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Identification and Characterization of Azo Dye Decolourizing Bacterial Strains, *Alcaligenes faecalis* E5.Cd and *A. faecalis* Fal.3 Isolated from Textile Effluents

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Abstract

The study was designed for isolation and characterization of azo dye decolourizing bacteria which is a prerequisite for developing a microorganism-facilitated treatment of polluting dyes. In this study nine types of bacteria which were able to decolourize three types of azo dyes (Blue H/C, Red 3B and Yellow 3R dye) were isolated from textile effluents collected from Gazipur industrial area in Bangladesh. Depending on 16S rDNA analysis, the most efficient decolourizing bacterium for the Blue H/C and the Red 3B dye was identified as *Alcaligenesfaecalis* strain E5.Cd while that for the Yellow 3R dye was identified as *Alcaligenesfaecalis* strain Fal.3. After characterization, both *A. faecalis* E5.Cdand *A. faecalis* Fal.3 were found to grow optimally at 35 ^{0}C and at pH 7 and pH 8, respectively. Both of these strains were sensitive to all antibiotics studied except for Bacitracin. Also, both strains showed maximum decolourization activities after 96 hours incubation in MS media at pH 7 (up to 93%) and pH 8 (up to 94%), at 35 ^{0}C temperature (up to 91%), at 50 ppm initial dye concentration (up to 92%), at 20% inoculum size (up to 93%), and at supplementation of 1% co-substrate (up to 93%).

Keywords: Textile effluent; Azo dye; Decolourization; *Alcaligenes faecalis.*

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1. Introduction

Large amount of effluents which is directly released in environment from the different industries without any proper treatment is now considering as a major threat to environment. In Bangladesh, dye-containing effluents released from textile and dyeing industries is one of the major sources of environment pollution [1, 2]. It is reported that different industries discharge around 280,000 tons of dyes per year into the environment worldwide [3]. Azo dyes, which are aromatic compounds with one or more (–N=N–) groups, are the most significant and major class of synthetic dyes discharged from various industries [4]. It has been reported that around one million tons of Azo dyes are produced annually for using commercial purposes worldwide [5]. Moreover, the dyes which are used in textiles and dyeing industries are mostly synthetic in nature that makes them more stable in nature and more challenging for biodegradation by natural process [6, 7]. Notably, very little amount of dye in water (10-50 mg L-1) can adversely affects different physical parameters of aquatic environment such as transparency and gas solubility of water, and the aesthetic value resulting in reduced dissolved oxygen and light intensity which ultimately affect the aquatic biodiversity [3]. It has been reported that textile dyes can adversely affect germination rates and biomass concentration of several plant species [3]. Moreover, these dyes are xenobiotic in nature as well as are mutagenic and carcinogenic in some circumstances [8, 9]. Similarly, allergic effects of these dyes have also been shown by many studies [10, 11]. Hence, treatment of dye-contaminated effluents prior to their final discharge is essential to prevent environment pollution. Recently, many scientific research have been focused on developing microorganism-facilitated treatment method for degradation of textile dyes [12]. To develop a microorganism-facilitated treatment, establishment of a pure culture of microorganism possessing high efficacy of biodegradation of dyes into colourless and nontoxic components is a prerequisite [13-17]. Hence, the present study was designed to establish pure cultures of bacteria having higher ability to degrade azo dye. Among the 9 pure cultures of azo dye decolourizing bacteria isolated from textile effluents, the most efficient decolourizing bacteria was *Alcaligenes faecalis* E5.Cd (IBR-5) for the Blue H/C and Red 3B dye, and that was *A. faecalis* Fal.3 (IY-3) for the Yellow 3R dye. However, different strains of *A. faecalis* such as *A. faecalis* SAG_5 [18] and *A. faecalis* PMS-1 [19] were reported for their decolourization efficacy, but *A. faecalis* E5.Cd and *A. faecalis* Fal.3 were novel regarding their higher ability for decolourization studied azo dyes. Moreover, their decolourization activities were dependent on incubation temperature, pH, initial dye concentration, inoculum size and amount of co-substrate used in culture medium.

2. Materials and Methods

2.1 Source of the sample and dyes

Effluents samples were collected in sterile plastic bottles from drainage canal of different Textile Dyeing Industries located in Gazipur, Bangladesh. Three azo dyes namely Blue H/C, Red 3B and Yellow 3R which were procured from Dysin-Chem Limited, Dhaka, Bangladesh were used in the present experiment.

