

Original Research Article

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Improvement of the Decolorization of Azo Dye (Direct Red 81) by Immobilized Cells of *Bacillus pumilus* and Free Cells of *Aspergillus clavatus* in Textile Waste Waters

Walaa A. Al-Shareef^{1*}, Salwa S.I. Afifi², Mohamed A. Ramadan³ and Nesma R. Sakr¹¹Microbiology and Immunology Department, Faculty of Pharmacy, October 6 University, Giza²Microbiology and Immunology Department, Faculty of Pharmacy-Girl, Al-Azhar University, Cairo³Microbiology and Immunology Department and Biotechnology Centre, Faculty of Pharmacy, Cairo University, Cairo

*Corresponding author

ABSTRACT

In this study, the effluent wastewater samples collected from dyestuff industrial area have been examined for biological treatment. The rate of decolorization was assessed using spectrophotometer at 508 nm and the percentage of decolorization was ascertained. The metabolites of Direct Red 81 (DR81) obtained after biodegradation were determined by HPLC. In order to improve the decolorization process, the effect of process factors like pH, temperature, agitation and dye wastewater concentration on dye decolorization was studied. From the results, a potential bacterium and fungus isolates, namely, *Bacillus pumilus* and *Aspergillus clavatus*, respectively, have been isolated and identified from dyestuff industrial area for the decolorization of DR81. After 5 days of incubation period with *Bacillus pumilus* and *Aspergillus clavatus*, the bacterium showed 17% of decolorization, while the fungus showed 95% of decolorization at a dye concentration of 100 mg/L at 30 ± 0.2 °C under aerobic processes. Mixed culture of *Bacillus pumilus* and *Aspergillus clavatus* were found to decolorize 88% of DR 81 after 5 days of incubation. *Bacillus pumilus* showed the best extent degradation of DR81 (97%) when supplemented with yeast extract after 5 days of incubation. Furthermore, supplemented cells of *Aspergillus clavatus* with yeast extract showed 99% of DR81 while it showed 98% of when supplemented with glucose, and non-supplemented cells showed 95% of degradation after 5 days of incubation too. Free cells of *Aspergillus clavatus* showed 95% decolorization of DR81 after 5 days of incubation compared to immobilized cells (74%) on activated charcoal (20 g/L) in mineral salt medium (MSM), while immobilized cells of *Bacillus pumilus* on kaoline (20 g/L) showed 71% decolorization of DR81 compared to free cells (17%). Therefore, the potential of these microbial isolates can be exploited for the removal of residual dyes from the industrial wastewater for environmental cleanup and restoration of the ecosystem.

Keywords

Biodegradation, Decolorization, *Bacillus pumilus*, *Aspergillus clavatus*, DR81, Immobilization, and wastewaters

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Introduction

Azo dyes represent a major group of aromatic chemical compounds which are extensively used in textile dyeing and many other industries. Azo dyes are used widely for dyeing purposes and constitute about 70%. Therefore, azo dyes are the most important and largest group of synthetic colorants released into the environment (Lade *et al.*, 2015; Kadam *et al.*, 2011). They are classified as reactive, direct, disperse, acid, basic and vat dyes (Sathian *et al.*, 2013). Among these various types, DR81 dyes represent more than half of the dyes present in the pulp and textile industries (Walger *et al.*, 2018) and they usually contain at least one azo group and some sulfonic acid groups thus making them water soluble. They have the same chromophore N=N and different auxochromes groups as NH₂ and OH (Olaganathan and Jamila 2010).

The releasing of such dyes in natural ecosystem results into azo group conversion to aromatic amines which could result in a toxic impact on aquatic life and even carcinogenic and mutagenic effect on humans. Therefore, wastewater containing such dyes must be treated before its release into the receiving water bodies. In addition, these dyes considered to be bio-recalcitrant contaminants which released to the environment during manufactures in the food, textile, and paper-making industries (Kim *et al.*, 2007). Hence, researchers have focused on the biological treatment of the dye wastewaters as the best alternative method when compared with physiochemical methods (Surwase *et al.*, 2013). The cost-effectiveness, ecological sociability of biological systems, and lower sludge production have made them more favorable technique (Dawkar *et al.*, 2010). Biological degradation can be performed under both aerobic and anaerobic conditions and aerobic degradation is more efficient than

anaerobic degradation (Shah 2014 & Ramadan *et al.*, 2014; Chengalroyen *et al.*, 2013; Saratale *et al.*, 2011). The treated industrial wastewater can be used for irrigation purpose, particularly for growing crops in degraded soils (Iqbal *et al.*, 2010). Furthermore, screening efficient fungal and bacterial strains that can cleave azo bonds of complex azo dye structures could be useful for the effective treatment of textile effluents.

