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# Decolorization of Different Azo Dyes and Detoxification of Dyeing Wastewater by *Pseudomonas stutzeri* (SB_13) Isolated from Textile Dyes Effluent

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***Authors' contributions***

*This work was carried out in collaboration between all authors. All authors read and approved the final manuscript.*

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## ABSTRACT

**Aims:** The present study aimed to estimate the decolorization of three individual azo dyes or in mixture, as well the decolorization of dyeing wastewater was evaluated using bacterial strain of *Pseudomonas stutzeri* (SB_13) or bacterial consortium isolated from textile dyes effluent. The cytotoxicity effect of dyeing wastewater and its biodegraded metabolites, as well the detoxification efficacy were evaluated.
**Study Design:** Soil and water samples were collected from the textile dyeing industrial area for bacterial isolation. Effect of different parameters on the dye decolorization by bacterial strains was optimized.
**Place and Duration of Study:** The study was performed in Botany & Microbiology Department, Faculty of Science, Al-azhar University, from July 2014 until January 2016.
**Methodology:** *Pseudomonas stutzeri* strain (SB_13) was isolated from textile dyes effluent and its ability for decolorization of different azo dyes and detoxification of dyeing wastewater samples was investigated. Comparison the decolorization effectiveness of bacterial strain (SB_13) which was used individually and in a bacterial consortium which contains two previously studied *Klebsiella*

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strains of (*Klebsiella pneumoniae* (Kp) and *Klebsiella variicola* (Kv)) was also observed. Decolorization of Disperse Blue (R16), Disperse Yellow (D4), and Reactive Red Synozol (R4) dyes which were used singly and in mixture was estimated under different concentrations and incubation conditions.

**Results:** The highest decolorization rates of single or mixtures of azo dyes were observed with 2% glucose or sucrose, 2% ammonium sulfate, and 3% (v/v) bacterial inoculum size, at pH of 5-7, temperature of 35ºC, and after 72-96 hrs. Mixed cultures of (SB_13 &Kp), (SB_13 &Kv), and (SB_13& Kp& Kv) significantly decolorized 59.5% of Disperse Blue (R16), 52% of Disperse Yellow (D4), and (18.3%) of Reactive Red Synozol (R4) dyes, respectively more than those found by individual strain (SB_13). Individual strain of (SB_13) showed the highest decolorization 61% capacity of azo dyes mixture compared to those observed by bacterial consortiums. The treatment of dyeing wastewater with SB_13 strain significantly reduced the phytotoxicity of wastewater (from 100% of abnormal mitosis to 23.6%) as compared with other treatments.

*Keywords: Azo dyes decolorization; detoxification; phytotoxicity; Pseudomonas; textile effluent; wastewater treatment.*

## 1. INTRODUCTION

Azo dyes are synthetic dyes which widely used in the industrial dyeing of cotton, wool, leather, paper, nylon, plastic and petroleum products [1,2]. Azo dyes characterized by their high ability to adhesive with cellulosic and cotton fibres; thus, they are commonly used in textile dyeing. Approximately $10^6$ ton of textile dyes has been produced annually all over the world including about 10,000 different dyes which are used in textile dyeing process [3,4]. The high wet stability characters of azo dyes in addition to their various and brilliant colors cause lower energy consuming in dying process [5].

However, the overuse of dyes causes the environmental pollution which mainly leads to increase the dyes concentration in soil and water. The dyes concentration into the textile dyeing effluent has been ranged from 10 to 200 mg $L^{-1}$ [4]. However, it has been recorded that the low azo dyes concentration of 10-50 mg $L^{-1}$ into the wastewater dyeing effluent is toxic and carcinogenic to organisms [6]. In addition, release of dyeing wastewater into the environment negatively affects the photosynthesis process and decrease oxygen content [7]. The persistence and hardly degradation of azo dyes contribute to the accumulation of these dyes into the soil and water in toxic levels with mutagenic effect on microorganisms, plants, and human [8,9].

The treatment of dyeing wastewater is mandatory required to avoid the environmental pollution, reduce toxicity of wastewater, reuse of dyeing water, and decrease the cost of dying process. The different physical and chemical methods such as filtration and precipitation followed by flocculation, chemical coagulation, ozonation and adsorption or neutralization and electro-dialysis are used to remediate dyeing wastewater [10]; however, these methods are economically high cost and cause water and soil pollution [11]. Alternative bioremediation technique is an innovative, cost-effective, and eco-friendly method allowing the utilization of microorganisms for decolorization and detoxification of dyes [9,12].

Microorganisms capable to remove colors of azo dyes under different conditions have been reported. Various bacterial species are potentially used in dyes decolorization based on their ability for degradation, transformation, and sorption of dye molecules [1,13]. However, further investigations are required to obtain novel bacterial strains with high ability for decolorization of different azo dyes individually or in mixtures. The present study aimed to estimate the decolorization of three individual azo dyes or in mixture, as well the decolorization of dyeing wastewater was evaluated using bacterial strain of *Pseudomonas stutzeri* (SB_13) isolated from textile dyes effluent. In addition, the capacity of bacterial consortium of *P. stutzeri* strain (SB_13) and two *Klebsiella* strains (*Klebsiella pneumoniae (*Kp*)* and *Klebsiella variicola (*Kv*)*) for dyes and wastewater decolorization was compared. The assessment of environmental risk of dyes decolorization on biological system is applicable required. Therefore, the cytotoxicity effect of dyeing wastewater and its metabolites, as well the detoxification efficacy were evaluated on the mitotic cell division of *Allium cepa* plant.

## 2. MATERIALS AND METHODS

### 2.1 Sample Collection and Bacterial Isolation

Soil and water samples were collected from the textile dyeing industrial area of 10th Ramdan, El Sharqia and Nasr City, Cairo, Egypt. The GPS positions were 30º 15' 54.51" N, 31º 45' 39.95" E and 30º03'02.1"N, 31º23'48.3"E, respectively. All samples were transported to the laboratory and screened for bacterial isolation. Three different azo dyes which used in this study are: Disperse Yellow (D4), Disperse Blue (R16), Reactive Red Synozol (R4) were provided as a gift from Startex for textile industry company in 10th Ramdan, El Sharqia.

Bacterial isolation conducted on mineral salt medium (MS) [14]. The cultural medium supplemented with 1 g/l of soil sample or 5 ml of water sample and incubated at 37ºC for 5 days on shaking incubator at 150 rpm. The purified bacterial isolates which grown on the mineral salt agar media supplemented with different concentrations of each dye were selected for further study. The ability of bacterial isolates to decolorize the different dye concentrations was estimated. The dye concentrations of (50 ppm, 100 ppm, 150 ppm, 200 ppm , and 250 ppm) were applied as a sole carbon source on solid media for selection of the most potent bacterial isolate according on the basis of growing on these dye concentrations.