2.2 Enrichment and isolation of dye decolourizing bacteria

All samples (untreated textile effluents) were used for isolation of dye decolourizing bacterial cultures by

enrichment culture techniques using enrichment medium amended with 20 ppm of the test dyes (Blue H/C, Red 3B and Yellow 3R) for the adaptation of the microorganisms [7]. For this, 1ml of sample of textile effluent was first diluted with 9ml sterilized water in test tubes separately. Then, 1ml of diluted sample was transferred into each single test tube containing 9 ml autoclaved enrichment medium. Required amount of respective dye was added to adjust the concentration 20 ppm and incubated to observe dye decolourization. After 24 –72 hours incubation, the bacteria from the decolourized test tube were streak plated on enrichment agar medium and mineral salt (MS) agar medium having 20 ppm of respective dye. Bacterial colonies that showed a clear decolourization zone around them on enrichment agar medium were picked and cultured for 24 hours at 35°C in MS medium amended with 1ml/l TE solution. Then, 1 ml of the culture of individual colony was reintroduced into 9 ml enrichment medium. To observe decolourization activity by individual bacteria, 1 ml of the culture of individual colony was added into 9 ml MS medium separately containing 20 ppm of respective dye, and then incubated for 16 hours at 35°C. Then, 2 ml of incubated media was taken out aseptically and centrifuged at 10,000 rpm for 10 minutes. The cell free supernatant was used to determine the percentage decolourization of the added dye. Isolate showing the most decolourization of the added dye was selected and preserved for further studies.

2.3 Determination of colour intensity and decolourization activity

The colour intensity of the sample was determined with standard spectroscopic methods [20]. Decolourization activity was expressed in terms of percentage decolourization and was determined by monitoring the decrease in absorbance at absorption maxima (λ_{max}) using UV-Visible spectrophotometer. The uninoculated MS medium supplemented with respective dye was used as reference. At different time intervals, the 2 ml samples were collected from reaction mixture and centrifuged at 10000 rpm for 10 min to separate biomass. The concentration of dye in the supernatant was determined by monitoring the absorbance at the maximum absorption wavelength (λ_{max}) at 660 nm for Blue H/C dye and 470 nm for Red 3B and Yellow 3R dye. [21].

The decolourization assay was calculated according to the following formula –

$$\text{Dye Decolourization activity (\%)} = \frac{Initial\ absorbance\ - Final\ abs\ orbance}{\text{Initial absorbance}} \times 100$$

2.4 Identification of dye-degrading bacteria by 16S rDNA gene sequence

Genomic DNA was extracted from dye decolourizing bacteria using CTAB method [22]. The PCR primers used to amplify 16S rDNA fragments were the bacteria-specific primers a forward primer F27 (5' – AGAGTTTGATCCTGGCTCAG – 3´; Tm: 61°C); and a reverse primer R1391 (5' – GACGGGCGGTGTGTRCA – 3´; Tm: 67.4°C). A total of 25 µl of reaction mixture consisted of – water 15µl, $MgCl_2$ 2.5µl, buffer 2.5, dNTPs 0.5µl, template 1µl, primer (forward 2 µl and reverse 2 µl). The PCR amplification was performed by Swift™ Minipro Thermal Cycler (Model: SWT-MIP-0.2-2, Singapore) using the following program: Denaturing at 95°C for 5 minutes, followed by 40 cycles of 40 seconds of denaturing at 95°C, 60 seconds of annealing at 65°C and 2 minutes of elongation at 72°C with a final extension at 72°C for 10 minutes. Then, the PCR products were subjected to 1% agarose gel electrophoresis, stained with ethidium

bromide and visualized on a UV transilluminator for the presence of about 1500 bp PCR products. The amplified PCR product was cleaned by using a AccuPrep® Gel Purification Kit (Bioneer corporation, Korea) in accordance to the manufacturer's protocol. PCR amplified 16s rDNA of the screened isolates was sent for automated sequencing (Applied Biosystem 3130) to the Centre for Advanced Research in Science (CARS) under Dhaka University, Bangladesh. The sequence generated from automated sequencing of PCR amplified DNA was analyzed through NCBI BLAST (http://www.ncbi.nlm.nih.gov) program to find out possible similar organism through alignment of homologous sequences. Finally, the isolates were identified based on alignment of partial sequence of 16S rDNA with the existing sequences available in the database.

2.5 Determination of optimum growth conditions

To determine the optimum pH of bacterial growth, culture medium was adjusted to pH 6.5, 7.0, 7.5 and 8.0. For determination of optimum temperatures, inoculated media were incubated at 25 ^{0}C, 30 ^{0}C, 35 ^{0}C and 40 ^{0}C. The growths of bacteria at different condition were determined at different time intervals by measuring optical density at 660 nm with photoelectric colorimeter.

2.6 Antibiotic sensitivity test

Sensitivity of antibiotic to the isolated bacteria was performed as described by Saha [23].

