive, mannitol fermentation-negative, convex, and white colony according to the Bergey's Manual of Determinative Bacteriology. Moreover, the bacteria codes 2 and 3 were gram-positive, catalase-positive, mannitol fermentation-negative, convex yellow pigments, and glucose fermentation-negative. Based on the biochemical tests, the bacteria code 1 is *Staphylococcus* sp. and bacteria codes 2 and 3 are *Micrococcus luteus*. Also the 16srRNA sequence showed that the bacteria code 1 was the closest phylogenetic neighbors to *Staphylococcus* sp. IMB003 at an accurate identity of 96%.(Figure 4.), bacteria code 2 was the nearest phylogenetic neighbors to *Micrococcus luteus* TY21SsR at an accurate identity of 100%, and bacteria code 3 was the nearest phylogenetic neighbors to *Micrococcus* sp. H5 with an accurate identity of 97% (Fig. 5.). The sequences of the 16srRNA gene of the strains were deposited in GenBank using accession numbers of KX180131, KX180132, and KX180133 for code 1, code 2, and code 3, respectively. Also, the strains were submitted to GenBank using laboratory identifier called *Staphylococcus* sp. MEH038S, *Micrococcus luteus* strain SEH038S, and *Micrococcus luteus* strain FEH038S, in order to which they processed.

In this study, after three days of incubation via *Micrococcus luteus* SEH038S and *Micrococcus luteus* FEH038S, the decolorization efficiencies for 200 mg/l concentration of RB5 were 90 and 89.9%, respectively. Nearly similar