### 2.2 Molecular Identification of Bacterial Isolate

For the 16S rRNA analysis, genomic DNA was extracted according to modified method (Miller et al. 1999). Briefly, individual colonies from an agar plate are picked using a sterile toothpick or inoculating loop and resuspended in 50 µl sterile deionized $H_2O$. The cell suspension is placed in a water bath at 97ºC and cooked for 10 minutes, centrifuge the cell lysate (15,000 × g, 10 minutes), remove the supernatant containing the DNA and add an aliquot (1 µl) to a PCR reagent mix. Following this: 16S rRNA was amplified in polymerase chain reaction (PCR) using the genomic DNA as template and bacterial universal primers, 27 f (5-GAGTTTGATCACTGGCTCAG-3) and 1492 r (5-TACGGCTACCTTGTTACGACTT-3) [15] to amplify an approximately 1.5 Kb of 16S rRNA gene. The PCR mixture (50 µL) contained 1× PCR buffer, 0.5 mM $MgCl_2$, 2.5 U Taq DNA polymerase (QIAGEN), 0.25 mM dNTP, 0.5µM of each primer, and 1 µl of extracted bacterial genomic DNA. The PCR was performed in a DNA Engine Thermal Cycler (PTC-200, BIO-RAD, USA) with a hot starting performed at 94ºC for 3 min, followed by 30 cycles of 94ºC for 0.5 min, 55ºC for 0.5 min, and 72ºC for 1 min, followed by a final extension performed at 72ºC for 10 min. The PCR products were sequenced at the Genome Quebec Innovation Center Facility (Montreal, Canada) with the two primers. The 16S rRNA sequence was compared against the GenBank database using the NCBI BLAST program. Sequences were then compared with 16S rRNA sequences in the GenBank database using BLASTN. Multiple sequence alignment was done using ClustalX 1.8 software package (http://www-igbmc.u-strasbg.fr/BioInfo/clustalx) and a phylogenetic tree was constructed by the neighbor-joining method using MEGA (Version 6.1) software. The confidence level of each branch (1,000 repeats) was tested by bootstrap analysis.

### 2.3 Optimization of Decolorizing Ability of Strain SB_13

The effect of various culture conditions such as pH, temperature, inoculum size, incubation period, and dye concentration on decolorization of Disperse Yellow (D4), Disperse Blue (R16), Reactive Red Synozol (R4) by the most potent bacterial strain was examined.

#### 2.3.1 Effect of different incubation periods and conditions on dyes decolorization

The effect of different incubation periods and conditions (static and shaking status) on dye decolorization process was investigated. Bacterial strain SB_13 was allowed to grow on mineral salt broth media containing individual dye as a sole carbon source, allowed to grow for 12, 24, 36, 48, 60, 72 and 96 hours at static and shaking (150 rpm).

#### 2.3.2 Effect of different incubation temperatures, pH and inoculum sizes on dyes decolorization

In order to test the effect of different incubation temperatures on the decolorization process, strain SB_13 incubated at different incubation temperatures of 25, 30, 35, 40 and 45ºC. Similarly, the effect of different pH values (5-9) on dye decolorization was tested. Separately, the dye decolorization percentages

were evaluated under different bacterial inoculum sizes of 1%, 1.5%, 2%, 2.5%, 3 % and 3.5% in culture media supplemented with dye concentration of 150 ppm for each individual dye.

At the end of each incubation period, the dye decolorization percentages (%) were assayed at 24h and 96h for Reactive Red Synozol (R4), and at 36h and 72h for Disperse Yellow (D4) and Disperse Blue (R16).

#### 2.3.3 Effect of different carbon and nitrogen sources on the dye decolorization by bacterial strain

In order to evaluate the effect of different carbon and nitrogen sources on dye decolorization, different carbon sources were introduced to mineral salt broth media at 2% concentration with equimolecular level for each sugar and supplemented by dye concentration of 150 ppm for each dye. The media without carbon source was used as a control (containing only dye concentration of 150 ppm as carbon source). The carbon sources were represented by glucose, sucrose, maltose, starch, fructose, mollase and bagasse. Similarly, with the equivalent amount of nitrogen level located at 0.5%, the effect of different organic and inorganic nitrogen source such as sodium nitrate, sodium nitrite, ammonium sulphate, urea, peptone, tryptophan, glutamic and yeast extract on dye decolorization were evaluated in media with dye concentration of 150 ppm. In each case, all previously mentioned optimal conditions of temperature, pH, and inoculation size were taken into the consideration.

#### 2.3.4 Dye decolorization (%) measurement

Two ml of each growing mineral salt media samples were withdrawn aseptically and centrifuged at 8,000 rpm for 15 minutes. The clear supernatant was used for measuring absorbance using UV-V spectrophotometer (721 spectrophotometer , M- ETCAL) at wave lengths of (600–700 nm), (410–460 nm), and (480–550 nm) for Disperse Blue (R16), Disperse Yellow (D4), Reactive Red Synozol (R4), respectively (Hunger, 2003). The un-inoculated mineral salt media supplemented with the same dye concentration was used as control. All dyes were prepared in triplicate and compared with control. The percent decolorization of effluent was determined by using the follwing formula [16].

$$D\ (\%) = [(dye(i)-dye(1)) / dye(i)]\times 100$$

where D, decolorization %; Dye (i), initial absorbance; Dye (1), final absorbance.

### 2.4 Wastewater Treatment

The ability of bacterial strain SB_13 for wastewater treatments was evaluated. Samples of textile wastewater effluent were taking from Textile Factory of Elshams, El Obour City. Textile wastewater treatment processes was done according to [17].

Neutralization by adjusting the pH of textile wastewater under treatment between 6 and 8 was done. Then, chemical precipitation of soild materials was conducted by adding coagulant factors of Alum ($Al_2SO_4$) and polymer of (RMCST01). The activated sludge was conducted, where the filtrate obtained from the previous step was treated with bacterial strain SB_13 or the commercial effective microorganisms (EM·1®) (a combination of approximately 80 different microorganisms developed by Professor Teruo Higa in the 1970s at the Ryukyus University, Okinawa, Japan). The filtrate was then aerated using a high efficiency low pressure blower and a medium-bubble diffuser assembly for 24h. Also, in order to test the efficiency of strain SB_13 for wastewater treatment, the wastewater samples were inoculated directly with strain SB_13 or the commercial effective microorganisms (EM·1®) without any chemical precipitation treatment.

#### 2.4.1 Analyses of treated wastewater

The values of pH, color, TDS (Total Dissolved Solids), TSS (Total Suspended Solids), COD (Chemical Oxygen Demand) and BOD (Biological Oxygen Demand) were measured for wastewater samples before and after the different treatments. The measures were carried out according to the standard methods recommended by Barbour et al., [18] and American public health association, [19].

### 2.5 Cytotoxicity Test

Cytotoxicity of wastewater samples which treated or untreated with bacterial inoculations was determined through the cytological changes in the normal cells of onion plant (*Allium cepa*).

Different treatments were: Control distilled water without bacterial inoculation (Ctrl), and dye

wastewater without treatment (Dy). Wastewater treated with: Chemical treatment (dy_Ch); Strain SB_13 plus chemical (Dy_Ch_SB13), EM plus chemical (Dy_Ch_EM), strain SB_13 (Dy_SB13), and EM (Dy_EM), only chemical solution (Ch). Seeds were placed in the distilled water for 2 hours and put on wet filter paper in Petri-dishes and kept in darkness at 23–25ºC. After seed germination, seeds treated with different treatments and root tips were carefully cut and used for cytological preparation. Control seeds were treated with distilled water.

The root tips (1-2 cm) of control and treated seeds were fixed in a freshly prepared Carnoy`s fixative (3:1 v/v absolute alcohol:glacial acetic acid) and kept in refrigerator for 24 hours. They were then stored at 4ºC in 70% ethyl alcohol as a preservative solution for cytological studies. Cytological preparations were carried out using the Feulgen squash technique [20] as the following: Root tips of onion were thoroughly washed in distilled water and hydrolyzed in 1N HCl at 60ºC for 4-6 minutes. Root tips were washed carefully with distilled water and stained with Fulgen reagent for 2 hours [21].

The deeply stained terminals of root tips were squashed in a drop of 45% acetic acid on a clean slide. After covering with cover slip and flattening of the plant material, the preparation was sealed with a ring of rubber solution. The slides were microscopically examined for counting and photographing by using XSZ-N 107 Research Microscope Fitted with Premiere MA88-900 Digital Camera.