2.7 Influence of different parameters on process of dye decolourization

The effect of temperature, pH, initial dye concentration, inoculum size and co-substrate (yeast extract) concentration on decolourization of Blue H/C dye, Red 3B dye and Yellow 3R by isolated bacteria, *A. faecalis* E5.Cd and *A. faecalis* Fal.3 was examined after 96 hours of incubation. To examine the effect of pH on dye decolourization, 1 ml of cell suspension (5×10^6 cells) was used to inoculate 50 ml test tubes containing 10 ml MS medium, supplemented with 100 ppm individual dye. Then, the media were adjusted to pH 6, 6.5, 7, 7.5, 8 and 8.5, and incubated at 35 ^{0}C for 96 hours. For determination of effect of temperature, MS medium supplemented with 100 ppm individual dye was adjusted to pH 7. Then, media inoculated with equal density of bacterial cells were incubated at 25 ^{0}C, 30 ^{0}C, 35 ^{0}C and 40 ^{0}C for 96 hours. To test the effect of different dye concentrations on their decolourization, MS media supplemented with 50, 100, 150 and 200 ppm individual dye were adjusted to pH 7. Then, the media inoculated with bacterial isolates were incubated at 35 ^{0}C for 96 hours. To determine the effect of inoculum size on decolourization, MS media supplemented with 100 ppm individual dye were adjusted to pH 7. Then, 9.5 or 9 or 8.5 or 8 ml of MS medium for each condition was inoculated respectively with 0.5 ml (5 %), 1 ml (10 %), 1.5 ml (15 %) and 2 ml (20 %) of suspension of bacterial isolates. Then, the inoculated media were incubated at 35 ^{0}C for 96 hours. To investigate the effect of yeast extract on decolourization, MS media supplemented with 0 %, 0.1 %, 0.5 % or 1 % of yeast-extract, and 100 ppm individual sye were adjusted to pH 7. Then, the media inoculated with bacterial isolates were incubated at 35 ^{0}C for 96 hours.

2.8 Statistical analysis

Unless indicated otherwise, all experiments were independently conducted three times and data were pooled for presentation as mean±SEM. All data were analyzed with Prism software (GraphPad, La Jolla, CA, USA) using two-tailed unpaired Student's t-tests. P-values <0.05 were considered significant.

3. Results

3.1 A. faecalis strain E5.Cd and A. faecalis strain Fa1.3 which were isolated from textile effluent were efficient azo dye decolourizing bacteria

The study was started by screening for potential textile dye decolourizing bacteria isolated from effluents of the textile industries. Colonies with decolourized zone were isolated and then tested for dye removal capability using 20 ppm Blue H/C, Red 3B and Yellow 3R dye as the sole carbon source in the MS medium. Five morphologically distinct bacterial isolates (labeled as IBR-1, 2, 3, 4 and 5) were identified for decolourization of Blue H/C and Red 3B dye. Among them, isolate IBR-5 showed highest decolourization activity for Blue H/C and Red 3B dye (Fig. 1A, B). Four morphologically distinct bacterial isolates (labeled as IY-1, 2, 3 and 4) were identified for decolourization of Yellow 3R dye. Among them, isolate IY-3 showed highest decolourization activity for Yellow 3R dye (Fig. 1C). Both IBR-5 and IY-3 were identified as gram-negative, rod-shaped and motile bacteria. As IBR-5 and IY-3 were most efficient azo dye decolourizing bacteria among the 9 bacterial isolates, these two isolates were selected for 16S rDNA based identification and characterization. For 16S rDNA based identification of IBR-5 and IY-3, the 16S rDNA isolated from two bacterial strains was amplified using bacteria specific universal primers 27F and 1391R. Then, the isolated 16S rDNA was used for sequencing. These sequences were submitted to NCBI. The highest similarity was shown for IBR-5 (99% similarity) to *A. faecalis* strain E5.Cd, and for IY-3 (98% similarity) to *A. faecalis* strain Fa1.3.

