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Biodegradation of Cibacron Redazo Dye and Industrial Textile Effluent by *Pseudomonas aeruginosa* Immobilized on Chitosan-Fe_2O_3 Composite

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Authors' contributions

This work was carried out in collaboration between all authors. Author NAHF designed the study, wrote the protocol, performed the biodegradation experiments and interpreted the data. Author AZAA performed the chemical analysis and interpreted the data. Author IMI identified the bacterial strain using molecular methods. Author TMS synthesized and characterized the magnetic nanoparticles. All authors managed the literature searches and produced the initial draft. All authors read and approved the final manuscript.

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Original Research Article

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ABSTRACT

A bacterial strain (*Pseudomonas aeruginosa*) having a high capacity for rapid decolourization of the azo dye Cibacron Red was isolated and identified by 16s-rRNA gene sequencing method. The decolorization of cibacron reactive red by free cell under experimental conditions was investigated. At initial dye concentration of 100 ppm, the maximum decolorization percentage (89%) was

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achieved at pH7, 37ºC under static conditions after 72 h of incubation. The bacterial cells were immobilized on chitosan-magnetite nanoparticles by using glutaraldehyde as a cross-linker. The immobilized cells were used for decolorization of both reactive red dye solution and textile industrial wastewater; a complete decolorization (100%) was achieved after 2 h and 12 h for each treatment, respectively. The dye biodegradation products were identified by GC/MS analysis and determination of laccase, peroxidase and dioxygenase enzyme activities in the bacterial supernatant. The obtained results from this work are expected to be a useful reference for development of effective decolorization bioprocesses utilizing immobilized bacterial cells as a biocatalyst and also to demonstrate feasible operation strategies to utilize the immobilized-cell as a fixed bed for efficient decolorization of dye-laden wastewaters.

Keywords: Pseudomonas aeruginosa; Cibacron Red; azo dye; textile wastewater; biodegradation.

1. INTRODUCTION

Contamination of soil, air, water, and food is one of the major problems facing the world today. The textile industry is one of them, which extensively use synthetic chemicals and dyes. Wastewaters from textile industries pose a threat to the environment, where large amount of chemically diverse dyes are used. A significant proportion of these dyes enter the environment via wastewater. Approximately 10,000 different dyes and pigments are used industrially and over 0.7 million tons of synthetic dyes are produced annually, worldwide [1]. A wide variety of azo dyes with anthraquinone, polycyclic and triphenylmethane groups are being increasingly used in textile dyeing and printing processes. They introduce toxicity in the form of genotoxicity and mutagenicity for aquatic organisms and bacteria as well as humans [2]. Therefore, physico-chemical, physical and biological methods have been investigated to overcome these problems. The physico-chemical methods have the limitations of high operational costs and generate large quantity of sludge for disposal; while the biological treatments have a lower cost.

The most generally accepted mechanism of azo reduction is the participation of redox mediators, acting as electron shuttles between the extra cellular dye and the intracellular redox enzymes. The microbial decolorization can be performed by anaerobic, aerobic or aerobic-anaerobic sequential treatments [3]. Both free and immobilize cells and enzymes can be used [4-8]. Nano-material's, typically range in size from 1 to 100 nm, and the application of nano-materials for environmental remediation is promising.

The application of nano zero valent iron on textile dye bioremediation is receiving great attention in the recent years because nZVI particles are highly reactive towards the pollutant, less toxic, and economical [9].

In the past several decades, magnetic nanoparticles of iron oxides (Fe_3O_4 NPs) have attracted much research interest due to their potential applications in magnetic storage, catalysis, electrochemistry, drug delivery, medical diagnostics, and therapeutics based on their unique magnetic, physiochemical, and optical properties [10]. Apparently, the magnetic nanoparticles possess the advantages of large surface area, high number of surface active sites and magnetic properties, which lead to high adsorption efficiency, removal rate of contaminants, easy and rapid separation from solution via magnetic field. Among the kinds of nano-sized iron oxides, magnetic iron oxides such as magnetite (Fe_3O_4) and maghemite (γ-Fe_2O_3) have been investigated intensively for environmental and bio-applications [11]. Hyunwoong and Wonyong [12] demonstrated photochemical remediation method for dye-polluted waters by using Fe^{+3} and visible light only. Acid Orange 7 (AO7) was selected as a target dye and it was successfully decolorized under visible light ($\lambda \geq 420$ nm) at a specific pH region. AO7 forms a complex with $Fe^{+3}$and subsequently undergoes a visible light-induced electron transfer from AO7 to Fe^{+3}, which initiates the dye degradation [12]. According to previous reports, the polyamine-saccharide chitosan presents a remarkable affinity to form coordination compounds between its amine and hydroxyl functional groups and metal ions, such as Fe^{+2}, Fe^{+3}, Co^{+2}, Cu^{+2}, Ni^{+2}, Pb^{+2}, Cd^{+2} and Cd^{+4}. Therefore, the synthesis of chitosan/ magnetite Nano composites could be possible from coordinated compounds between ferric and ferrous ions and chitosan [13]. Therefore, we expected a synergetic effect of a novel system combining the biodegrading bacteria and Fe_2O_3 immobilized on chitosan matrix.

In the present study *Pseudomonas aeruginosa* was isolated and identified and their biodegradation capability of Brilliant Red azo dye was investigated. Furthermore, the free cells were immobilizedon chitosan-magnetite-nanoparticles composite and tested for decolorization and biodegradation of synthetic Cibacron Brilliant Red azodyeas well as a textile industrial wastewater.

2. MATERIALS AND METHODS

2.1 Chemicals and Culture Medium

All reagents, $FeCl_2$-$4H_2O$, $FeCl_3$-$6H_2O$, NaOH, HCOOH (88 % w/v) and low molecular weight chitosan (degree of deacetylation of 84.5% and molecular weight of 50-190 kDa) and Cibacron Brilliant Red (Mw 802 g mol^{-1}, λ-max: 517 nm) were obtained from Aldrich Company.