Mitotic indices were determined using the following equation:

> MI = (Number of dividing cells in all phases of mitosis / Total number of counted cells) X 100.

The frequency of the total abnormalities of mitosis was calculated using the following equation:

> Abnormalities of mitosis (%) = (Total number of abnormal cells / Total number of dividing cells) X 100.

### 2.6 Statistical Analysis

Data were statistically analyzed by SPSS v17, one-way and two way analyses of variance (ANOVA) test were used for multiple sample comparison, when normality and homogeneity of variance were satisfied, followed by multiple comparison Tukey test.

## 3. RESULTS

The molecular identification based on 16S rRNA gene amplification showed that the bacterial strain (SB_13) had 97% similarity to *Pseudomonas stutzeri* with GenBank accession numbers of EU531806. The topology of *P. stutzeri* strain SB_13 to gammaproteobacteria was retrieved from the phylogenetic tree including various bacterial 16S rRNA gene sequences of the common bacterial families (Fig. 1). The sequence was obtained from the present study deposited in GenBank under accession number of KU053821.

The highest dye color removal rates by strain SB_ 13 were found at dye concentration of 50 ppm; where 61.7% of Disperse Blue (R16), 42% of Disperse Yellow (D4), and 28.03% of Reactive Red Synozol (R4), were removed by strain SB_13 after 72h-96h. Dyes color removing significantly decreased at dye concentrations above 50 ppm, where dye concentrations of 200-250 ppm were hardly removed by strain SB_13. Physical degradation of azo dyes was found in control treatment. The highest dyes concentration which could be decolorized by strain SB_13 was 150 ppm, where 26.9% of Disperse Blue (R16), 12.1% of Disperse Yellow (D4), and 12% of Reactive Red Synozol (R4), were decolorized (Fig. 2). Thus, dye concentration of 150 ppm was selected for further analyses in the present study.

The results showed that shaking condition is better than static condition for dye decolorization. The highest color removal of Disperse Blue (R16) (21.2% after 72h), Disperse Yellow (D4) (32.1% after 72h), and Reactive Red Synozol (R4) (9.1% after 96h) dyes by strain SB_13 were found under shaking conditions compared to those (13% of Disperse Blue (R16), 18.8% of Disperse Yellow (D4), and 4.3% of Reactive Red Synozol (R4)) observed under static conditions (Table 1).

Different pH values significantly affected the decolorization rates, where the maximum Disperse Blue (R16) dye decolorization of 23% was recorded at pH 6.0 after 72h. While the highest decolorization of Disperse Yellow (D4) (30.9% after 72h) and Reactive Red Synozol

**Table 1. Effect of static and shaking conditions on azo dyes decolorization by bacterial strain SB_13**

| Isolate code | Decolorization (%) of Disperse Blue (R16) | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Static time (hrs) | | | | | | | Shaking time (hrs) | | | | | | |
| | 12 | 24 | 36 | 48 | 60 | 72 | 96 | 12 | 24 | 36 | 48 | 60 | 72 | 96 |
| Ctrl | 3.59±0.24 | 6.33±0.26 | 7.04±.25 | 7.30±0.15 | 7.83±0. 11 | 8.26±0.10 | 8.57±0.22 | 2.11±0.16 | 2.54±0.03 | 7.06±0.0 9 | 8.95±0.05 | 11.12±0.15 | 13.41±0.15 | 15.95±0.22 |
| SB_13 | 4.20±0.57 | 5.41±0.07 | 8.11±.39 | 9.24±0.14 | 10.73± 0.17 | 13.09±0.45 | 13.09±0.49 | 1.96±0.42 | 3.94±0.49 | 9.85±0.55 | 8.08±0.40 | 9.88±0.37 | 21.17±0.67 | 18.00±.87 |
| | Decolorization (%) of Disperse Yellow (D4) | | | | | | | | | | | | | |
| | Static time (hrs) | | | | | | | Shaking time (hrs) | | | | | | |
| | 12 | 24 | 36 | 48 | 60 | 72 | 96 | 12 | 24 | 36 | 48 | 60 | 72 | 96 |
| Ctrl | 2.39±0.07 | 3.60±0.20 | 3.73±0.05 | 3.94±0.06 | 4.36±0 .13 | 4.61±0.11 | 5.57±0.30 | 1.59±0.09 | 6.08±0.57 | 6.83±0. 18 | 9.05±0.47 | 9.60±0.26 | 11.11±0.52 | 12.07±0.41 |
| SB_13 | 5.18±0.21 | 7.20±0.18 | 14.94±0.05 | 15.62±0.44 | 14.94±0.05 | 18.77±0.58 | 17.88±0.38 | 11.01±0.16 | 13.98±0.19 | 21.93±0 .34 | 16.90±0.43 | 21.93±0.34 | 32.07±1.08 | 32.07±1.08 |
| | Decolorization (%) of Reactive Red Synozol (R4) | | | | | | | | | | | | | |
| | Static time (hrs) | | | | | | | Shaking time (hrs) | | | | | | |
| | 12 | 24 | 36 | 48 | 60 | 72 | 96 | 12 | 24 | 36 | 48 | 60 | 72 | 96 |
| Ctrl | 0.39±0.04 | 1.22±0.04 | 1.43±0.04 | 1.57±0.10 | 2.29±0 .14 | 2.54±0.13 | 3.29±0.15 | 0.57±0.72 | 1.47±0.04 | 2.61±0. 13 | 3.29±0.03 | 3.43±0.10 | 4.25±0.13 | 4.51±0.06 |
| SB_13 | 0.82±0.001 | 2.33±0.09 | 2.81±0.09 | 3.36±0.18 | 3.32±0.17 | 4.42±0.20 | 4 .31±0.21 | 2.05±0.003 | 7.92±0.21 | 6.45±0.05 | 5.60±0.11 | 7 .68±0.24 | 8.06±0.23 | 9.08±0.26 |

*Different letters between columns denote that mean values are significantly different (p≤0.05) by Tukey LSD test, means ± SE (n=3). Control without bacterial inoculation (Ctrl), and Pseudomonas stutzeri strain (SB_13)*

(R4) (7.7% after 96h) dyes were measured at pH 5.0 (supplementary supporting information, Table 1S). Variation in temperature range resulted in significant differences in dyes color remove. The optimal temperature of 35ºC was observed to reach the best decolorization rate of Disperse Blue (R16) (23.6% after 72h), Disperse Yellow (D4) (29% after 72h), and Reactive Red Synozol (R4) (5.2% after 96h). The results showed that any increase or decrease in temperature than the optimum degree leaded to reduce the bacterial activity of dyes color removal (supplementary supporting information, Table 2S). The bacterial inoculation size of 3% (v/v) significantly caused the highest dyes decolorization of Disperse Blue (R16) (26% after 72h), Disperse Yellow (D4) (34.8% after 72h), and Reactive Red Synozol (R4) (12.2% after 96h) (supplementary supporting information, Table 3S).