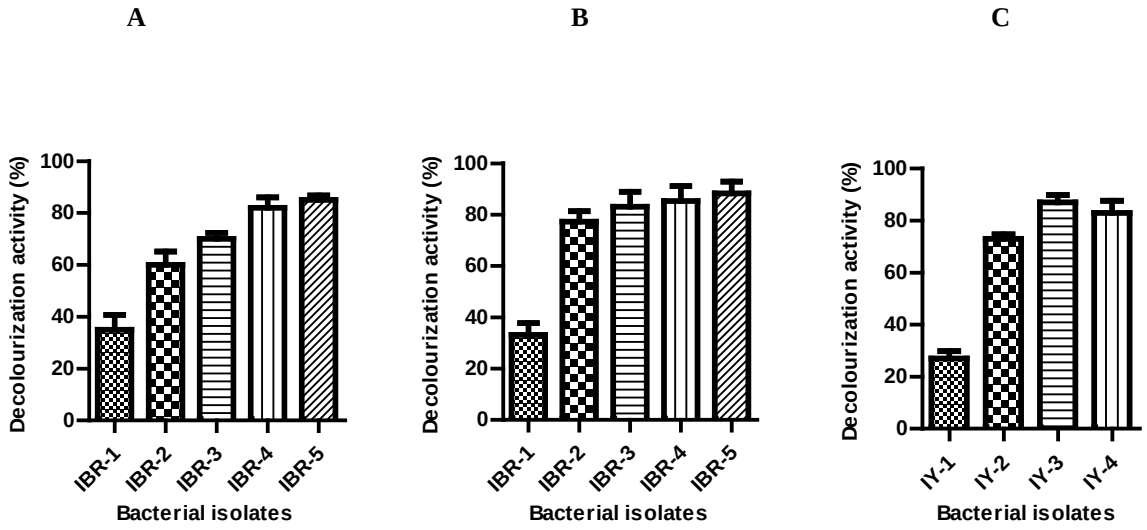

Figure 1: Azo dye decolourization efficacy by different bacterial isolates grown in MS media supplemented with 20 ppm Blue-H/C dye (A), Red-3B dye (B) and Yellow-3R dye (C). The decolourization activity was measured after 16 hours incubation at 35 ^{0}C. The data is a mean±SEM from three independent experiments

3.2 Both strains of A. faecalis grow optimally at 35 ^{0}C

Optimum temperature for growth of both *A. faecalis* strain E5.Cd and *A. faecalis* strain Fa1.3 was determined at pH 7 in nutrient broth medium. The optimum temperature for growth of both studied strain was found to be 35 ^{0}C (Fig. 2A, B). For *A. faecalis* strain E5.Cd, the maximum growth rate (OD 0.7) was observed at 35 ^{0}C while the minimum growth rate (OD 0.37) was observed at 25 ^{0}C (Fig. 2A). Similarly, the maximum growth rate (OD 1.5) of *A. faecalis* strain Fa1.3 was observed at 35 ^{0}C while the minimum growth rate (OD 0.75) was observed at 25 ^{0}C (Fig. 2B).

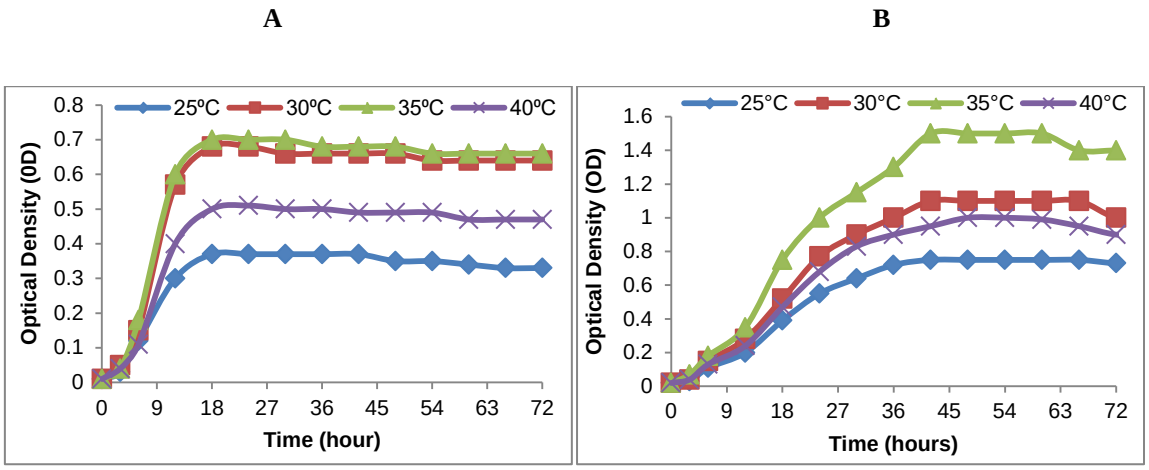

Figure 2: Optimum temperature for growth of the bacterial strains *A. faecalis* E5.Cd **(A)** and *A. faecalis* Fa1.3 **(B)** at pH 7.0. The optimum temperature of bacterial growth was determined at every 3-hours interval up to 72 hours incubation at 25 ^{0}C, 30 ^{0}C, 35 ^{0}C and 40 ^{0}C by measuring optical density at 660 nm

3.3 A. faecalis strain E5.Cd and A. faecalis strain Fa1.3 grow optimally at pH 7 and pH 8 respectively

Optimum pH for growth of Blue H/C and Red 3B dye decolourizing bacteria (*A. faecalis* strain E5.Cd) and Yellow 3R dye decolourizing bacteria (*A. faecalis* strain Fa1.3) was determined at 35 ^{0}C temperature in liquid broth medium.

As shown in Fig. 3A, *A. faecalis* strain E5.Cd exhibited maximum growth (OD 0.68) at pH 7 and the minimum growth (OD 0.61) at pH 8 while *A. faecalis* strain Fa1.3 showed the maximum growth rate (OD 1.5) at pH 8 (Fig. 3B).