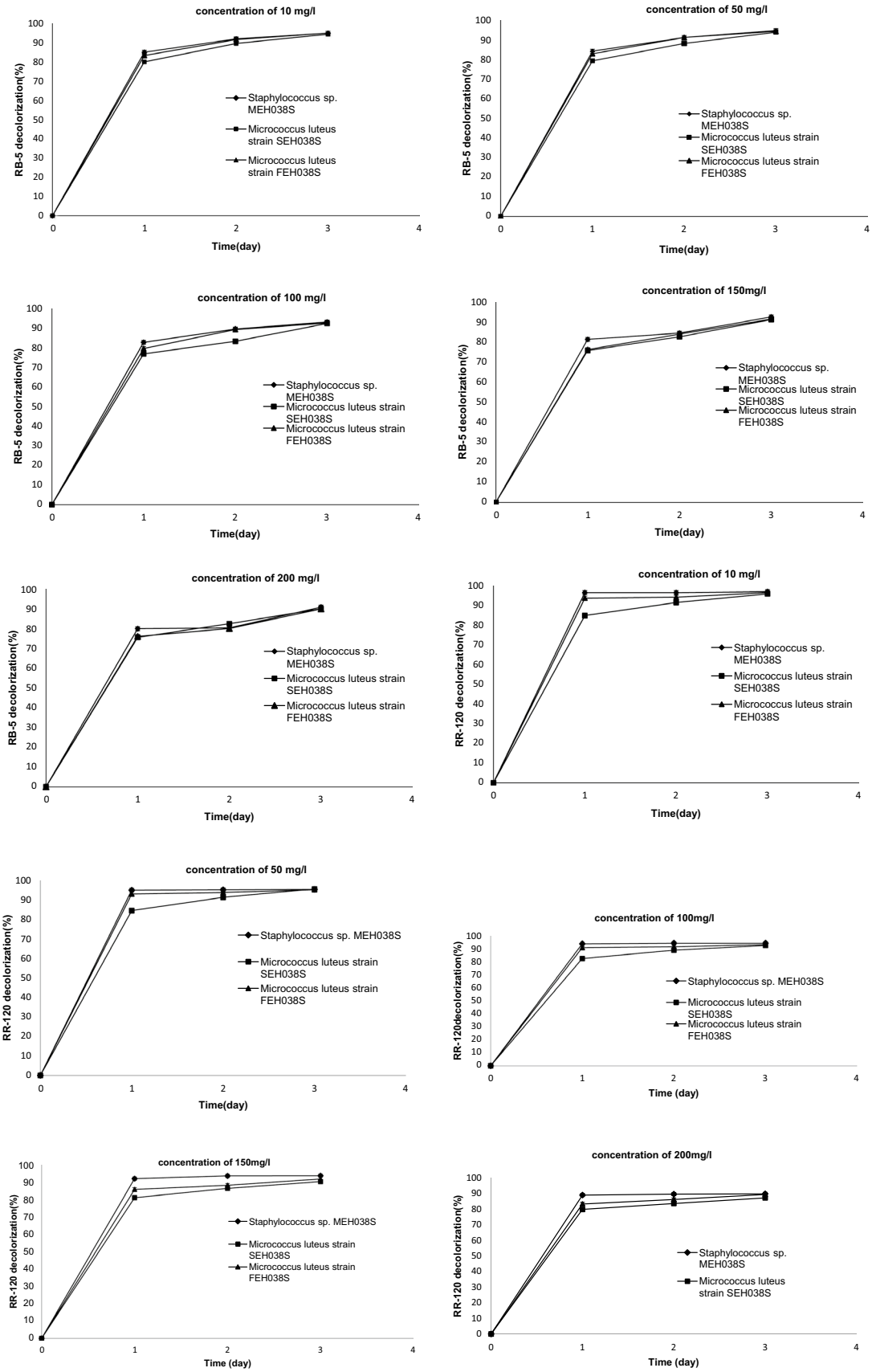

Fig. 3 Decolorization efficiencies of RR120 and RB5 dyes at 10, 50, 100,150, and 200 mg/l concentrations by isolated strains

Table 2 Number of decolorization tests

Dye	Bacterial strain	Maximum initial concentration (mg/l)	Incubation time (h)	Efficiency (%)	Reference(s)
RB5	*Pseudomonas aeruginosa* strain ZM130	100	48	>90	Maqbool et al. (2016)
RB5	*Shewanella* sp. Strain IFN4	200	4	>90	Imran et al. (2014)
RR120	*Pseudomonas aeruginosa* strain ZM130	100	48	>90	Maqbool et al. (2016)
RR120	*Bacillus lentus* BI377	1500	12	92	Oturkar et al. (2011)
RR120	*Acinetobacter junii* strain FA10	150	48	94.8	Anwar et al. (2014)
RB5	*Micrococcus luteus* strain SEH038S	200	72	90	This study
RB5	*Micrococcus luteus* strain FEH038S	200	72	89.9	This study
RR120	*Micrococcus luteus* strain SEH038S	200	72	87.1	This study
RR120	*Micrococcus luteus* strain FEH038S	200	72	89.3	This study
RB5	*Staphylococcus* sp. MEH038S	200	72	90.8	This study
RR120	*Staphylococcus* sp. MEH038S	200	72	89.7	This study

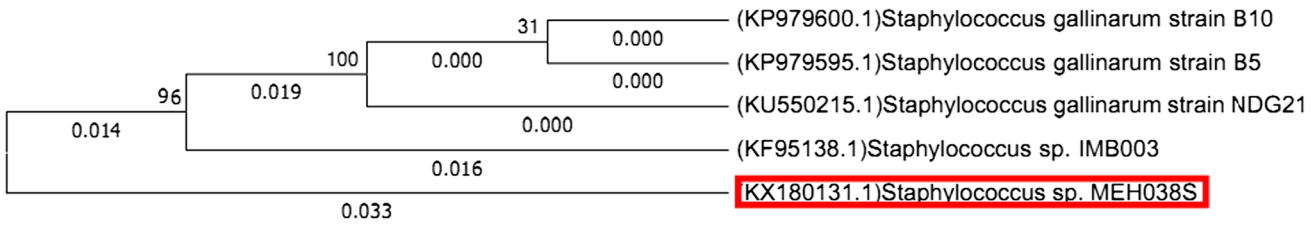

Fig. 4 Phylogenetic tree of Staphylococcus strain MEH038S constructed via neighbor-joining algorithm