The data presented in (supplementary supporting information, Table 4S) revealed that glucose and sucrose were the best carbon sources for dyes decolorization by strain SB_13. Glucose and sucrose significantly increased the decolorization of Disperse Blue (R16) (25% and 23.7%), Disperse Yellow (D4) (35.3% and 33.8%), and Reactive Red Synozol (R4) (12.2% and 10.3%), respectively. Without the carbon source addition, the decolorization of Disperse Blue (R16), Disperse Yellow (D4), and Reactive Red Synozol (R4) dyes were 2.3%, 12%, and 2.8%, respectively. At the absence of nitrogen source, the Disperse Blue (R16) (8% after 72h), Disperse Yellow (D4) (11.5% after 72h), and Reactive Red Synozol (R4) dyes (5.6% after 96h) were decolorized by strain SB_13. Addition of ammonium sulfate as a nitrogen source significantly increased the dyes color removing, where strain SB_13 decolorized 27.4% of Disperse Blue (R16), 36.6% of Disperse Yellow (D4), and 5.6% of Reactive Red Synozol (R4) dyes. Moreover, yeast extract caused that strain SB_13 decolorized about 27.8% of Disperse Blue (R16) after 72h, 35.6% of Disperse Yellow (D4) after 72h, and 9.2% of Reactive Red Synozol (R4) after 96h. Also, ammonium nitrate and sodium nitrate addition resulted in strain SB_13 removed 26.4% and 36.1% of Disperse Yellow (D4) dye, respectively (supplementary supporting information, Table 5S). The summary of data which showed the decolorization rates by strain SB_13 before and after optimization of the inoculated conditions are presented in (Table 2).

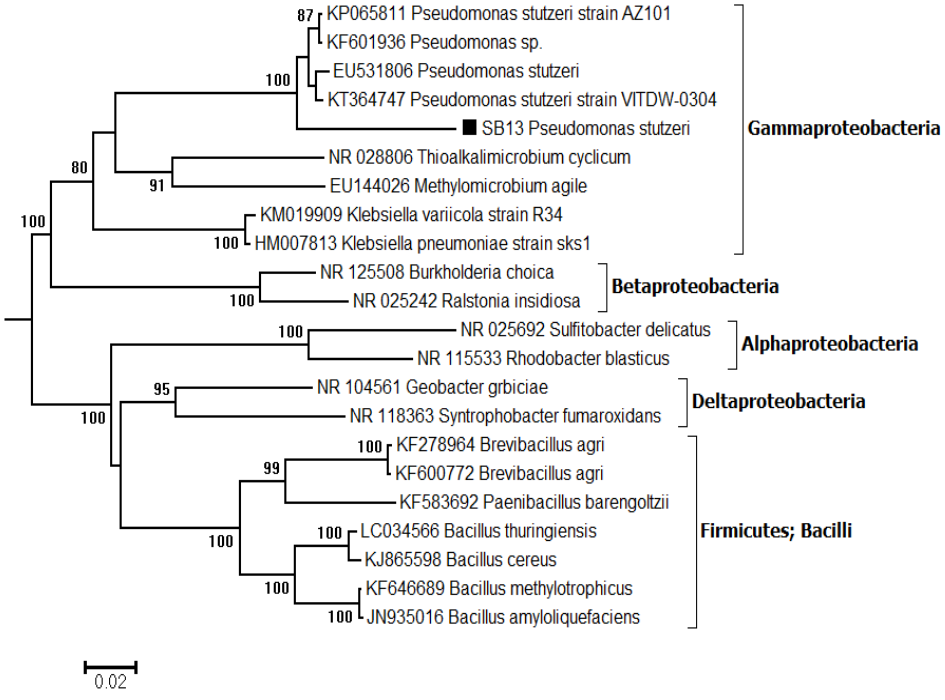


**Fig. 1. Phylogenetic analysis of 16S rRNA sequences of *P. stutzeri* strain (SB_13) with the sequences from NCBI. Symbol ■ refers to 16S rRNA gene fragments retrieved from this study. The analysis was conducted with MEGA6 using neighbor-joining method. Bootstrap value (1000 replicates)**

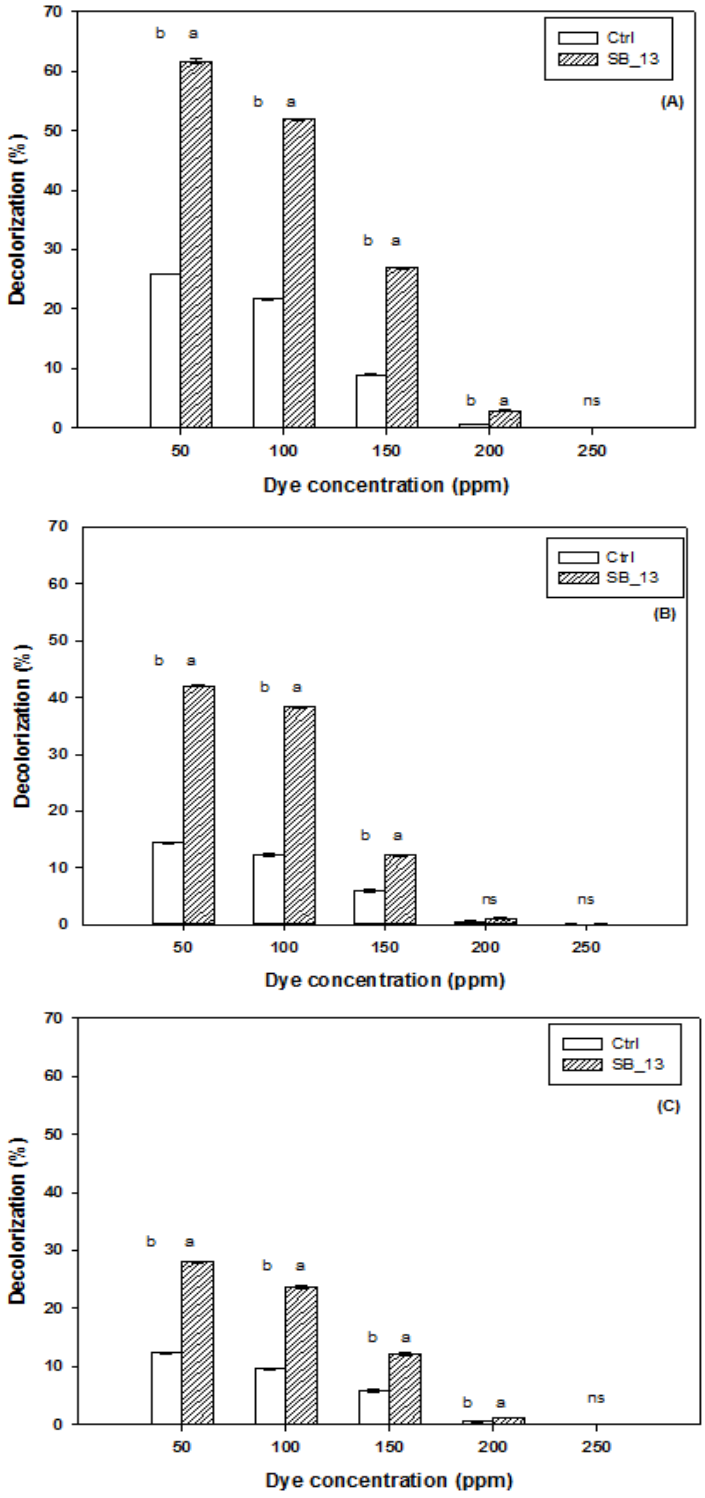


**Fig. 2. Effect of different dye concentrations on azo dye decolorization by *P. stutzeri* strain (SB_13). (A) Disperse Blue dye (R16) at 72h; (B) Disperse Yellow dye (D4) at 72h; (C) Reactive Red synozol dye (R4) at 96h. Ctrl, control without bacterial inoculation.**

*Error bars are means ± SE (n=3). Different letters on bars denote that mean values are significantly different (p≤0.05) by Tukey LSD test. ns, not significantly different*