3.4 Both strains of A. faecalis were sensitive to all studied antibiotics except Bacitracin

Study of antibiotic sensitivity pattern is vital to maintain pure culture of a bacterial isolate as well as to take a decision for using it safely in any environmental application. In this study, the patterns of antibiotic sensitivity of two isolated bacterial strains were tested by disk-diffusion method using nutrient agar medium.

It was found that both strains were resistant to Bacitracin while they were sensitive to 8 other antibiotics (data not shown). However, both of the isolated strains showed intermediate sensitive to Cephradine (data not shown).

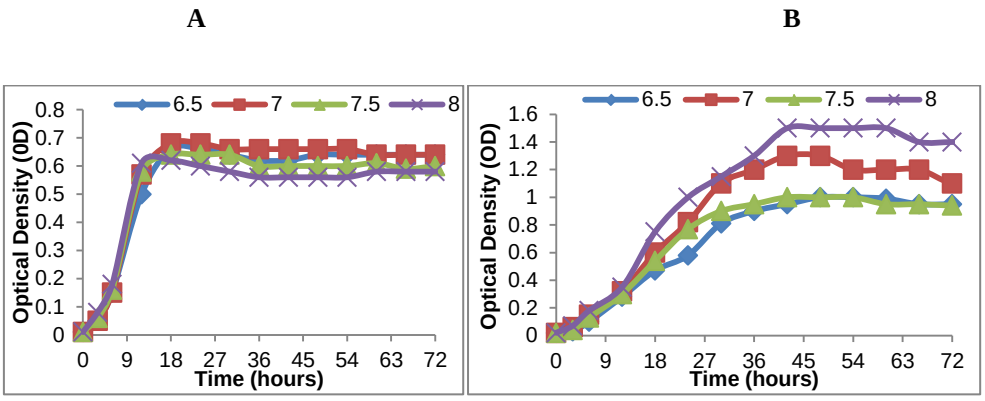

Figure 3: Optimum pH for growth of the bacterial strains *A. faecalis* E5.Cd **(A)** and *A. faecalis* Fa1.3 **(B)** at 35 ^{0}C. The optimum pH of bacterial growth was determined at every 3-hours interval up to 72 hours incubation at pH 6.0, 6.5, 7.0, 8.0 and 8.5 by measuring optical density at 660 nm

3.5 Dye-decolourization efficacy of both strains of A. faecalis were dependent on incubation temperature, pH, initial dye concentration, inoculum size and supplementation of co-substrate

It was observed that the percentage of dye decolourization activity varied with change in pH of the medium. The optimal pH for decolourization of Blue H/C and Red 3B dye by *A. faecalis* strain E5.Cd was pH 8 and 7 respectively (Fig. 4A, B), and for decolourization of Yellow 3R dye by *A. faecalis* strain Fa1.3 was pH 7 (Fig. 4C). However, the optimum pH of growth of *A. faecalis* strain E5.Cd and *A. faecalis* strain Fa1.3 was pH 7 and 8 respectively (Fig. 3A, B). For all of studied dye viz. Blue H/C, Red 3B and Yellow 3R dyes, the decolourization activity was increased as the temperature increased up to 35^{0}C, but the colour removal ability was decreased at 40^{0}C temperature (Fig. 5A, B, C). Thus, the results revealed that the optimal incubation temperature for the decolourization of the studied dyes was 35^{0}C (Fig. 5A, B, C).

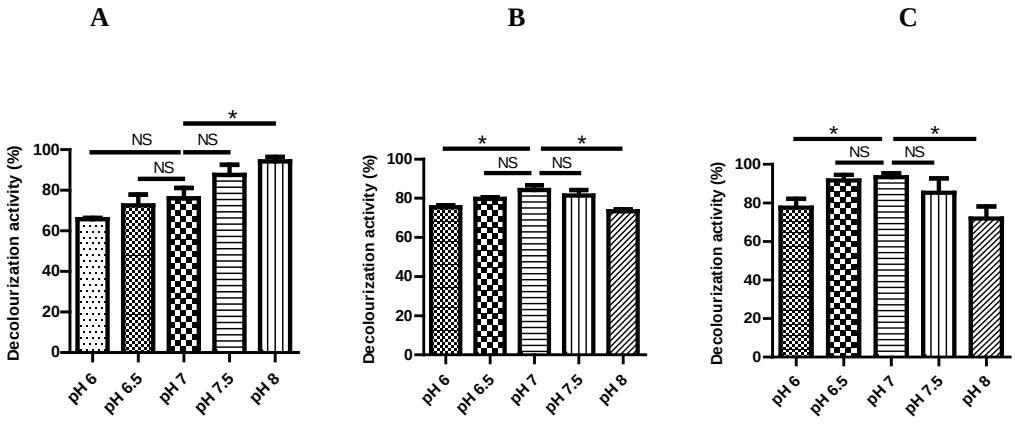

Figure 4: Effect of pH on decolourization of azo dyes by bacterial strain *A. faecalis* E5.Cd **(A, B)** or strain *A. faecalis* Fa1.3 **(C)** in MS media supplemented with 100 ppm Blue-H/C dye **(A)** or Red-3B dye **(B)** or Yellow-3R dye **(C)** at pH 6, 6.5, 7, 7.5 and 8. The decolourization activity was measured after 96 hours incubation at 35 ^{0}C. Data is a mean±SEM from three independent experiments. *P<0.05, ^{NS}P≥0.05, all relative to the pH 7 (control)