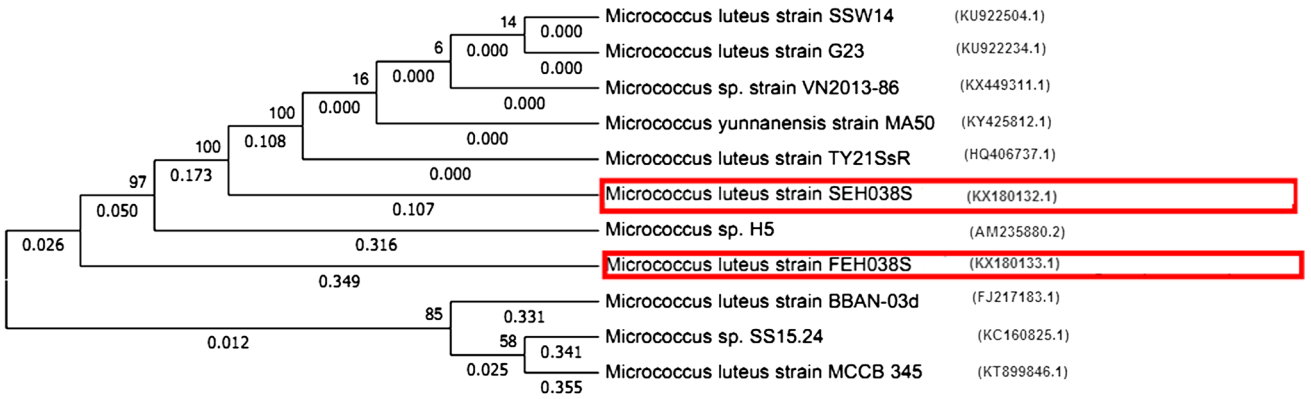

Fig. 5 Phylogenetic tree of *M. luteus* strains FEH038S and SEH038S constructed via neighbor-joining algorithm

efficiencies for the same concentration of RR120 were 87.1 and 89.3%, respectively. The removal of RR120 via *Micrococcus luteus* strains has not yet been reported.

In a study, Hassan et al. applied the *Micrococcus luteus*, to remove the yellow FN2R, navy WB, orange W3R, red FNR, and blue FNR dyes. Seven days into incubation, the removal efficiencies for yellow FN2R and navy WB were 60% for both, and for orange W3R, red FNR, and blue FNR were between 85 to 90% (Hassan et al. 2013). The two studies showed that the incubation time in the Hassan's study was 2.3 times higher than that of ours in comparison. In another study done by Singh et al., the removal efficiency of

direct orange 16 at 5 mg/l concentration was 96% via *Micrococcus luteus* strain SSN2 (Singh et al. 2015). Although the removal efficiency in Singh study is very high, the highest removed concentration in our study is over 40 times higher than that done by Singh.

In this study, the removal efficiencies for the RB5 and RR120 concentrations of 200 mg/l via *Staphylococcus* sp. MEH038S, within three days of incubation, were 90.8 and 89.7%, respectively. Yan used *Staphylococcus cohnii* to remove 100 mg/l of acid red B concentration. Yan reported that the removal efficiency after 24 h of incubation was 90 percent (Yan et al. 2012). In another study, Chaieb et al. applied Staphylococcus lentus to remove Congo red, Evans blue, and Eriochrome Black T at 100 mg/l dye concentration (Chaieb et al. 2016). The dyes removal efficiency has been reported to be 100%. In the above studies, dye removal efficiencies were high; however, the dye concentration in our study was twice higher than that used in Chaieb et al. and Yan's. According to Table 1, it is found that the decolorization efficiency, especially in the case of RR120, occurs mainly during the first 24 h. More research needed to determine the decolorization mechanisms by obtained strains and to identify the cause of major decolorization in the first 24 h. The decolorization pattern varies; it depends on the type of color, the type and strain of microorganism, the mechanism of decolorization, the enzymatic activity, and the toxicity of the intermediate products. In some cases, it has been reported that the enzymatic activity of the organisms was high in the first 24 h and depleted in the following days. As an example, Uhnakova's study explained that laccase utilized oxygen molecules to oxidize various types of xenobiotic compounds without the need for redox mediator and secondary metabolites (Uhnáková et al. 2011). This theory was supported by Hadibarata who explained that laccase reached the maximum activity during 24 h of incubation (Hadibarata et al. 2012). In other cases, the kinetics of the enzymes and intermediate products can play an inhibition role in decolorization. In a study done by Hu, the toxicity of metabolic products of azo dyes was analyzed, resulted in the kinetics of azoreductase on azo dyes, emphatically suggesting an inhibition model (Hu 2001).

Finally, by comparing our study and previous studies, it is clear that *Micrococcus luteus* strains and *Staphylococcus* sp. have high efficiencies in azo dyes removal; however, no studies have yet reported the 90% biological RB5 and RR120 decolorization efficiency at 200 mg/l concentration with applicability of non-indigenous bacteria strains in textile industrial effluents.

Conclusion

Bacterial strains of. *Staphylococcus* sp. MEH038S, *Micrococcus luteus* SEH038S, and *Micrococcus luteus* FEH038S which are capable of decolorizing RR120 and RB5 were isolated from dairy effluents. Discovered strains were identified with 16S rRNA and some biochemical tests. This study was conducted at 10, 50, 100, 150, and 200 mg/l azo dye concentrations for three consecutive days of incubation. These strains were biodecolorized at 200 mg/l concentration of RR120 from 87.1 to 89.7% and that RB5 from 89.9 to 90.8%. Our results suggest that the mixed application of dairy and textile wastewaters in a bioreactor system is congenial in decolorizing of textile effluents. Based upon these findings, future attempts will be performed to isolate more microorganisms from dairy effluents which are capable of azo dye decolorization.

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