**Table 2. Azo dyes decolorization by bacterial strain SB_13 before and after the optimization of incubation conditions**

| Isolate code | Decolorization (%) of Disperse Blue (R16) | |
|---|---|---|
| | **Before optimization** | **After optimization** |
| Ctrl | 2.54±0.03 | 15.1±0.39 |
| SB_13 | 3.94±0.49 | 41.6±0.59 |
| | **Decolorization (%) of Disperse Yellow (D4)** | |
| | **Before optimization** | **After optimization** |
| Ctrl | 6.08±0.57 | 5.1±0.21 |
| SB_13 | 13.98±0.19 | 39.9±0.08 |
| | **Decolorization (%) of Reactive Red Synozol (R4)** | |
| | **Before optimization** | **After optimization** |
| Ctrl | 1.47±0.04 | 4.4±0.19 |
| SB_13 | 7.92±0.21 | 12.1±0.1 |

*Incubation shaking condition were at pH value of 7.0, 37 ºC, and inoculum size of 1% (v/v) before optimization, and were at pH value of 5.0-7.0, 35 ºC, and inoculum size of 3% (v/v) after optimization. Control without bacterial inoculation (Ctrl), and Pseudomonas stutzeri strain (SB_13)*

The main experiments were conducted under the optimal conditions mentioned above: dye concentration of 150 ppm, pH of 5-7, temperature of 35ºC, and 3% (v/v) inoculum size of individual strain SB_13 or bacterial consortiums of *P. stutzeri* strain (SB_13) and *Klebsiella* strains of (*Klebsiella pneumoniae (Kp) and Klebsiella variicola (Kv*)). After 36h, the analysis of variance showed that the maximum decolorization (59.5%) of Disperse Blue (R16) dye was observed by bacterial consortium of SB_13& Kp compared to color removal of 41.6% caused by individual inoculation by strain SB_13. In the case of Disperse Yellow (D4) dye and after 72h, bacterial mixed culture of SB_13 & Kv significantly decolorized 52% of the dye higher than 39.9% removed by the strain SB_13. Moreover, ANOVA data analysis revealed that inoculation by strain SB_13 significantly decolorized 12.1% of Reactive Red Synozol (R4) dye lower than those caused by bacterial consortium of SB_13 & Kp & Kv which decolorized 18.3% of dye after 96h (Fig. 3). By using the three azo dyes in a mixture and after 72h, the analyses of variance showed that the maximum decolorization (61%) of dyes mixture was reported by inoculation with individual strain SB_13, followed by decolorization of 44.8% which caused by inoculation with bacterial consortium of SB_13 & Kp. The minimum decolorization (2%) of dyes mixture was observed in control treatment without bacterial inoculation, where bacterial consortium of SB_13 & Kp & Kv caused the color removal (28.6%) of dyes mixture (Fig. 4).

Data analyses showed that the highest decolorization percentage (53.5%) of dying wastewater was found after chemical plus bacterial treatment by strain SB_13. Moreover, the lowest values of BOD and COD were found for wastewater treated with chemical plus strain SB_13; While, the highest values of TDS and TSS were recorded for the same treatment (Table 3).

The effect of dye wastewater on mitotic index (MI%) of cell division in root rips of *A. cepa* indicated that wastewater sharply reduced MI (0.1%), where inoculation by strain SB_13 plus chemical treatment caused significant increase in MI (4.4%) followed by MI of 7.7% which was observed in control samples treated with distilled water (Fig. 5). On the other hand, the cytotoxicity test showed that dye wastewater treatment fully caused 100% of abnormal mitotic cell division compared to control distilled water treated samples in which the 2.6% of abnormal mitosis was observed. Interestingly, inoculation of wastewater by strain SB_13 plus chemical treatment significantly reduced mitotic abnormality to 23.6% compared to abnormalities of 56.6% and 35.8% was found by chemical treatment and EM plus chemical treatments, respectively (Fig. 5). Some of the abnormal mitotic cell division of *A. cepa* meristematic cells which caused by wastewater treatments are shown in (Fig. 6).

## 4. DISCUSSION

At the present study, it was indicated that the decolorization of the different azo dyes was depend on the dye concentration, where the highest decolorization rates by *P. stutzeri* (SB_13) was observed at dye concentration of

50 ppm in liquid medium. The decolorization rate of these azo dyes reduced when the dye concentration increased above 50 ppm in the media. The high dye concentrations are toxic to bacterial strain SB_13 and minimize the bacterial dye decolorization; Thus, it is suggested that dye concentrations above 50 ppm inhibit bacterial growth and its enzymatic activities. The results presented in this study showed that the dye concentration of 150 ppm was the sub lethal dose which inhibited the bacterial activity of SB_13 for dye decolorization; Therefore, that dye concentration was chosen for further analyses. Hassan et al. [13] observed that when dye concentration increased from 50 ppm to 250 ppm, the capacity of *Klebsiella* species for dyes removal was significantly decreased. The dye structure and concentration in addition to the site of bacterial isolation significantly contribute to the decolorization effectiveness [22]. The *Pseudomonas fluorescens* strain SDz3 decolorized 90% of crystal violet and brilliant green dyes only at the concentration of 0.05 g $L^{-1}$.

The results showed that shaking condition significantly increased biodegradation by SB_13 strain; moreover, color removal under shaking condition was higher than that found in static condition. Shaking increases oxygen dissolving in the medium, facilitates oxygen transfer between the medium and bacterial cell, and introduces better chance for dye molecules contact with the bacterial cell. These results suggest that strain SB_13 prefers aerobic status and high oxygen concentration for azo dye decolorization. Similarly, Hassan et al. [13] found that the higher ability of *Klebsiella* strains for dye decolorization under shaking condition compared to static condition indicates that shaking status is favourable to enhance bacterial biomass and oxygen transfer between bacterial cells and the surrounding substrates. Lade et al. [9] reported that *Bacillus* spp. decolorized azo dyes in the presence of oxygen and found that decolorization of azo dyes increased by the oxidation process of bacterial cells. Bacterial enzymatic degradation and dye molecules adsorption on bacterial cell wall are the mechanisms responsible for microbial dye decolorization [12]. The shaking condition may indicate the biosorption mechanism achieved for bacterial dye decolorization [23].

The pH and temperature importantly affect the bacterial cell growth, enzymatic action, and overall the physiological activities; Therefore, optimization of these factors significantly influence on bacterial capacity for dye decolorization. At the present study, the pH 5-7 was the best value which potentially caused the highest azo dyes decolorization by SB_13, and any shift in the pH from the optimum pH range (5-7) significantly decrease the decolorization performance by SB_13. Prasad et al. found that *Bacillus endophyticus* strain VITABR 13 had high ability to decolorize azo dyes at a wide range of pH (6-9). Singh et al. [24] observed that the pH range of 6-8 was the best value for acid orange decolorization by *Staphylococcus hominis* RMLRT03. High acidic or alkaline pH values minimized dye decolorization by bacterial isolates of *Klebsiella* [13]. Phugare et al. [25] recorded that the maximum decolorization of azo dye Red HE3B by a bacterial consortium consisting of *Providencia* sp. SDS and *Pseudomonas aeuroginosa* strain BCH was found at pH 7.0. The pH actually affects the transportation of dye molecules through the bacterial cell membrane and subsequently relates to decolorization capacity [26]. The maximum azo dyes decolorization rate was found at 35ºC, while the temperature above 35ºC caused the minimum decolorization percentage. The minimum decolorization percentage which was observed at high

**Table 3. Decolorization of wastewater under different treatments**

| Treatments | Decolorization (%) | pH | Measurements (mg/L) | | | |
|---|---|---|---|---|---|---|
| | | | BOD | COD | TDS | TSS |
| Dy | 4.5 | 6.5 | 100 | 262 | 205 | 20 |
| dy_Ch | 48 | 3.6 | 45 | 131 | 804 | 240 |
| Dy_Ch_SB13 | 53.5 | 3.6 | 20 | 100 | 925 | 260 |
| Dy_Ch_em | 50.6 | 3.6 | 35 | 118 | 1110 | 340 |
| Dy_SB13 | 11.4 | 6.5 | 50 | 145 | 238 | 60 |
| Dy_em | 6.5 | 6 | 95 | 171 | 455 | 100 |