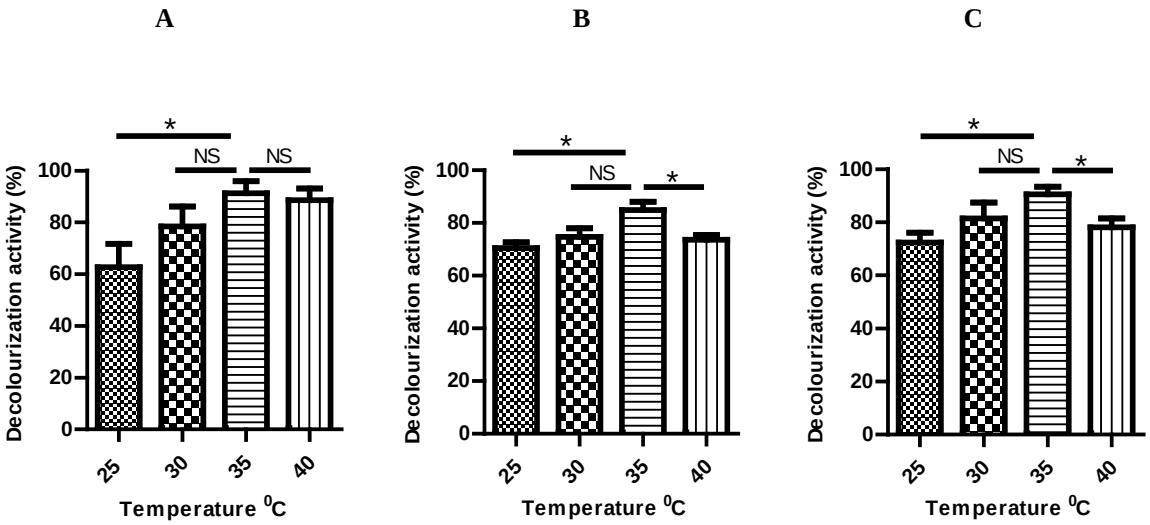

Figure 5: Effect of temperature on decolourization of azo dyes by bacterial strain *A. faecalis* E5.Cd **(A, B)** or strain *A. faecalis* Fa1.3 **(C)** at pH 7 in MS media supplemented with 100 ppm Blue-H/C dye **(A)** or Red-3B dye **(B)** or Yellow-3R dye **(C)**. The decolourization activity was measured after 96 hours incubation at 25 ^{0}C, 30 ^{0}C, 35 ^{0}C and 40 ^{0}C. Data is a mean±SEM from three independent experiments. *P<0.05, ^{NS}P≥0.05, all relative to the 35 ^{0}C (control)

Effect of dye concentration on dye-decolourization by both strains of *A. faecalis* was measured. It was found that the percentage of decolourization activity decreased as the dye concentration increased (Fig. 6A, B, C) with an exception for 100 ppm Red-3B dye (Fig. 6B). However, the difference between decolourization activities for 50 ppm and 100 ppm dye concentration were statistically insignificant for all three studied dyes (Fig. 6A, B, C).