Though most of the research work on dye decolonization have been carried out using individual fungal and bacterial cultures but the work pertained to fungal-bacterial synergism for biodegradation of DR81 is missing. In this regards, cell immobilization technique is studied for its potential to improve bioremediation and fermentation processes. There are two immobilization methods that are widely used which are is entrapment and adsorption but the adsorption method is more simple and better physiological conditions (Kattimani *et al.*, 2009). It was found that bacterial cultures able to utilize dyes as carbon or nitrogen sources, but mostly microorganism cannot utilize dyes as a sole carbon or nitrogen source so it needs organic carbon and nitrogen sources for dye degradation (Palanivelan *et al.*, 2014).

In this study, microbial decolorization of DR81 has investigated aerobically with pure and mixed culture and because of DR81 is an azo dye extensively used in textile dyeing; this dye has been selected for the present study. The purpose of this study was examined for their ability to decolorize the recalcitrant DR81 in industry wastewaters, and how to enhance the activity of decolorization with immobilizing cells in different solid supports as activated charcoal, marine sand, ash, and kaolin. *Bacillus pumilus* as a bacterium isolate and *Aspergillus clavatus* as a fungal isolate were used as a specific strain that could be applied to a pure or mixed culture for the

decolorization of synthetic wastewater that contained DR 81.

Materials and Methods

Azo dye and chemical reagents

The direct azo dye, Direct Red 81 (DR81) was used as a target dye for the synthetic wastewater and purchased from Sigma-Aldrich®. The initial concentration of the azo dye was fixed at approximately 100 mg/L. DR81 ($\lambda_{max} = 508$ nm), has a complicated molecular formula (C₂₉H₁₉N₅Na₂O₈S₂) (Heravi *et al.*, 2015; Hossein and Behzad 2012). The stock solution of DR81 (5000 mg/L) was prepared in distilled water and further diluted to various low concentration solutions. The initial concentration of the azo dye was fixed at approximately 100 mg/L. All chemicals and medium components were obtained from Hi Media Laboratories Pvt. Ltd., Mumbai, India. All the other chemicals used in the present study were of an analytical grade and highest purity available.

Samples collection

A total of 44 effluent samples were collected from in and around the Textile Industrial Zone (EL-Salam and Micado and other factories), in the form of untreated liquid effluent, 6th of October City, Cairo, Egypt as well as 10th of Ramadan, El Sharkeya, Egypt. The collected effluent was transported to the laboratory in sterile glass-screw cap tubes. The pH of the filtered effluent was maintained at 7.0 and stored at 4°C temperature until processing to prevent contamination by non- indigenous microbes; however, samples commonly used within 24 h of sample collection.

Screening of microbial isolates

Ten ml of each textile wastewater sample was inoculated into 250 ml volumetric conical

flask containing 100 ml of sterile Mineral Salt Medium (MSM) as a source of carbon and nitrogen (Ramdan *et al.*, 2014). MSM had a composition of (g/L) KH₂PO₄, (1); K₂HPO₄, (1); MgSO₄, (0.5); CaCl₂, (0.1); NaCl, (1); NH₄NO₃, (1) and DR81, (0.1). The mixture was incubated at 30°C for 21 days under static condition before being transferred to fresh medium. After incubation, 2 ml of cell suspensions were plated onto MSM with 2.5 % (w/v) agar-agar medium and incubated at 30 °C for 48 h. Microbial colonies that appeared on the agar medium were re-suspended into the flask containing freshly prepared MSM broth spiked with the DR81. A total of 25 bacterial isolates and 18 fungal isolates were recovered from effluent samples and further streaked three times to get purified isolates. The bacterial and fungal isolates that showed the highest decolonization of the dye in a liquid medium in 120 h were selected. Out of 43 isolates, two potential isolates (one bacterium and one fungus) were selected and examined again for their potential to decolorize the dye in wastewaters. The purified cultures were preserved at -80 °C in 15% (v/v) glycerol for subsequent studies.

Identification of degrading organisms

Bacterial identification of bacterial isolates

After purification by successive single colony isolation on the agar plate, the bacterial isolate that showed the highest decolorization was selected and identified by the conventional microbiological methods. In this regard, the traditional method was obtained such as (i) Gram stain technique and (ii) Biochemical tests such as catalase and oxidase test, and commercially available multi test identification system: analytical profile index (API). API® identification products are identification test kits for Gram positive and Gram negative bacteria and yeast. API® strips identifications based on extensive databases

and are standardized, easy-to-use test systems and it gives accurate identification. API[®] strips contain up to 20 miniatures, quick biochemical tests, and all are safe and easy to perform. Test strips were used as the instructions provided.

Fungal identification

PCR and sequencing technique was performed fungal isolate identification by Clinilab, 160 St., El-Etihad Square Riham Tower, El-Maadi, Cairo, Egypt.