*Wastewater without treatment (Dy).Wastewater treated with: chemical treatment (dy_Ch); strain SB_13 plus chemical (Dy_Ch_SB13), EM plus chemical (Dy_Ch_em), strain SB_13 (Dy_SB13), and EM (Dy_em). BOD, biological oxygen demand; COD, chemical oxygen demand; TDS, total dissolved solids; TSS, total suspended solids*

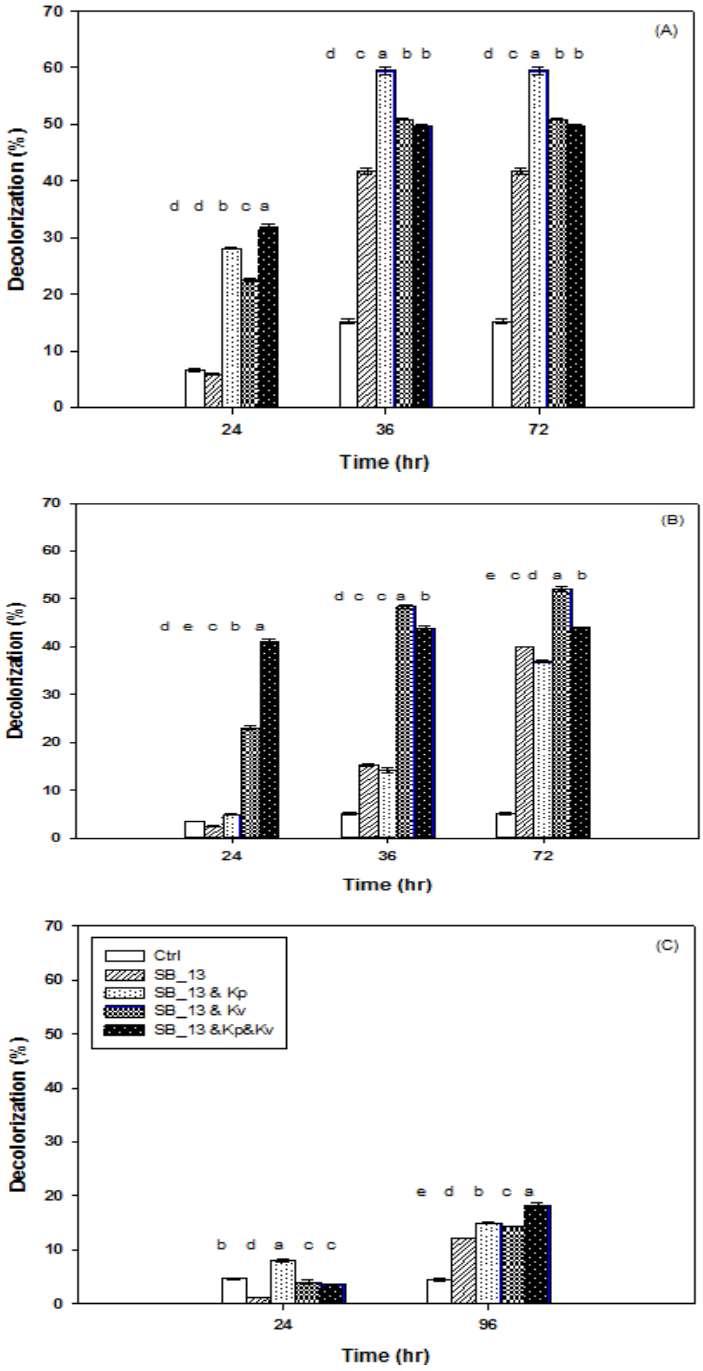


**Fig. 3. Effect of individual strain of SB_13 and bacterial consortiums inoculation on azo dyes decolorization. A, Disperse Blue dye (R16); B, Disperse Yellow dye (D4); C, Reactive Red synozol dye (R4). Ctrl, control without bacterial inoculation; SB_13 strain of *P. stutzeri*; Kp strain of *Klebsiella pneumoniae*; Kv strain of *Klebsiella variicola*.**

*Error bars are means ± SE (n=3). Different letters on bars denote that mean values are significantly different (p≤0.05) by Tukey LSD test*

temperature might be explained by thermal inhibition of bacterial activity for dye decolorization. The effect of high temperature on microbial dye decolorization and degradation

might be because the suppress of bacterial enzymes which are responsible for azo dyes degradation [27,28]. The results presented here showed that the optimal inoculum size of 3% (v/v) of bacterial strain SB_13 was required to decolorize the highest amount of different azo dyes. Similarly, Hassan et al. [13] showed that *Klebsiella* spp inoculum size of 3% (v/v) significantly increased the decolorization of azo dyes, while Mohan et al. [10] who reported that decolorization percentage of Coractive Blue P-3R was the highest at 10% inoculum size of *Bacillus* sp. and *Planococcus* sp.

Azo dyes are imperfect sources for carbon and nitrogen; therefore, bacterial degradation of azo dyes mainly activated in the presence of carbon and nitrogen sources. Glucose and sucrose were the best preferable carbon sources utilized by SB_13 to achieve the maximum decolorization performance, in contrast to other carbon sources such as starch, fructose, maltose, molass, and bagasse which reduced the azo dyes degradation. In addition, color removal of azo dyes by SB_13 was potentially activated in the presence of glucose or sucrose compared with the absence of these carbon sources. In this study, it is assumed that glucose and sucrose equip favourable conditions for bacterial growth, enzymatic activity, as well azo dyes degradation by SB_13. Mohan et al. [10] reported that glucose caused decolorization of azo dyes by *Bacillus* sp higher than those found with mannitol addition; At the same line, Wang et al. [27] observed that the highest decolorization rate of reactive red 180 dye by *Citrobacter* sp was found with glucose supplementation (4 g/L). The highest decolorization percentage of azo dye disperse Orange S-RL by *Acinetobacter sp*. SRL8 was observed under 0.2% of glucose concentration. Moreover, SB_13 strain preferred to utilize ammonium sulfate and yeast extract as nitrogen sources and exhibited the maximum azo dyes degradation. Hassan et al. [13] indicated that the enhancement of azo dyes decolorization by *Klebsiella* species required the addition of carbon and nitrogen sources.

Obviously, it was recorded that the two species of *Klebsiella* had ability to decolorize azo dyes [13]; Therefore, the bacterial consortium including SB_13, Kp and Kv has been conducted and subjected for dye decolorization of different azo dyes with multi comparison analyses to each individual or mixed bacterial culture under previously optimum conditions (pH, temperature, inoculation size, and dye concentration) and at time course of 24-96 hrs. Our data multi-comparison revealed that the bacterial mixed culture is better than individual SB_13 strain for azo dyes decolorization capacity. For example, mixed culture of SB_13 &Kp, SB_13 &Kv,