Yeast extract-glucose (YG) medium composed of (g/L): glucose (Difco), 1.25, peptone (Difco), 5.0 and yeast extract (Difco), 3.0, pH 7 [14]. The mineral salt medium (MSM) used in the degradation study composed of (g/L): K_2HPO_4, 1.73; KH_2PO_4, 0.68; $MgSO_4.7H_2O$, 0.1; NaCl, 0.1; $FeSO_4.7H_2O$, 0.03; NH_4NO_3, 1.0; $CaCl_2.2H_2O$, 0.02 and supplemented with glucose (3 g/L). The pH of the medium was adjusted to 7.5.

2.2 Samples Collection

Wastewater was collected from the waste disposal site of small scale textile dying and printing processing unit located at Shoubra (Kaliobia, Egypt) in a clean high-density polyethylene bottles. These bottles were rinsed earlier with a metal-free soap and then soaked in 10% HNO_3 overnight and finally washed with deionized water [15]. Samples were stored at 4℃ until analysis.

2.3 Isolation and Screening of Dye Degrading Microorganism

A nutrient broth contains Cibacron Brilliant Red (100 mg/L) was inoculated with 10% (w/v) of the wastewater sample. The flask was incubated at temperature 30℃ ± 0.2 under the static condition. After 5-daysof incubation, serial dilutions (10^{-1}–10^{-6}) were prepared and inoculated into nutrient agar medium using pouring-plate technique. The grown colonies were inoculated into liquid MSM supplemented with a low glucose concentration (3.0 g/L) and supplemented with azo dye (100 mg/L). The flasks were incubated at 30℃ for 7 days. The decolorization efficiency was determined by measuring the OD at 517 nm by using Shimadzu UV spectrophotometer (Shimadzu UV 1800, Japan). The bacterial isolate that showed the maximum decolorization was selected for further experiments in this study.

2.4 Molecular Identification of the Isolate

2.4.1 DNA extraction

The genomic DNA was extracted from a pure culture of the bacterial isolate using the Wizard® Genomic DNA Purification Kit (Promega, Cat#A1120) with minor changes.

2.4.2 Amplification of the 16S-rRNA gene

The amplification of the 16S rRNA gene fragment was performed using the universal forward and reverse primers (8F:5'-AGAGTTTGATCCTGGCTGAG-3' and 1492R:5'-ACGGCTACCTTGTTACGACTT-3') [16]. The mixture of the PCR reaction encompassed DNA template (20 ng), dNTPs (250 mM each), primers (25 pmol each), MgCl2 (2.5 mM), PCR buffer (5 µl of 5X), Taq DNA polymerase [1.5 U (Promega)] and the total volume was set to 25 µl using di-distilled water. The reaction was placed on an automatic thermal cycler (GeneAmp1 PCR System 9700, Perkin-Elmer) under the following conditions: initial denaturation at 94℃ for 3 min; followed by 35 cycles [94℃ for 1 min, 55℃ for 1 min, 72℃ for 1.5 min] and final extension cycle at 72℃ for 7 min. The PCR product was analyzed using the electrophoresis apparatus in 1% agarose gel. This PCR fragment was purified using Promega Wizard SV Gel and PCR Clean Up-system Kit Cat#A9282, and cloned using the pGEM-T easy cloning kit (Promega, Madison, USA). White positive clones were selected and confirmed for successful insertion using PCR. Plasmid DNA isolation was performed on one confirmed positive clone using Wizard plus SV Mini-preps DNA purification system (Cat#A1460).

2.4.3 DNA sequencing

The cloned PCR fragment was sequenced using the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, USA) in conjunction with ABI PRISM (310 Genetic Analyzer). Approximately, a sequence of 1.5 kb was obtained. Sequence

data was aligned using the BLASTn protocol of the NCBI GenBank database (www.NCBI.com/BLASTn). The top five homologous hits were selected for Neighbor-Joining, multiple alignments (Clustal W), and construction of phylogenetic tree using DNASTAR software [17].

2.5 Effect of Dye Concentration on the Decolorization Efficiency

All decolorization experiments were performed in triplicates. The inoculum was prepared by growing the bacterium in nutrient broth for 24 h at 28℃, on rotary shaker 150 rpm. 100 ml of MSM was inoculated by 1mL of the seed inoculum. The dye was added at concentrations of 100, 200, 300 and 400 mg/L. After 24 h, aliquots (3 mL) of the culture were withdrawn, centrifuged at 5000 rpm for 15 min to separate the bacterial cell mass. The absorbance of the supernatant was measured by UV spectrophotometer at 517 nm and compared with un-inoculated medium, which was used as a control for calculation of the decolorization percentage.

2.6 Effect of Physicochemical Parameters on Dye Decolorization

Various physicochemical parameters including static and shaking condition, temperature and pH were monitored to study their effect on decolorization of Cibacron Brilliant Red. To study the effect of static and shaking conditions, the selected bacterial isolate was grown 250 ml Erlenmeyer flasks containing 100 mL of MSM broth amended with Cibacron Brilliant Red (100 mg/L) and incubated under static as well as shaking conditions (120 rpm) on orbital shaker for 72 h. The optimum temperature and pH were determined by evaluating the dye decolorization at 25, 30,37and 42℃, and at different pH values ranged from 4 to 12.

2.7 Enzyme Assays

The bacterial cells were grown in 50 ml MSM broth supplemented with 50 mg/LofCibacron Brilliant Red for 48 h. Culture without dye addition was used as control. The grown cells were harvested by centrifugation (10,000 × g for 20 min at 4℃). The extracellular enzymes (laccase, peroxidase anddioxygenase) were determined in the obtained supernatant. Laccase activity was determined by ABTS oxidation method as described by Hatvani and Mecs [18]. Peroxidase was assayedby the rate of pyrogallol decomposition [19]. Dioxigenase activity was determined spectrophotometrically at 260 nm by quantifying the formation of cis,cis-muconic acid (CCMA) (εCCMA = 16 800 M–1 cm–1) at 40℃. [20]. All enzyme assays were carried out in triplicate.