DNA extraction

Fungal mycelia were scraped directly from the surface of an agar culture. The final mycelial mat was weighed before placing it into a ceramic mortar. Liquid nitrogen was added to the tissue for grinding. After sufficient grinding extraction buffer [100 mM Tris- HCl (pH 8.0), 20 mM Na₂EDTA, 0.5 M NaCl, 1 % sodium dodecyl sulfate and proteinase K (3 mg/mL proteinase K (added fresh))] was added to the mortar so that the homogenate became ready. A mixture of buffer saturated phenol/chloroform/isoamyl alcohol was added at a ratio of 0.5 ml per gram of tissue.

The mixture was ground vigorously for ~30 sec with a vortex to form a thick paste. Two ml of extraction buffer and 1 ml of buffered phenol/CHCl₃/ isoamyl alcohol per 0.5 g of starting tissue were then added, and the solution was mixed thoroughly. The mixture was transferred into several microfuge tubes using a micropipette. The microfuge tubes were capped and centrifuged at 14,000 rpm for 5 min at room temperature. The aqueous phase was transferred to a new tube and was mixed with 0.1 volume of 3M sodium acetate (pH=5) and 2.5 volume absolute ethanol. The solution was gently mixed by inversion until the white thread-like strands of DNA forming a visible mass. Samples were incubated at -20⁰ C for

30 min and centrifuged for 15 min at 14,000 rpm to recover the precipitate. The supernatant was decanted and 500 µl of room temperature 70 % ethanol was added to the DNA pellet. The tube was gently inverted several times to wash the DNA pellet. The sample was centrifuged at 14,000 rpm for one min at room temperature. The ethanol was carefully aspirated using micropipette tip. The tube was inverted on clean tissue paper and the pellet was air-dried for 15 min. Also, 50-100 µl of TE buffer (10 mM Tris-HCl, one mM Na₂ EDTA, pH 8.0) was added to the tube and DNA was dissolved by incubating at 65°C for an hour and then stored at (2–8) °C for subsequently used.

For determination of DNA quality, quantity and integrity; samples were characterized using a UV spectrophotometer (Model: UV – 1800 240V) by measuring absorbance at 260/280 nm followed by visualization in gel electrophoresis stained by ethidium bromide. Ratios between 1.8 and 2.3 were considered satisfactory.

Agarose gel electrophoresis (0.8 %) for genomic DNA integrity determination

Agarose (0.8 grams) (Catalog number: 9012-36-6, Sigma, USA) were added to 100 ml 1X TAE buffer (Tris-Acetate-EDTA) prepared from 50X TAE buffer (Catalog number: B52, Thermo Scientific, USA) where it was diluted 50 times by double distilled water. This solution was heated in microwave oven until agarose was dissolved. Then the solution was cooled to 50°C, and 2.5 µl (10 mg/ml) ethidium bromide was added to the agarose solution, mixed well and then poured into the appropriate tray. After the agarose solidified, it was transferred to the horizontal electrophoresis submarine (Biometra, Compact multi_wide). The submarine was filled with 1X TAE buffer till the buffer totally covered the agarose gel, 5 µl of each

sample was mixed with 1 µl (6X) loading dye (Catalog number: R0611, Thermo Scientific, USA) and loaded in the gel wells, 5 µl of 1kb DNA ladder (Catalog number: 10787-018, Invitrogen, USA). Also, five µl of uncut lambda (λ) DNA ladder (35 ng/µl) (Catalog number: SD0011, Invitrogen, USA) was loaded to the agarose gel. The submarine was closed with its lid and connected to power supply (Biometra, Standard Power Pack P25 T); a voltage of about 120 volts was applied for about 30 min. After the run was completed, the gel was visualized using gel documentation system (Biometra GmbH BDA Box 2).

Amplification of Internal Transcribed Spacer (ITS) regions of fungal ribosomal DNA (rDNA)

Polymerase Chain Reaction (PCR)

The primers used for universal fungal DNA amplification from all isolates were for intervening ITS1, and ITS2 rRNA gene regions. The size of the product generated varies according to the organism tested. Primers used for direct sequencing were ITS 1; 5'-TCC GTA GGT GAA CCT GCG G-3' (position corresponding to small subunit 1769-1787), and ITS4; 5'-TCC TCC GCT TAT TGA TAT GC-3' (position corresponding to large subunit 41-60). All primers were synthesized by Invitrogen by Thermo Fisher Scientific Co.

PCR was conducted using GoTaq Flexi DNA Polymerase, 100u (units), Catalog number: M8291, Promega Corporation Madison, WI 53711-5399 USA. 25 µl reaction mixtures were prepared to consist of GoTaq® Flexi DNA Polymerase, 5X Colorless GoTaq® Flexi Buffers, Magnesium Chloride Solution and specific primers which were ordered from Invitrogen Company, 3175 Staley Road Grand Island, NY 14072, USA.