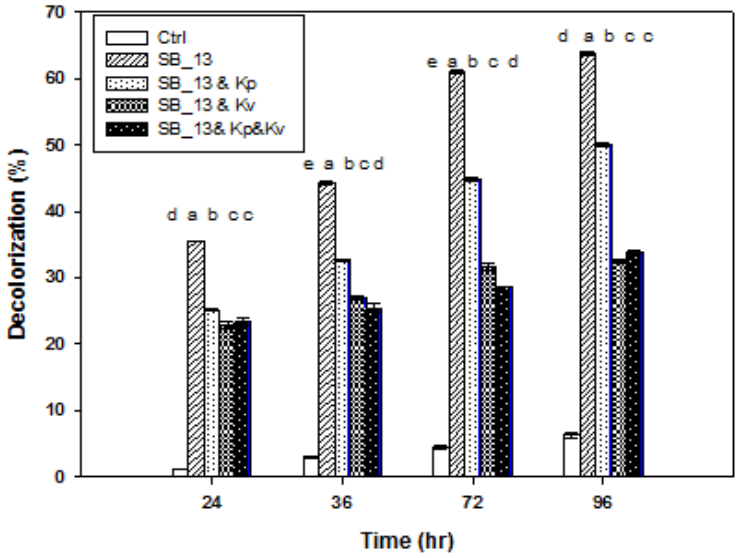


**Fig. 4. Effect of bacterial inoculations on decolorization of azo dyes mixture. Ctrl, control without bacterial inoculation; SB_13 strain of *P. stutzeri*; Kp strain of *Klebsiella pneumoniae*; Kv strain of *Klebsiella variicola***

*Error bars are means ± SE (n=3). Different letters on bars denote that mean values are significantly different (p≤0.05) by Tukey LSD test*

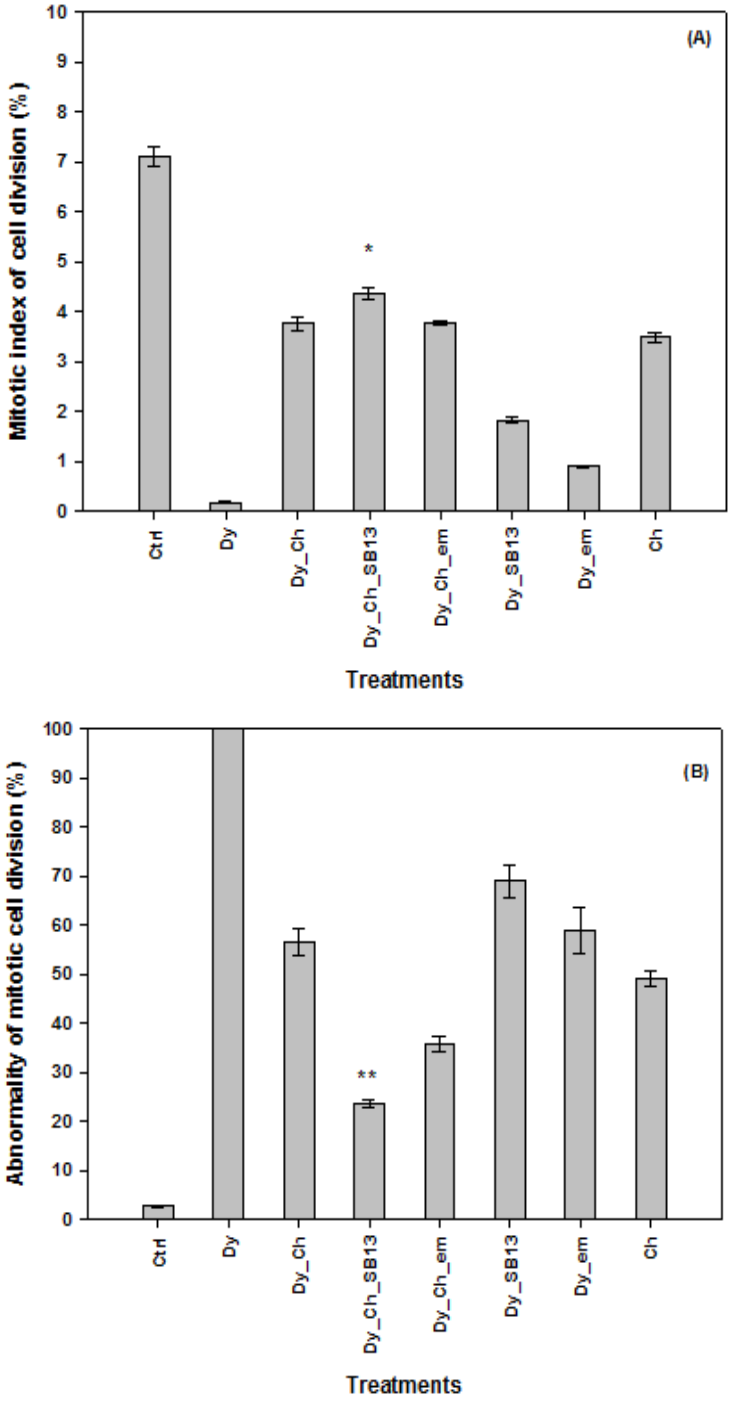


**Fig. 5. Phytotoxicity test; (A) effect of treatments on mitotic index of cell division (%); (B) effect of treatments on abnormality of mitotic cell division (%). Control distilled water without bacterial inoculation (Ctrl), and dye wastewater without treatment (Dy). Wastewater treated with: chemical treatment (dy_Ch); Strain SB_13 plus chemical (Dy_Ch_SB13), EM plus chemical (Dy_Ch_em), strain SB_13 (Dy_SB13), and EM (Dy_em). Only chemical solution (Ch).**
*Error bars are means ± SE (n=3). * on bars denote that the best treatment which significantly (p≤0.05) increased mitotic index % compared to wastewater without treatment (Dy). ** on bars denote that the best treatment which significantly (p≤0.05) decreased abnormalities of mitosis % compared to wastewater without treatment (Dy).*

and SB_13 & Kp&Kv significantly decolorized (59.5%) of Disperse blue (R16), (52%) of Disperse yellow (D4), and (18.3%) of Reactive red synozol (R4) dyes, respectively more than those found by individual SB_13 (which decolorized 41.6% of Disperse blue (R16), 39.9% of Disperse yellow (D4), and 12.1% of Reactive red synozol (R4)) after 72-96 hr.

The enhancement of azo dye decolorization and degradation which are preceded by bacterial consortium in contrary to individual strains may be explained by the synergistic interaction of individual bacterial strains in the mixed culture. Moreover, further biodegradable action can be occurred through the metabolites resulted from the activity of one strain which can be utilized by other strains in mixed culture [29]. Each individual bacterial strain has its own morphological and physiological characters (such as: Cell wall composition, spore formation, capsule presence, and enzymes activity) which control the tolerance of bacterial strain to xenobiotic conditions as well dye removal effectiveness. In the bacterial consortium, various routes can be found to illustrate the enhancement of dye decolorization and degradation; for instance, each individual bacterial strain can differentially tolerate, sequestrate, immobilize, adsorb, or precipitate dye molecules into different cellular locations [23,30,31,32]. For example, *Pseudomonas* strains showed better tolerance to higher brilliant green concentrations than *Klebsiella* strains [33].