2.8 Synthesis of Magnetic Nanoparticles (MNP)

MNP was prepared according to the methodology described by Salem et al. [21]. Briefly, 0.5 g of zeolite was mixed with a 6.1 g $FeCl_3.6H_2O$ and 4.2 g $FeSO_4.7H_2O$ and then dissolved in 100 mL of distilled water using an ultrasonic bath. Mean while, the pH of the mixture was adjusted to 10.0 using 0.1 M NaOH. The mixture was agitated in a rotary shaker at 160 rpm for 24 h. Thereafter, 25 mL of the 6.5 M NaOH was then slowly added and mixed with the above solution. The system was mixed for an hour following the addition of NaOH. The formed black precipitates were washed with ultra-pure water several times; an external magnetic field was used to enhance the washing process. This procedure leads to form αFe_3O_4-Zeolite nano-particles with a size of around 10–20 nm. The αFe_3O_4-Zeolite was then oxidized at 300ºC for 3 h to obtain γFe_2O_3-Zeolite (MNCZ).

2.9 Cell Immobilization on Chitosan-magnetite-nanoparticles Composite

The cells were immobilized on chitosan-magnetite nanoparticles cross-linked by glutaraldehyde [22]. Powdered chitosan ((Mw 750,000 Da, 87.6% deacetylation) was dissolved in 2% acetic acid. Twenty mL of 1% chitosan solution was mixed with 50-150 mg of prepared magnetite powder followed by gradual addition of 13.2 mL of 0.5M KOH at 50℃ under stirring. After 10 minutes, 0.5 g of glutaraldehyde (25% solution) was added. Stirring was continued for additional 30 minutes. Chitosan-magnetite-nanoparticles composites were washed with 0.1 M sodium acetate buffer, pH 5. A solution of bacterial cells (5 x 10^8cfu /g particles) was added and the mixture was stirred at 20℃ for 30 min and then left at 4℃ overnight. The particles were then washed until no cells were detected in the washes. Immobilized cells were stored at 4℃ in 0.1 M sodium acetate buffer, pH 7.Cell-free immobilization particles were prepared following the same procedure except that the cells were excluded to be used as control in the following experiments.

2.10 Characterization of Chitosan Magnetic Nanoparticle (CHMN)

The morphology of magnetite NPs and chitosan-magnetite nanoparticles coated cells were determined by transmission electronic microscopy (TEM) (Hitachi 8100, 200 kV). In order to prepare TEM sample, a drop of suspension was placed on a carbon-coated copper TEM grid and then left to dry in air. Scanning electron microscopy (SEM) was used to verify the bacterial entrapment in magnetite nanoparticles coated cells. The sliced gel after fixation was dried in a Balzers CPD 030 device and then coated with carbon in aJEE-4X vacuum evaporator. Observations were made with a JSM 840-A SEM and images were captured digitally. The size and Zeta (f) potential of NPs were analyzed by DelsaNano C Particle Size and Zeta Potential Analyzer (Beckman Coulter Inc.).

2.11 Dye Decolorization by the Immobilized Cells

Biomass-chitosan magnetite nanoparticles (3g) were placed in a 100 mL MSM containing Cibacron Brilliant Red (200 mg/L).The culture was then statically incubated at 37℃ for decolorization. Decolorization with an identical amount of cell-free immobilization matrix was also performed as a control.

2.12 Decolorization of Textile Industrial Wastewater by the Immobilized Cells

Three g of Biomass-chitosan magnetite nanoparticles were added into 250 mL of the effluent from actual waste disposal site of textile processing units, pH were adjusted to 7 using 0.1 M NaOH. Since the enzymatic decolorization activity is strongly inhibited in presence of oxygen [23]. The interaction was tested under static-incubation for 2 h. After 2 hours of incubation at 37℃, the solution was filtered and the supernatant was collected for O.D. measurementat 520 nm using spectro-photometer. The cell-free immobilization matrix was also tested as a control. Measurement of soluble COD was performed according to the procedure described in Standard Methods for the Examination of Water and Wastewater [24]. The COD reduction percentage (CR%) was calculated as follows:

$$CR\% = COD0 - COD_t / COD_0$$

Where COD_0 and COD_t are the initial COD value (at 0 h) and the observed COD value after a particular reaction time (t), respectively. All experiments were carried out in triplicates. Data obtained were subjected to statistical analysis to determine means and standard deviations of means.

2.13 GC/MS Analysis

The GC/MS analysis was performed to identify the degradation products. Fifty mL of the supernatant was extracted with equal volume of ethyl acetate after 1 and 2 days of incubation. The ethyl acetate extract was concentrated to 3 mL by rotary evaporator at 40℃. One µL was injected into GC/MS HP-5890 GC equipped with HP-5972 mass detector. The analysis was performed by using HP-5MS column 30m X 0.25 mm id X 0.25 µm film thickness. Mobile phase: helium, flow rate 1mL/ min. Oven temperature starts at 80℃ for 1minute up to 320℃ at a rate of 15℃/min. The mass detector temperature was 300℃. The fragmentation pattern in mass spectra was automatically compared with that in the GC-MS software (Wiley 7N mass library).

2.14 Statistical Analysis

Data were analyzed by one-way analysis of variance (ANOVA) with the Tukey–Kramer multiple comparisons test [25].

3. RESULTS AND DISCUSSION

3.1 Isolation and Identification of Decolorizing Bacteria

The bacterial isolate (named NS) was selected and identifieddue to its high decolorization activity of Cibacron Brilliant Red. t showed decolorization efficiency of 63% after 24 h and increased to 97.6% after 72 h of incubation. The microscopic examination using gramstaining revealed that NS isolate is a gram-negative, rod-shaped, and non-spore forming bacterium. The amplified fragment of 16S-rRNA gene was obtained at the expected size of 1500bp (Fig. 1). The DNA fragment was sequenced and aligned to find the best homologues sequences using NCBI/BLASTn protocol. The multiple alignments showed significant similarity (99 - 100%) with the 16S-rRNA gene identified in *Pseudomonas aeruginosa*. The maximum score against the top best five hits, total score, sequence coverage, e-value, and maximum identity were all presented

in Table 1. To decipher the relative genetic kinship between the NS sequence and the previously isolated sequences on the gene bank, we applied neighbor-joining method. The analysis showed that the closest gene sequence was that of *P aeruginosa* strain IHB B-686 (Fig. 2). Both sequences (NS and IHB B-686) were separated in a discrete kinship distant from the top sequences of the other strains presented in Table 1. Therefore, we can conclude that NS was belonging to one of the *Pseudomonas aeruginosa* strains illustrated in Table 1.