DNA sequencing

All PCR-amplified products were sequenced at Clinilab by automated dye termination sequencing. Each PCR-amplified product was purified with High Pure PCR Product Purification Kit (Catalog Number: 11 732 668 001, Roche Applied Sciences, Germany) and sequenced using a 16-capillary 3100 Genetic Analyzer (Applied Biosystems). The ABI Prism BigDye Terminator Cycle Sequencing Ready Reaction Kit Version 3 (Applied Biosystems) was used with protocols supplied by the manufacturer. PCR-amplified products from cultures were sequenced in a forward and reverse direction using ITS1 primer and the ITS4 primer respectively. Sequences were visualized and edited using SEQSCAPE Version 1.1 (Applied Biosystems).

Data Analysis

GenBank search Sequence was performed using the BLAST standard nucleotide-nucleotide primary local alignment search tool [National Center for Biotechnology Information (NCBI), Library of Medicine, Bethesda, MD, USA (<http://www.ncbi.nlm.nih.gov/BLAST/>)].

Maintenance of stock cultures

The bacterial isolate was maintained on slants of agar-agar (2%) supplemented with 50 mg/L of DR81, while the fungal isolate was maintained on Czapek Dox agar slants. Isolates were incubated at 28°C for five days and then stored at 4°C and sub-cultured every two months.

Decolorization studies

Plate assay

Plate assay was performed for the detection of decolorizing activity of the recovered isolates.

The nutrient agar was autoclaved at 121°C for 15 min. then DR81 was added after agar became warm. Isolates were plated on nutrient agar for bacterial isolate and sabouraud agar for fungal isolate (Vinodhkumar *et al.*, 2013), or inoculated into agar well plates containing DR81 (100 mg /L). The plates were wrapped with parafilm and were incubated at 37 °C for 7 days. The plates were observed for clearance of the dye surrounding the colonies (Vinodhkumar *et al.*, 2013) or surrounding the wells.

Decolorization assay of direct red 81 in liquid medium

Decolorization assays of the recovered isolates were done in MSM amended with 100 mg/L of the tested dye. The decolorization test was then performed by adding 5 % (v/v) of the inoculum into Erlenmeyer flasks containing 50 ml of the MSM and pH adjusted to be 6.5 with 0.1M phosphate buffer. After incubation (30°C under aerobic and agitation condition where incubator shaker worked at 150 rpm [Model Innova® 42R Inc/Ref Shaker, New Brunswick, US]) broth cultures were withdrawn daily at specified time interval for successive five days. Moreover, they were centrifuged at 10,000 rpm for 15 min. at room temperature for removal of biomass.

Then the supernatants were collected to obtain their optical density (OD) to be measured at 508 nm under visible light in the spectrophotometer (Model: UV – 1800 240V).

The optical density of the control (uninoculated) flasks was also measured. Finally, the rate of decolorization was calculated as the percent of decolorization (%)

Decolorization activity (%) = $(A_i - A_t)/A_i \times 100$

Where A_i = initial absorbance

A_t = observed absorbance (the concentration at cultivation time (0, 1, 2, 3, 4 and 5 days)

Each test was performed triplicate and mean values expressed as a result.

Decolorization of dye in industrial wastewaters

This experiment was carried out to reveal the effect of recovered isolates on industrial wastewaters. Cell suspension (2 ml) with a density of 10^6 was used to inoculate 100 ml flask containing 50 ml industrial wastewater with 100 mg/L of DR81, using abiotic control with DR81. The fungal mass was determined along with decolorization reaction at the same time intervals of samples withdrawn to determine fungal survival.

High Performance Liquid Chromatography (HPLC)

This method was carried out according to (Sahasrabudhe *et al.*, 2014) with some alteration. The removal of Direct Red 81 azo dye and a generation of decolorization products was monitored using an HPLC system equipped with an ODS C18 column (Shimadzu SPD 20A) with HPLC grade methanol 70 % + 30 % deionized water as mobile phase at the flow rate of 1.0 mL/min for 10 min at 508 nm. A portion of the sample (10 microliters) was manually injected into the injector port, and then the metabolites were analyzed using a dual absorbance UV detector.

Effect of carbon and nitrogen sources on the extent of degradation

Overnight incubation for microorganisms, the growing colonies was suspended in standard saline solution to obtain the optical density of $10^6 - 10^7$ CFU/ml (0.6 at 610 nm wavelength). A portion of two milliliters of cell suspension

were used to inoculate 100 ml flask containing 50 ml MSM with 1 % of each yeast extract or glucose. The used dye concentration was 100 mg/L. Flasks incubated for five days at 37°C at 150 rpm (Sahasrabudhe *et al.*, 2014; Palani Velan *et al.*, 2012). The percentage of azo dye decolorization was determined for each flask daily as previously mentioned.