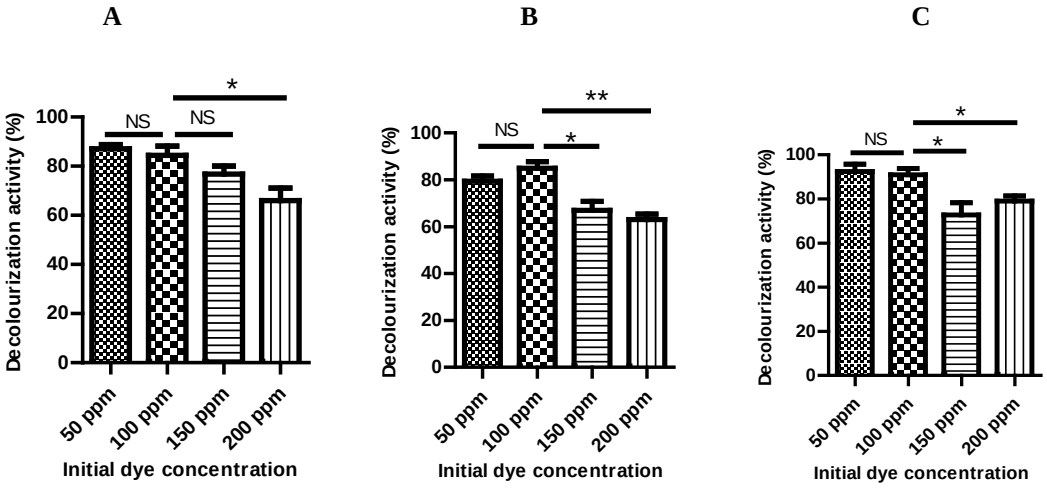

Figure 6: Effect of dye concentration on decolourization of azo dyes by bacterial strain *A. faecalis* E5.Cd **(A, B)** or strain *A. faecalis* Fa1.3 **(C)** at pH 7 in MS media supplemented with 50, 100, 150 and 200 ppm Blue-H/C dye **(A)** or Red-3B dye **(B)** or Yellow-3R dye **(C)**. The decolourization activity was measured after 96 hours incubation at 35 ^{0}C. Data is a mean±SEM from three independent experiments. *P<0.05, ^{NS}P≥0.05, all relative

to the 100 ppm dye concentration (control)

Conversely, the percentage of decolourization increased as the inoculum size increased, but these increases of decolourization activity were statistically insignificant for all studied dyes (Fig. 7A, B, C). Thus, it indicates that the effect of inoculum size on decolourization of the studied dyes by isolated bacteria was obscure.

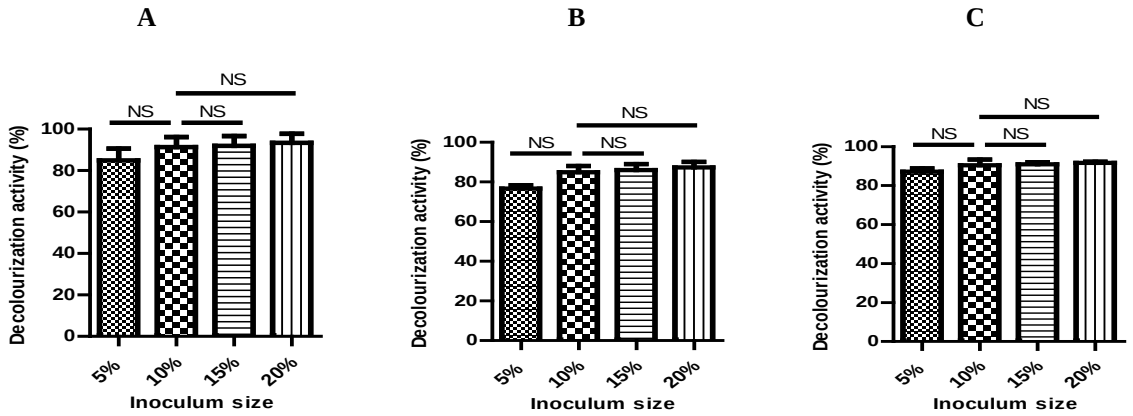

Figure 7: Effect of inoculum size on decolourization of azo dyes by bacterial strain *A. faecalis* E5.Cd **(A, B)** or strain *A. faecalis* Fa1.3 **(C)** at 5 %, 10 %, 15 % and 20% inoculum size at pH 7 in MS media supplemented with 100 ppm Blue-H/C dye **(A)** or Red-3B dye **(B)** or Yellow-3R dye **(C)**. The decolourization activity was measured after 96 hours incubation at 35 ^{0}C. Data is a mean±SEM from three independent experiments. $^{NS}P \geq 0.05$, all relative to the 10% inoculum size (control)

Effect of co-substrate (yeast extract) on bacteria-facilitated decolourization of studied dyes was observed. For all three studied dyes, the decolourization activity was increased as the concentration of the co- substrate (yeast extract) was increased, but the difference is statistically significant only when decolourization activity for 1% of co-substrate compared with 0% of co-substrate (control) (Fig. 8A, B, C).