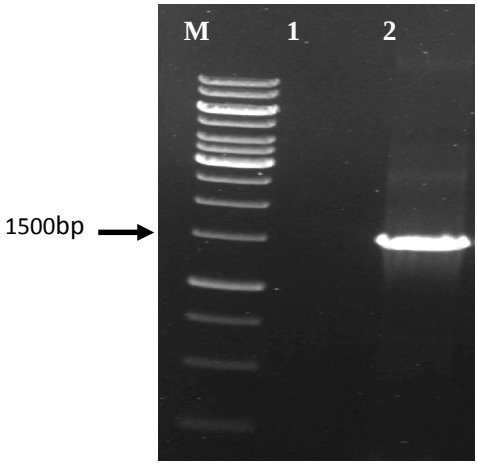


Fig. 1. Visualization of amplified 16S-rDNA fragment of 1500 bp from NF-isolate. M: 1K bp DNA ladder 1: (-) ve control and 2: the 16S-rDNAof the NF- isolate

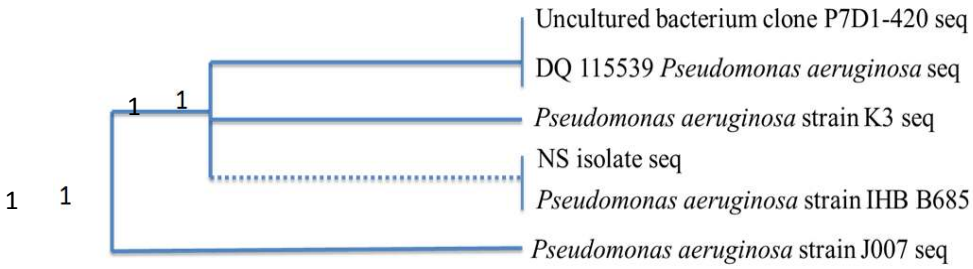


Fig. 2. Phylogenic tree of the 16S-rDNA isolate with the selected best homologous known bacterial strains

Table 1. The bacterial isolate homology to the nearest neighborhood joining bacterial strains

Accession number	Bacterial strain	Max score	Total score	Sequence coverage	E value	Max identity
KF668476.1	*Pseudomonas aeruginosa* strain IHB B 6863 16S ribosomal RNA gene	2765	2765	100%	0.0	100%
EF509253.1	Uncultured bacterium clone P7D1-420 16S ribosomal RNA gene	2765	2765	100%	0.0	100%
FJ227280.2	*Pseudomonas aeruginosa* strain J007 16S ribosomal RNA gene	2761	2761	99%	0.0	100%
EF064786.1	*Pseudomonas aeruginosa* strain K3 16S ribosomal RNA gene	2761	2761	99%	0.0	100%
DQ115539.1	*Pseudomonas aeruginosa* 16S ribosomal RNA gene	2761	2761	99%	0.0	100%

3.2 Decolorization of Cibacron Reactive Red by Free Cell under Various Physicochemical Conditions

3.2.1 Effect of dye concentration and incubation time

As shown in Fig. 3, the decolorization percentage of various Cibacron Brilliant Red concentrations by the free cell at different time intervals. It was observed that at a concentration of 50 ppm, 72 h was adequate to achieve a complete color removal. However, when the initial dye concentration increased (100, 200, 300 and 400 mg/L), the maximum decolorization percentages were achieved after longer incubation times (Fig. 3). It was also noticed that the cell pellets were not pigmented at all dye concentrations. This result indicated that *P. aeruginosa* can effectively tolerate the high dye concentration up to 300 mg/L and at the higher concentration of 400 mg/L the efficiency was decreased to 35.7% after 168 h. It was reported that dye decolorization could be strongly inhibited when a high concentration dyestuff was used to examine the poisonous effect of the dye on the degrading microorganisms [26,27]. An isolate of *Pseudomonas* sp. ETL-B showed 100% decolorization of Orange 16 (200 mg/L) after 12 h of incubation, while the higher concentration (1000 mg/L) was decolorized after 84 h [28]. *Pseudomonas* sp. showed 78.04% decolorization of Alizarin red S after 48 h of incubation in MSM supplemented with 1% glucose, 1% peptone, pH 7.0, 37℃, 500 mg/L dye concentration [29]. *Pseudomonas stutzeri* strain (SB-13) showed the highest decolorization 61% capacity of azo dyes mixture (Disperse Blue (R16), Disperse Yellow (D4), and Reactive Red Synozol (R4) dyes) [30]. *P. aeruginosa* isolate isolated from Wastewater from textile industries showed 86% decolorization of Reactive Red HE8B (100 mg/L) after 48 h [31].

3.2.2 Effect of pH and incubation temperature

Fig. 4A and 4 Brepresent the Effect of pH and temperature on the Cibacron Brilliant Red decolorization by *P. aeruginosa*. It was found that the optimum pH and temperature for complete Cibacron Brilliant Red decolorization were 7.0 and 37℃ ± 0.2, respectively. The decolorization percentages were much lower (11.4% and 8. 14%) at strongly acidic (pH4.0) and strongly alkaline (pH 12.0) conditions, respectively). Therefore, pH has a strong effect on the efficiency of dye decolorization, and the optimal pH for color removal is often between 6.0 and 10.0% [32]. This could be due to the fact that the optimum pH for the growth of *P. aeruginosa* was neutral or slightly alkaline. *P. aeruginosa* NCIM 102 was also capable of decolorizing the Acid Orange-10 dye over a pH range of 7-9 with a good efficiency [33].