Microbial degradation of azo dyes by mixed cultures

A liquid medium (MSM) amended with 100mg/L of DR81 was used for mixed culture assay. Mixed culture of bacterial and fungal isolates was inoculated (10^7 CFU/ml) into the medium. Flasks were incubated at 37°C under aerobic and agitation condition at 150 RPM. Samples were detected by UV-Vis Spectrophotometer at 508 nm wavelength for determination of DR81 percent of decolorization at different time intervals. Control was performed with the same procedure but without inoculation.

Microbial Degradation of DR81 by Immobilized Cells

Immobilization experiment was performed to increase the potential capacity of the cells for decolorization. Different solid supports (20 g/L) of ash, marine sand, activated charcoal (AC) or Kaolin of Aldrich were added to a liquid MSM. Recovered isolates were immobilized in solid supports according to the procedure given by (Pokharia and Ahluwalia 2016; Kattimani *et al.*, 2009) with some modification. All solid supports were sieved and sterilized at 121°C for 15 min. One gram of solid support was submerged in 250 ml Erlenmeyer flask containing 50 ml MSM containing a proper concentration of tested isolates, then after 10 days of incubation under agitation condition (150 rpm), the flasks supplemented with 100 mg/L of DR81 to determine the immobilization effect. Samples

were detected at different time intervals by UV-Vis Spectrophotometer at 508 nm wavelength for determination of DR81 percent of degradation. All results were compared with control (uninoculated) which had zero percent of decolorization.

Decolorization of dye in industrial wastewaters

This experiment was carried out to reveal the effect of recovered isolates on industrial wastewaters. Cell suspension (2 ml) with density of 10^6 was used to inoculate 100 ml flask containing 50 ml industrial wastewater with 100 mg/L of DR81, using abiotic control with DR81. The fungal mass was determined along with decolorization reaction at the same time intervals of samples withdrawn to determine fungal survival.

Statistical analysis

Values of data were expressed as means of triplicates \pm SD; statistics were analyzed by one way ANOVA followed by Tukey-Kramer multiple comparisons test; *: Reading were considered significant when $P < 0.05$.

Results and Discussion

Isolation and identification of decolorizing isolates

A total of 43 microbial isolates (25 bacterial isolates and 18 fungal isolates) were recovered as mentioned before. These isolates were then screened to figure out potential decolorizer of DR81 in a medium having 10% of the respective dye. Through the screening process, two isolates (one bacterial isolate and one fungal isolate) were selected as potential dye decolorizer and preserved for further study. Bacterial isolate was identified as *Bacillus pumilus*, and the fungal isolate was identified as *Aspergillus clavatus*. The bacterial isolate

with the strongest decolorizing ability was identified by the conventional microbiological methods to the genus level and by commercially available multitest identification system: analytical profile index (API) (Figure 1). Test strips were used as the instructions provided. Fungal isolate identification was performed by Identification Unit of Fungi, Regional Center of Mycology and Biotechnology Azhar University, Cairo, Egypt. A fungal isolate *Aspergillus clavatus* was re-identified by PCR and sequencing. The gene sequence of *Aspergillus clavatus* has been deposited in GenBank with the accession number HQ026749.1 as shown in figure 2.

Decolorizing assay

Detection of decolorization of DR81 by plate assay

Decolorizing activity of degradable isolates was detected by plate assay. Clearing zone was formed surrounding the growing cultures on nutrient agar or Sabouraud agar plates containing 100 mg/L DR81 within 7 days. Isolates decolorization ability was shown in figure 3. The diameter of decolorization zone was calculated after 7 days of incubation, *Bacillus pumilus* and *Aspergillus clavatus* showed the highest decolorization activity on DR81 compared to the other recovered isolates.

Spectrophotometer analysis

DR81 solution was analyzed by UV-Vis Spectrophotometer. Figure 4A describes that 100 mg/L of DR81 solution absorbed light maximally at 508 nm. Decolorization results with *Bacillus pumilus* after 5 days showed that the main peak at 508 nm decreased approximately (Figure 4B) while the peak was completely disappeared with *Aspergillus clavatus* after 2 days (Figure 4C). In this regard, the main peak at 281 nm was

decreased after 5 days with bacteria while the peak was shifted to 335 nm with *Aspergillus clavatus*. The marked decrease of the peak at 508 nm by fungi indicates almost complete decolorization of DR81. Decolorization percentage of DR81 by both *Bacillus pumilus* and *Aspergillus clavatus* was shown in Table 1.

High Performance Liquid Chromatography analysis (HPLC)

After 5 days of aerobic treatment process, HPLC analysis for biodegradation products was carried out. HPLC analysis of DR81 as a control showed a peak at the retention time of 1.588 min. (Figure 5A), and there was no new peak was observed with *Bacillus pumilus* indicating that decolorization was due to its degradation into unidentified intermediates, while *Aspergillus clavatus* showed two metabolites peaks at the retention times of 2.036 and 4.257 min. (Figure 5B). All samples showed the appearance of dye peak in the metabolites along with appearance of new peaks with different retention times which supported dye biotransformation to different metabolites.