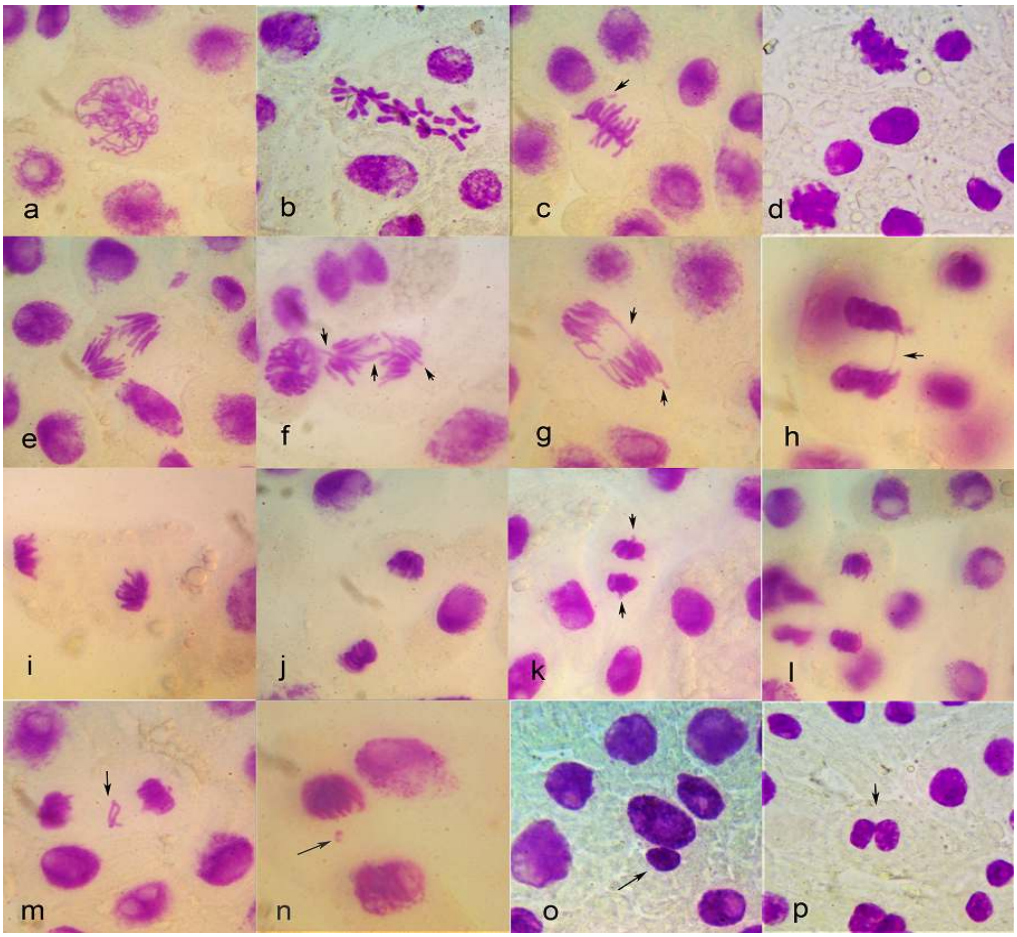


**Fig. 6. Abnormalities of mitotic cell division of *A. cepa* plant were obtained by dyeing wastewater. (a) Irregular prophase, (b) C-metaphase, (c) metaphase with fragment, (d) sticky metaphase, (e) tripolar anaphase, (f, g) anaphase with bridges and forward chromosomes, (h) sticky anaphase with single bridge, (i) diagonal anaphase, (j) diagonal telophase, (K) telophase with forward chromosomes (l) sticky telophase, (m) telophase with lagging chromosome (n) telophase with fragment, (o) micronucleus, (p) binucleated cell**

Little is known about the decolorization efficacy of bacterial strains for mixtures of azo dyes. The capacity of SB_13 for decolorization of azo dyes mixture was estimated due to textile dye effluents contain various dyes and in order to evaluate the efficiency of SB_13 in wastewater treatment. In the present study, multi-comparison analyses revealed that decolorization of azo dyes mixture by bacterial inoculation after 72hrs was significantly increased in the following order: Control < SB_13 & Kp & Kv < SB_13 & Kv < SB_13 & Kp< SB_13 which decolorized 2.2%, 28.7%, 31.7%, 44.9%, and 61.1%, respectively. Our results showed that when the dyes are in a mixture, the dyes removal by strain SB_13 was significantly increased. Variation in dye decolorization and degradation is based on the difference in physical and chemical dye composition in a mixture [34]. Zabłocka-Godlewska et al. [12] observed that the dye structure was changed in a mixture and confirmed that using UV-Vis scan of the dyes. Previously, the highest decolorization percentage (50%) of azo dyes mixture was achieved by *Klebsiella* species [13]. Differences in color removal of dye mixture and individual dyes were previously reported for different bacterial and fungal strains [12,35]. The ability of SB_13 for color removal of azo dyes mixture (61%) suggests the potentially applicable role of SB_13 in biological treatment of dyeing industrial wastewater.

The phytotoxicity test was conducted on the meristematic cells of *Allium cepa*, where the mitotic index (normal cell division percentage) and mitotic abnormalities (abnormal cell division percentage) were estimated. At the present study, the tested wastewater was highly toxic to root tip cells of *A. cepa*, where it caused the completely inhibition of mitotic cell division of *A. cepa* meristematic cells. It was observed that the maximum reduction of mitotic index of meristematic cells (0.17% MI) and the production of the highest value of chromosomal aberrations (100%) was found after the treatment of *A. cepa* root tips with wastewater for 24h. The inhibition of mitotic cell division could be attributed to: blocking of mitotic cycle during interphase or at early prophase [36], increasing the number of non-dividing cells [37], and retarding the entrance of cells into the mitotic division by a marked prolongation of the cell cycle [38]. In addition, another reason to suppress mitotic cell division is inhibition the formation of various metabolic events which necessary for mitosis [37]. Interestingly, the treatment of wastewater with SB_13 strain combined with chemical treatment significantly reduced the phytotoxicity of wastewater as compared with other treatments. At the present study, inoculation with SB_13 strain plus chemical treatment caused the lowest abnormal cell division index (23.6%), followed by 35.7% value which was found after EM_chemical treatment, in contrast to wastewater without any treatment which fully inhibited the mitotic cell division (with 100% of abnormalities). The results presented here suggest that inoculation with SB_13 strain potentially integrate to biological transformation of toxic compounds in the tested wastewater to less toxic products through the biodegradation or sorption processes. *Pseudomonas* strain SDz3 decreased the zootoxicity of Evans blue dye under static or shaking conditions, and the phytotoxicity only reduced in the static condition [22]. The decolorization of dyes by *Klebsiella* strain Bz4 significantly decreased the phytotoxicity of the dye mixture, while the color removal by *Klebsiella* strain Rz7 did not change the phytotoxicity of dye mixture [12]. Bacterial decolorization of TPM methyl red acuity decreased the phytotoxicity, dye color removal by bacterial treatment resulted in 90% *Brassica pekinensis* seed germination compared to no seed germination was observed for control dye without bacterial treatment [1].

## 5. CONCLUSION

*Pseudomonas stutzeri* strain of SB_13 which was isolated from textile dyeing effluent has potential capacity for decolorization of different azo dyes as single dyes or in mixtures. The maximum decolorization rate was recorded for Disperse Blue (R16) and the lowest color removal percentage was found with Reactive Red Synozol (R4) dye. Dyes concentration above 50 ppm apparently reduced the capacity of SB_13 strain for azo dyes decolorization. Under shaking conditions and at dye concentration of 150 ppm, the best decolorization rates of single or mixtures of azo dyes were observed with glucose or sucrose, ammonium sulfate, and 3% (v/v) bacterial inoculum size, at pH of 5-7, temperature of 35ºC, and after 72-96 hrs. Individual *P. stutzeri* strain of SB_13 showed decolorization capacity of azo dyes mixture higher than those observed by bacterial consortium contains *P. stutzeri* strain of SB_13 and *Klebsiella* strains of Kp and Kv. While, the best color removal rates of single azo dyes was conducted by bacterial consortium compared with individual SB_13 strain. The cytotoxicity of

wastewater decreased in samples treated with strain SB13_chemical compared with other treatments or untreated wastewater samples. The decolorization effectiveness of SB_13 strain incorporated with its ability for detoxification of wastewater samples suggests the applicable role of SB_13 strain in wastewater treatment at large scale and the possibility of its application for decolorizing the effluents resulted from textile dyeing industries.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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