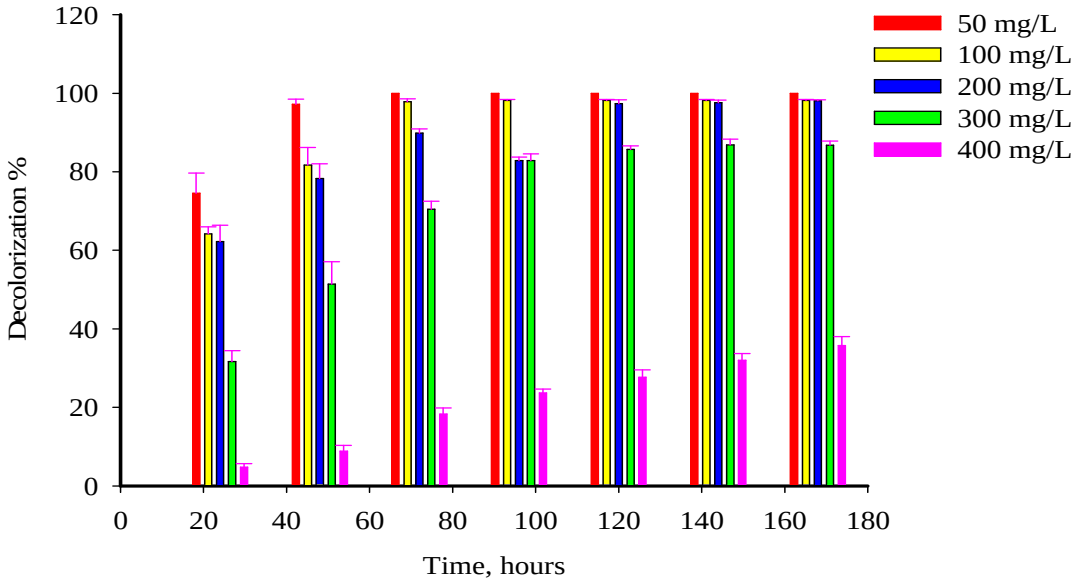


Fig. 3. Decolorization (%) of different Cibacron Brilliant Red concentrations by *P. aeruginosa* at different incubation times

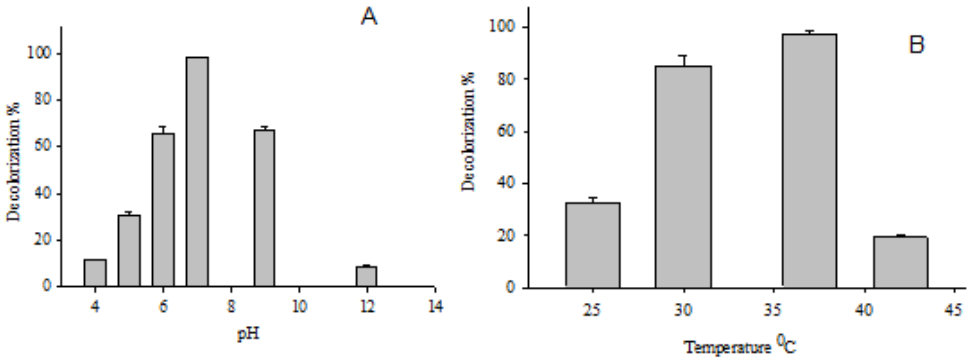


Fig. 4. Effect of pH (A) and temperature (B) on the Cibacron Brilliant Red decolorization percentage by *P. aeruginosa* free cells

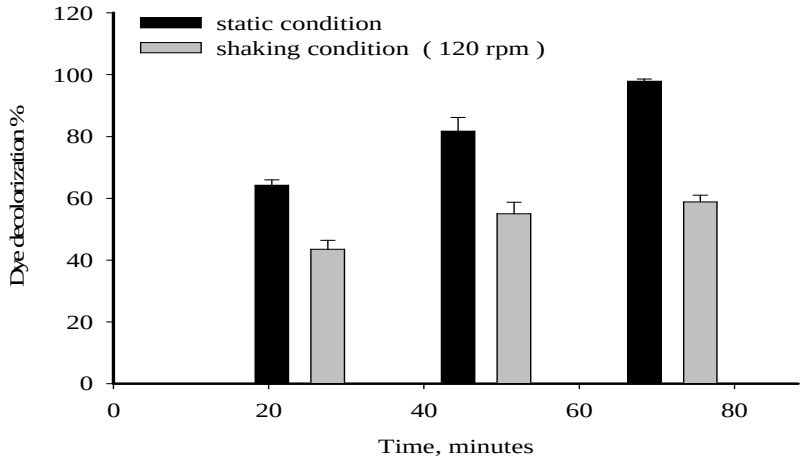


Fig. 5. Effect of shaking on the decolorization (%) of Cibacron Brilliant Red by *P. aeruginosa*

3.2.3 Effect of shaking and static culture

The decolorization percentages under static condition were higher than that under shaking conditions at all of the tested incubation periods (Fig. 5). This reflects that static condition was necessary as is the case for most of the microbial species for azo-dyes reduction. The result bears similarity with those of studies on *P. putida* MTCC 102, *P. desmolyticum* and *P. luteola*. It was found that under agitation conditions, presence of oxygen deprives the azoreductase from obtaining electrons needed for cleavage of azo dyes under static anoxic conditions. These electrons are available to azoreductase from $NADH+H^+$ to decolorize azodyes [33-35].

3.3 Characterization of Bio-magnetic Nanoparticle Composite

The SEM imageof the bio-magnetic nanoparticle-chitosan composite (BMC) is presented in Fig. 6(a). The figure shows homogenously dispersed immobilized bacterial-magnetic nanoparticles within the chitosan matrix without aggregation. Fig. 6(b) shows the TEM imageof BMC. It can be seen that the magnetic nanoparticles (Fe_2O_3NPs) were intensively surrounded the microbial cells and adhered to its surface. This phenomenon is due to the large specific surface area and the high surface energy of the Fe_2O_3 NPs. Moreover, when the BMC washed out by deionized water or saline solution (0.85% w/w) or phosphate buffer (0.1 M, pH 7), the cells were still attached to the surface of NPs with slight loss. Therefore, the interaction between the NPs and bacterial cells could not be only by the direct electrostatic force to Fe_2O_3 NPs but also by the adsorption to chitosan-conjugated magnetite [36].