Effect of carbon and nitrogen sources on the extent of biodegradation

In this study, 1% of each glucose and yeast extract was used as a carbon and nitrogen source, respectively. Figure 6 shows the effect of different nitrogen and carbon sources on decolorization of DR81 by *Bacillus pumilus* and *Aspergillus clavatus*. After two days of incubation, the percentage of DR81 decolorization for *Bacillus pumilus* and *Aspergillus clavatus* without any supplement was 8% and 73%, respectively. While the percentage of DR81 decolorization for *Bacillus pumilus*, when supplemented with yeast extract was 91% and 98% for *Aspergillus clavatus*. The decolorization

percent of DR81 with glucose was found to be 20% for *Bacillus pumilus* and 98% for *Aspergillus clavatus* (Table 2). *Aspergillus clavatus* obtained the high extent of decolorization after 2 days of incubation. Yeast extract and glucose had the same effect with *Aspergillus clavatus* while yeast extract was more efficient than glucose with *Bacillus pumilus*.

Degradation of DR81 by mixed culture

This experiment was performed to see the effect of mixed culture on decolorization of DR81. *Bacillus pumilus* and *Aspergillus clavatus* used together as a mixed culture. Percent of dye degradation increased from 17.48% to 87.69% by *Bacillus pumilus* and *Aspergillus clavatus* mixed culture with regard to *Bacillus pumilus* degradation as shown in Table 3.

Effect of immobilized cells

The efficiency of immobilized microbial cells to decolorize the DR81 was investigated at agitated conditions. Immobilized bacterial cells on ash were able to degrade about 56% of initial dye concentration while immobilized fungal cells were degrading about 65%. The percentage of DR81 decolorization when using marine sand as solid support with *Bacillus pumilus* was 31% while it was 52% with *Aspergillus clavatus*. Furthermore, immobilized *Bacillus pumilus* cells on activated charcoal were degrading 52% of the dye while immobilized *Aspergillus clavatus* cells were degrading 74% of initial dye concentration. And when using kaolin as a solid support, bacterial degradation was 71% while fungal degradation was 70%. The results obtained in this method indicate that the percentages of DR81 decolorization for free bacterial and fungal cells were 17% and 95%, respectively of initial dye concentration within 5 days of incubation. *Bacillus pumilus*

immobilized cells had the best decolorization effect on kaolin (71%) (Table 4), while *Aspergillus clavatus* immobilized cells had the best decolorization effect on activated charcoal (74%) of the initial dye concentration at the same time interval. Nevertheless, the unexpectedly result was obtained with *Aspergillus clavatus* free cells that had the highest extent of DR81 decolorization (94.93%) compared to their immobilized cells on different solid supports, as shown in Table 5.

DR81 decolorization in industrial wastewaters

This experiment was carried out to determine the capacity of the microbial degraders to function in industrial wastewaters. Low inoculum density of *Aspergillus clavatus* showed more significant DR81 decolorization in sterile condition than in non-sterile one. High inoculum density had a better effect than low concentration. These results illustrated after five days of incubation as shown in figure 7.

Survival of *Aspergillus clavatus* in industrial wastewater

This experiment determined the survival rate of the dry weight of *Aspergillus clavatus* as shown in figure 8. The present data showed that *Aspergillus clavatus* could not survive in industrial wastewater for a long time. Discharge of textile azo dyes to the environment is an issue of health concern while the use of the biological system has proved to be the best option for remediation. The effluents of industrial dyes are the major sources of water pollution, threatening the existence of aquatic biodiversity; alternate eco-friendly approaches are needed to remediate the dye-contaminated wastewaters discharged from a range of textile industries (Aruna *et al.*, 2015).

Table.1 Decolorization percentage of DR81 (100 mg/L)

Microbial isolates	Absorbance at 508 nm	Percentage of dye decolorization	Percentage of dye remaining
<i>Bacillus pumilus</i>	1.8 ± 0.15	17.479%	82.52%
<i>Aspergillus clavatus</i>	*0.69 ± 0.72	94.929%	5.07%

Values are expressed as means of triplicates ± SD; statistics was carried out by one way ANOVA followed by Tukey test; *: significantly different from control value at P<0.05.