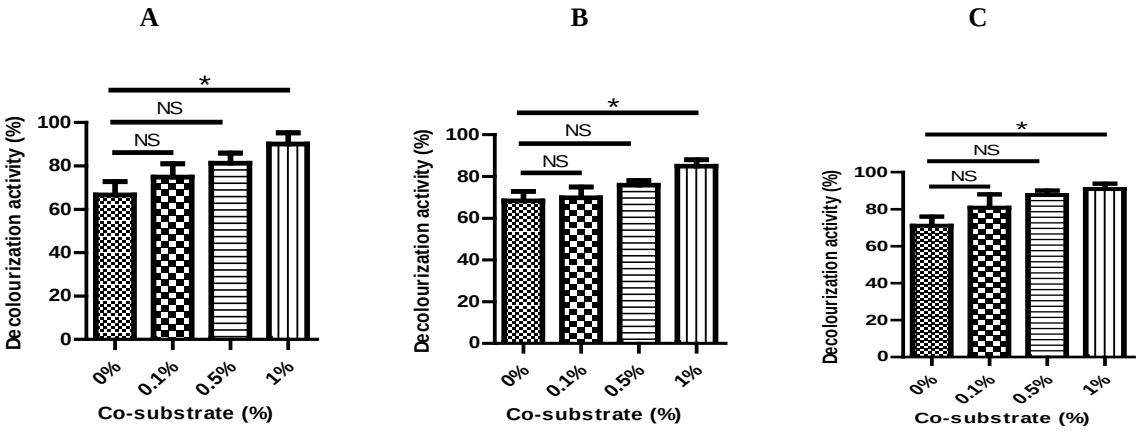

Figure 8: Effect of co-substrate on decolourization of azo dyes by bacterial strain *A. faecalis* E5.Cd **(A, B)** or strain *A. faecalis* Fa1.3 **(C)** at 0 %, 0.1 %, 0.5 % and 1 % of yeast-extract as co-substrate at pH 7 in MS media supplemented with 100 ppm Blue-H/C dye **(A)** or Red-3B dye **(B)** or Yellow-3R dye **(C)**. The decolourization

activity was measured after 96 hours incubation at 35 ^{0}C. Data is a mean±SEM from three independent experiments. *P<0.05, ^{NS}P≥0.05, all relative to the 0% co-substrate (control)

4. Discussion

In this study, two strains of *Alcaligenes faecalis* viz. *A. faecalis* E5.Cd and *A. faecalis* Fa1.3 were identified as azo dye decolourizing bacteria. Similarly, it has been reported that *A. faecalis* is an efficient dye decolourizing bacteria for Direct Blue-15 dye, Acid Orange-7 dye, Melanoidin and Reactive Orange 13 [18, 19, 24, 25]. However, some strains of *A. faecalis* can decolourize textile dyes efficiently when they are in a bacterial consortium [24, 25]. Treatments by consortium of anaerobic and aerobic microbes have been suggested to enhance the degradation of azo dyes [26]. Though, the pure culture of some strains of *A. faecalis* has been shown to be efficient decolourizer of textile dyes in many studies as we found in this study. Besides, use of microbial enzymes for dye decolourization has been reported in recent years for its effective application in treatment of textile dyes [27]. However, reactive azo dyes usually have a synthetic origin and intricate aromatic molecular structures, which make them steady and hard to biodegrade and generally considered as xenobiotic [28]. Both *A. faecalis* E5.Cd and *A. faecalis* Fa1.3 showed better growth and decolorization efficacy in the temperature range from 30 °C to 35 °C which was supported by similar results found in another study [29]. In this study, optimum pH for growth and decolorization efficacy of both strains was observed between pH 7 and 8. Similarly, it was reported that *A. faecalis* can grow optimally at pH 7[30]. We found that the color removal percentage of most dyes increased sharply with the addition of yeast extract which is also supported by other studies [31]. Metabolism of yeast extract is considered vital for the restoration of NADH, which is the electron donor for the azo bond reduction [32]. However, we found that decolourization activity was not significantly dependent on inoculum size which may be due to measuring the decolourization activity after long incubation period (96 hours). In this study, isolated bacterial strains showed maximum decolourization activity at lower dye concentration. Decrease in decolourization ability at high dye concentration might be due to the toxicity of the dye [31]. In addition, azo dyes generally contain one or more sulphonic-acid groups on aromatic rings, which might act as detergents to inhibit the growth of microorganisms [31]. Although the decolourization efficacy of *A. faecalis* E5.Cd and *A. faecalis* Fal.3 was prompting there were few limitations of this study. Decolourization efficacy of the isolated bacteria was studied only for three types of azo dyes but their efficacy to decolourize many other types of azo dyes remained obscured. Likewise, decolourization efficacy of the isolated bacteria was tasted in small-scale in lab which do not ensure their similar decolourization capacity in large-scale in industrial bioreactor. Notably, the higher rate of decolourization of azo dyes by the isolated bacteria does not confirm the higher detoxification rate of azo dyes. Hence, it should be recommended that toxicity of the decolourized azo dyes have to be tested before considering the *A. faecalis* E5.Cd and *A. faecalis* Fal.3 as efficient microbial agents for using in an industrial bioreactor to neutralize the textile effluents.

5. Conclusion

Altogether, the results of this study indicate that the isolated and the identified strains *A. faecalis* E5.Cd and *A. faecalis* Fal.3 were bacitracin-resistant efficient azo dye decolourizing bacteria which can be used to develop an effective microorganism-facilitated treatment method following the observed optimal conditions (pH between 7

and 8, temperature 35^0C, 50 ppm initial dye concentration, and 1% co-substrate supplementation) for their highest efficacy of decolourization of azo dye.

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