Bacterial cell surfaces are anionic due to the presence of ionized groups such as carboxylate, hydroxyl and phosphate in the various cell wall

polymers [37]. The strength and extent of this cell wall-metal interaction will depend on the chemical composition of the cell wall, the distribution and number of ligand groups and the affinity of the particular metal ion for these groups. Uncharged groups such as peptide N atoms may also function as ligands to complete the coordination number requirements of the metal ion. This process is described as surface binding purposely for subsequent translocation into the cell. TEM measurements also supported the notion of non-single layer adsorption (Fig. 6b).

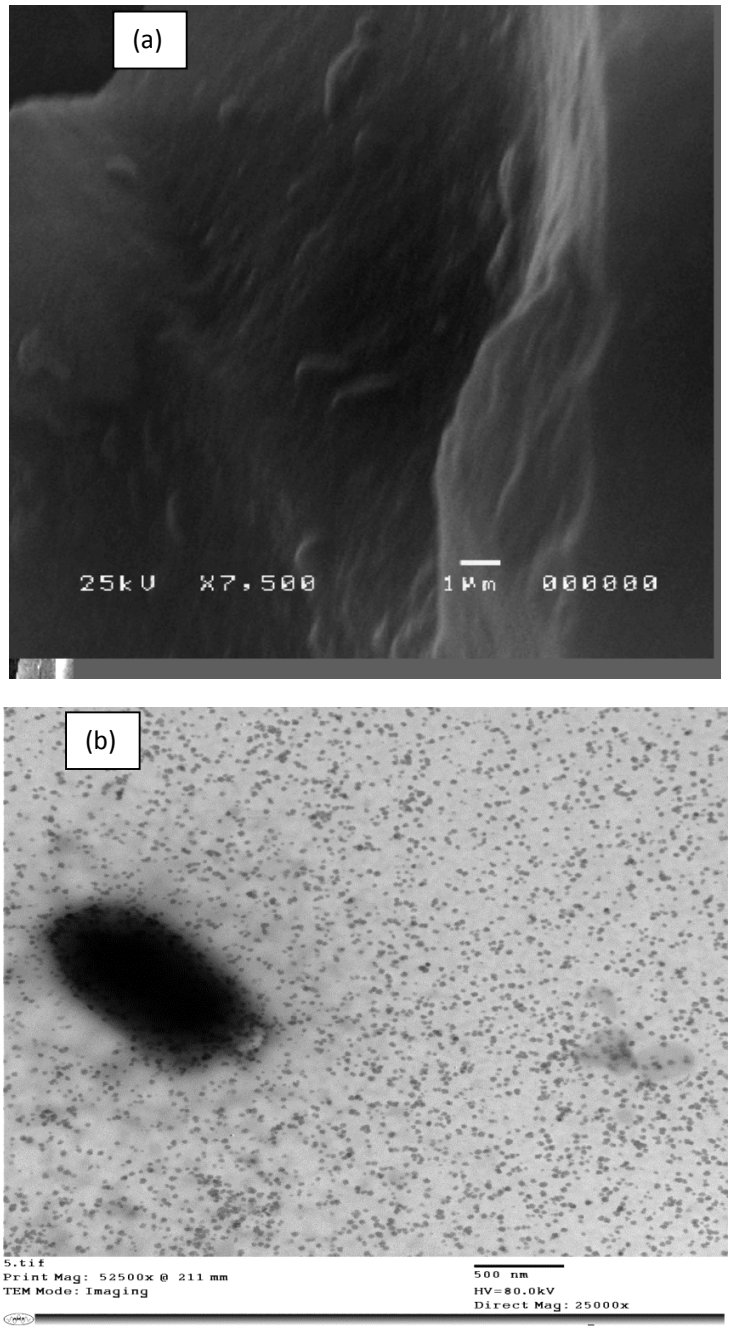


Fig. 6. SEM photomicrograph of BMC (a); TEM photomicrograph of BMC, showed the magnetic nanoparticles density around the bacterial cell (b)

3.4 Decolorization of Cibacron Brilliant Red Using BMC

As the matrix used for cell immobilization may contribute to decolorization via abiotic process (such as adsorption), color-removal experiments with fixed beds solely containing cell-free matrix of chitosan-magnetite nanoparticles (MC) was conducted for comparison. The color removal by cell-free MC at Cibacron Brilliant Red dye concentration of 200 mg/L reached to maximum removal (98.8%) after 168 hours, while 100% decolorization was obtained by the immobilized cells (BMC) after only 2 hours. In comparison with the free cells, the immobilized cells of *P. aeruginosa* showed a higher decolorization percentage than free cells. Since the BMC matrix have a high adsorption capacity for the dye molecules, which resulted in an adsorption phase before the enzymatic decolorization became dominant. This finding suggests that adsorption of dye molecules on BMC matrix saves the contact of the dye molecules with the cells. Similar results were obtained in studies of the decolorization kinetics of the immobilized *P. luteola* cells in a synthetic polymer matrix of polyacrylamide (PAA). PAA-immobilized cells had a significantly higher decolorization of C.I. Reactive Red 22 than those obtained from the free cells [38].

3.5 Batch Decolorization for Real Textile Effluent with Immobilized Cells

Results of batch study of textile effluent decolorization using the immobilized cells (BMC) and the cell free composite MC are shown in Table 2. The BMC has completely removed the color from the effluent within 8 hr at pH 7.0 and temperature of 37℃ under static conditions; while only 74.52% color removal was achieved by the MC. Consequent reduction in COD (from 149 to11 mg O_2/L), BOD (from 120 to 58 mg O_2/L) and TSS (from 105 to 74 mg/L) of effluent were obtained by the BMC.