Table.2 Effect of glucose and yeast extract on decolorization percent of 100 mg/L DR81 by bacterial and fungal isolates after 2 days incubation

Supplements	Extent of DR81 degradation by <i>Bacillus pumilus</i> and <i>Aspergillus clavatus</i>	
	<i>Bacillus pumilus</i>	<i>Aspergillus clavatus</i>
Supplement-free	8%	73%
With Glucose	20%	98%
With Yeast extract	91%	98%

Table.3 Decolorization % of DR81 by *Bacillus pumilus* /*Aspergillus clavatus* mixed culture

Microbial isolates	Absorbance at 508	Percentage of dye decolorization	Percentage of dye remaining
<i>Bacillus pumilus</i>	1.8 ± 0.15	17.48%	82.52%
<i>Aspergillus clavatus</i>	*0.69 ± 0.72	94.93%	5.07%
<i>Bacillus pumilus</i> with <i>Aspergillus clavatus</i>	*0.7 ± 0.7	87.69%	12.31%

Values are expressed as means of triplicates ± SD; statistics was carried out by one way ANOVA followed by Tukey test; *: significantly different from control value at P<0.05.

Table.4 Decolorization % of DR81 by free and immobilized cells of *Bacillus pumilus*

Free/ immobilized cells	Absorbance at 508 nm	Percentage of dye decolorization	Percentage of dye remaining
Free cells	1.8 ± 0.15	17.48%	82.52%
Immobilized cells with ash	*1.3 ± 0.48	55.93%	44.07%
Immobilized cells with marine sand	1.73 ± 0.29	32.05%	67.95%
Immobilized cells with activated charcoal	*1.56 ± 0.49	51.58%	48.42%
Immobilized cells with kaoline	*1.58 ± 0.63	70.91%	29.09%

Values are expressed as means of triplicates ± SD; statistics was carried out by one way ANOVA followed by Tukey test; *: significantly different from control value at P<0.05.

Table.5 Decolorization % of DR81 by free and immobilized cells of *Aspergillus clavatus*

Free/ immobilized cells	Absorbance at 508 nm	Percentage of dye decolorization	Percentage of dye remaining
Free cells	*0.69 ± 0.72	94.93%	5.07%
Immobilized cells with ash	1.18 ± 0.54	64.75%	35.25%
Immobilized cells with marine sand	1.53 ± 0.44	51.69%	48.31%
Immobilized cells with activated charcoal	1.3 ± 0.65	73.76%	26.24%
Immobilized cells with kaoline	1.3 ± 0.66	70.11%	29.89%

Values are expressed as means of triplicates ± SD; statistics was carried out by one way ANOVA followed by Tukey test; *: significantly different from control value at P<0.05.

Fig.1 API 50 Ch: for identification of *Bacillus pumilus*



Fig.2 Phylogenic tree of *Aspergillus clavatus* strain. This tree was produced using BLAST pairwise alignments

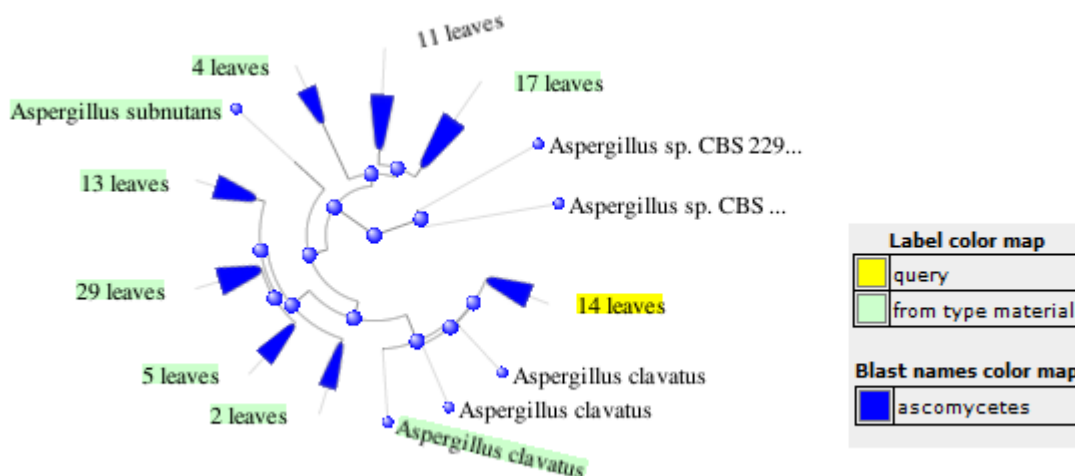


Fig.3 Decolorization activity of potent isolates by plate assay of DR81 (100 mg/L). (A) *Bacillus pumilus* and (B) *Aspergillus clavatus*