3.6 Identification of Degradation Products

In order to understand the degradation mechanism of Cibacron Brilliant Red by *P. aeruginosa*, the GC/MS analysis was conducted after degradation experiment, which is presented in Fig. 7. There are ten major degradation products were detected on the GC/MC chromatogram. The chemical structure of these products was elucidated from the mass library as well as from their mass fragmentation patterns (Table 3). Based on these structures, the biodegradation mechanism can be anticipated. The biodegradation of the Cibcron Brilliant Red by *P. aeruginosa* is catalyzed by four major enzymes: desulfinase, azo-reductase, dioxygenase and laccases. The sulfonate groups are removed by the desulfinase at either the initial degradation steps or after the reaction of the other enzymes by removing the sulfonate group from the products of azo-reductase or laccases reactions. Absence of any sulfonate derivative in the detected degradation products reflects the desulfinase activity.

The proposed biodegradation pathway of the red dye by *P. aeruginosa* is explained in Fig. 7. The basic reaction causes fragmentation of the molecule is the azo-reductase reduction of the di-azo bonds into two amino groups. Upon the azo-reductase activity, the ring (E) resulted in formation of aniline, which in turn oxidized by a peroxidase enzyme to form the nitrosobenzene (MW 107, Rt. 11.1 min.). The remaining naphthalene moiety (rings C and D) was degraded mainly by the reaction of laccases and dioxygenase resulted in formation of the other products. The ring (A) attached with fragments of ring (B) resulted in formation of two compounds: 1-phenyl-1,4-dihydrotriazete (MW 133, R.t.11.8 min.) and 1-ethyl-1-phenylguanidine (MW 163, R.t. 12.3 min.); however, formation of these two compounds couldn't be interpreted based on the available data. The degradation products of an azo-dye varyas the substrates vary. For example, if the azo reduction worked firstly, its degradation products will be substrate for the subsequent enzymes (laccases and dioxygenase), which will result in different degradation products if another enzyme worked firstly.

Furthermore, the laccases enzymes target the hydroxyl phenolic group of the phenolic azo dye, generating a phenoxy radical, which is sequentially followed by oxidation to a carbonium ion. A nucleophilic attacked by water on the phenolic ring carbon bearing the azo linkage to produce 3-diazenyl-benzenesulfonic acid and 1, 2-naphthoquinone derivatives [39,40].

3.7 Enzymes Assay

To confirm production of the enzymes expected in the biodegradation pathway, all of these enzymes were determined in the bacterial culture of *P. aeruginosa*. The laccases, peroxidases and

dioxygenases enzymes were determined in the bacterial culture of *P. aeruginosa* grown in MSM in absence (control) and presence of azo dye. As shown in Table 4, the enzymes activities were induced in the cells grown in MSM supplemented with the azo dye. This result reflects the induction effect of the azo dye for enzymes production by *P. aeruginosa.*

Table 2. The industrial wastewater parameters before and after treatment with cell-free (MC)and immobilized cells (BMC) chitosan-magnetite nanoparticles

Tested parameters	COD (mgO_2/L)	BOD5 (mgO_2/L)	TSS (mg/L)	Decolorization %
Raw industrial wastewater	149	120	105	0.0%
After treatment with MC	37	45	78	25%
After treatment BMC	11	58	74	100%

Table 3. The chemical structure and fragments of the Cibacron Brilliant Red degradation products by *P. aeruginosa*

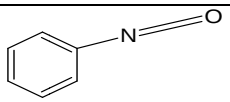
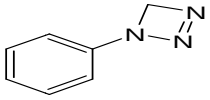
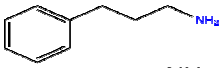
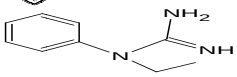
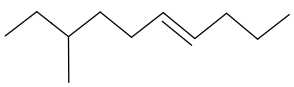
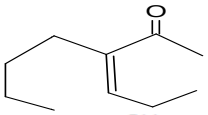
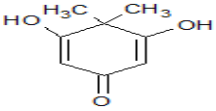
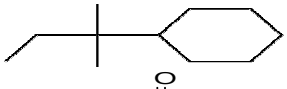
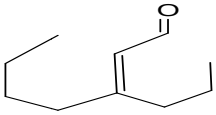
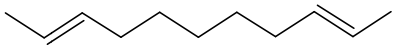
R.t.	Compound name	Structure	Fragments	MW
11.1	Nitrosobenzene		77,91, 107	107
11.8	1-phenyl-1,4-dihydrotriazete		77,91,105,133	133
12.0	Propylamine,3-phenyl		77, 91, 105,119, M+ absent	135
12.3	1-ethyl-1-phenylguanidine		77,91, 105,120,163	163
16.2	4-decene,8-methyl		70,97,110,125, 138,154	154
16.6	2,heptanone-3,propylidene		70, 110, 126, 154	154
17.1	2,5-Cyclohexadien-1-one, 3,5-dihydroxy-4,4-dimethyl		70,86,96,112,125,139, 154	154
17.3	1,1-dimethylpropyl-Cyclohexane		70,91,110,125,154	154
17.4	3,propyl-2,heptenal		70, 97, 110, 154	154
17.8	2,9-unidecadiene**		56, 67, 85, 123, 152	152

Table 4. The enzymes production (U/mL) by *P. aeruginosa* cells grown in MSM in absence (control) and presence of the azo dye

Enzyme	Enzyme production In absence of azo dye	Enzyme production in presence of azo dye
Laccases	0.2	0.69
Peroxidases	0.21	0.72
dioxygenases	0.09	0.15

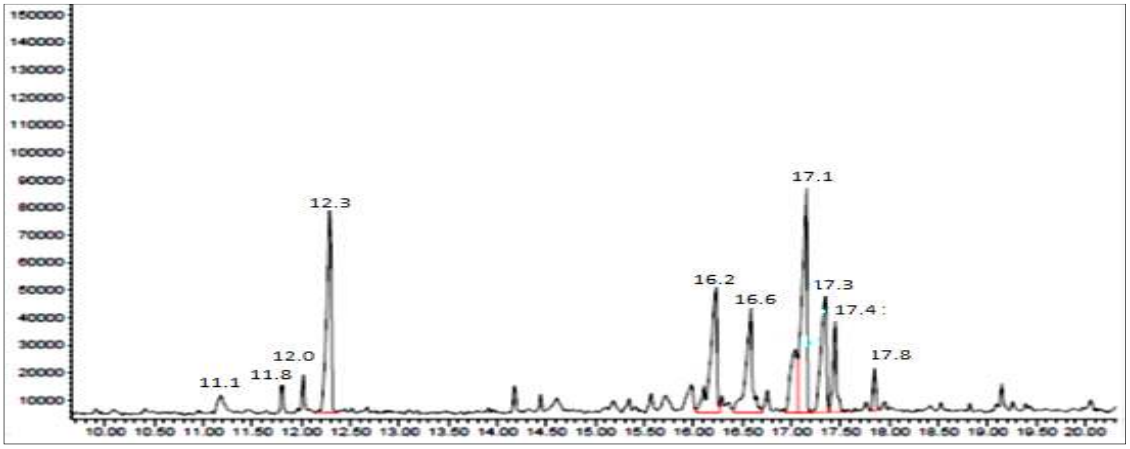


Fig. 7. The GC/MS chromatogram for the biodegradation products of Cibcron Brilliant Red dye by *P. aeruginosa*

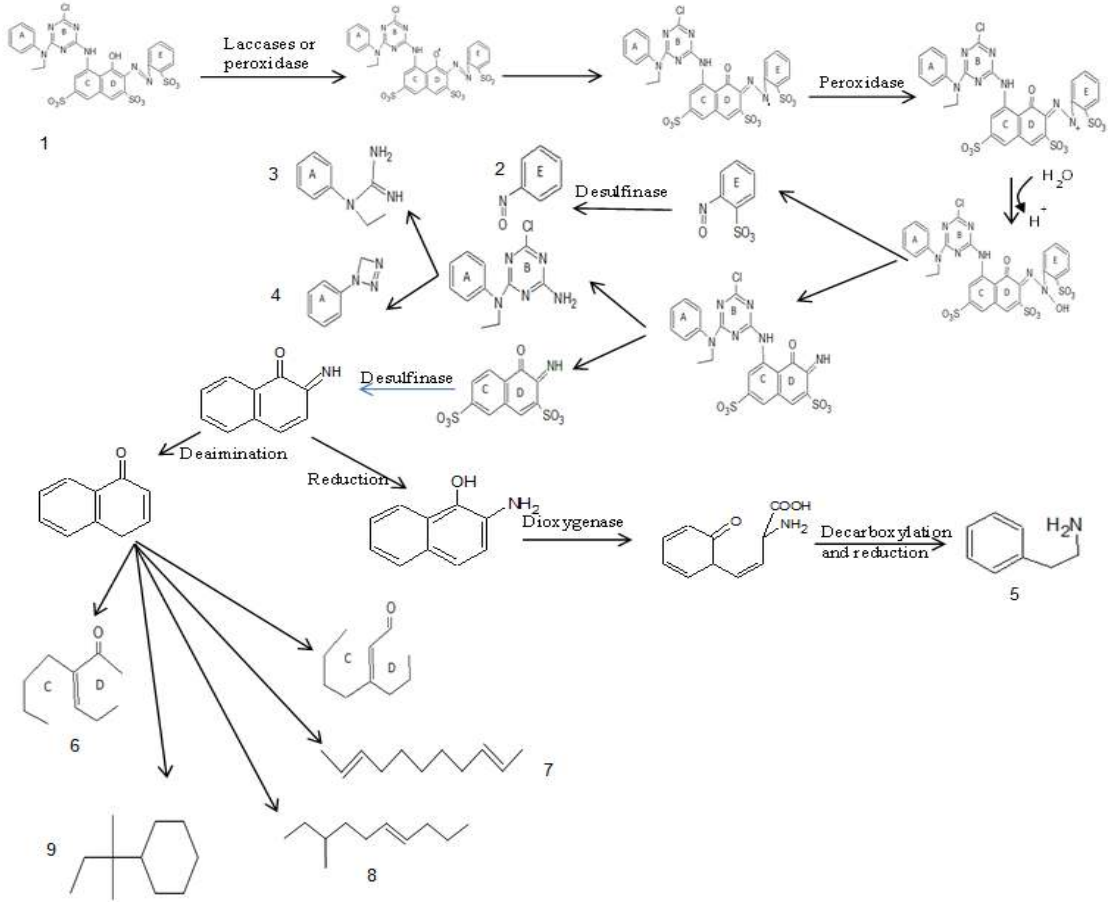


Fig. 8. The Proposed biodegradation pathway of Cibacron Brilliant Red by *P. aeruginosa*

1) Cibacron Brilliant Red, 2) Nitrosobenzene, 3) 1-ethyl-1-phenylguanidine, 4) 1-phenyl-1,4-dihydrotriazete, 5) Propylamine,3-phenyl, 6) 2,heptanone-3,propylidene, 7) 2,9-unidecadiene, 8) 4-decene,8-methyl, 9) 1,1-dimethylpropyl-Cyclohexane

4. CONCLUSIONS

Pseudomonas aeruginosa was isolated from textile wastewater sample and identified by the 16s-rRNA gene sequencing. It showed high decolorization efficiency of Cibacron Brilliant Red azo dye. The immobilized cells on Chitosan-magnetite matrix exhibited enhanced decolorization efficiency because of the higher adsorption capacity of the nanoparticles combined with enhanced bacterial activity. At initial dye concentration of 100 mg/L, the maximum decolorization percentage (89%) was achieved by free cell sat pH7, 37ºC under static conditions after 72 h of incubation; while the immobilized cells showed 100% decolorization after only 2 h of incubation at initial dye concentration of 200 mg/L. The biodegradation pathway was elucidated by GC/MS identification of the degradation products as well as determination of laccase, peroxidase and dioxigenases, which arethe enzymes included in the biodegradation process. The application of immobilized cells in decolorizing real textile wastewater is a major aim for the textile industries. Therefore, through this investigation, we strongly recommend use of immobilized *P. aeruginosa* on Chitosan-magnetite nanoparticles for treatment of brilliant red azo dye-containing wastewater.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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