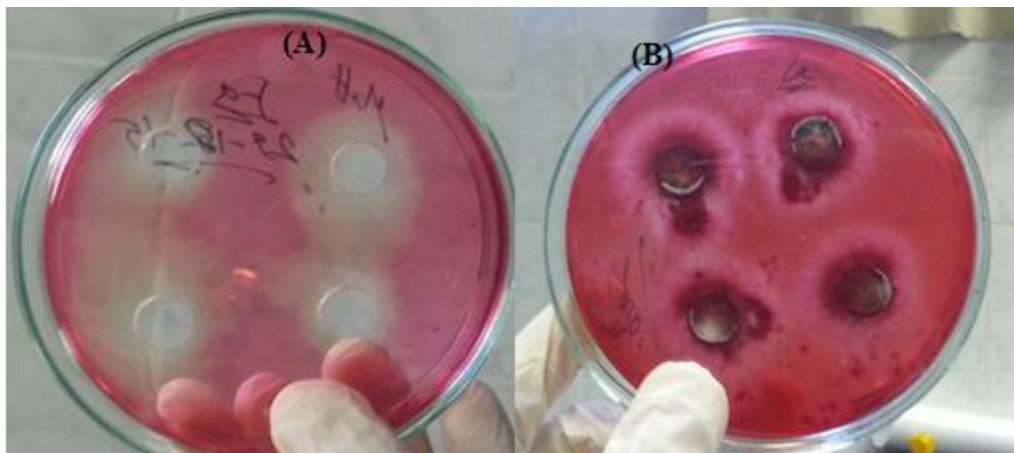


Fig.4 (A) UV-vis spectra (200-800 nm) of DR81 (100mg/L) in MSM, (B) UV-vis spectra of DR81 (100 mg/L) in MSM, before and after decolorization by *Bacillus pumilus* and (C) UV-vis spectra of DR81 (100 mg/L) in MSM, before and after decolorization by *Aspergillus clavatus*

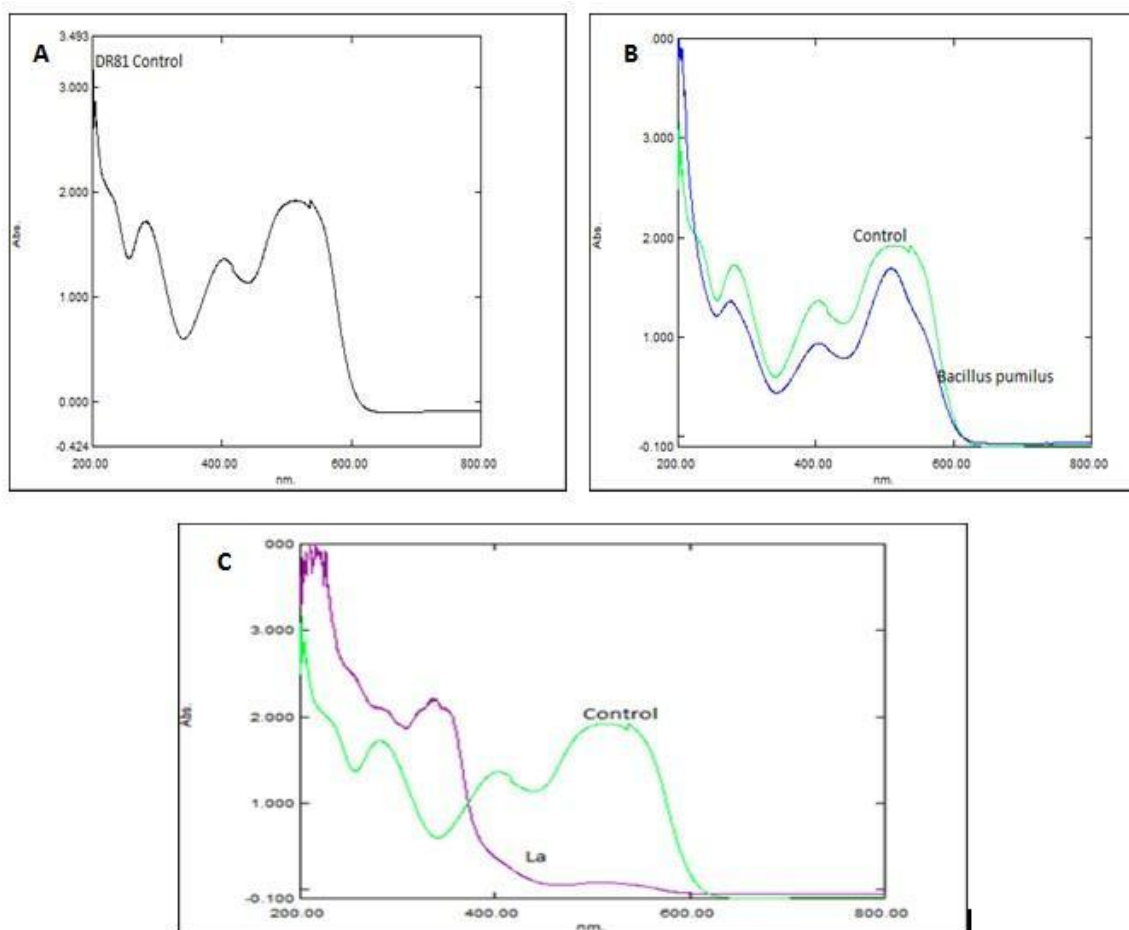


Fig.5 (A) HPLC profile of 100 mg/L DR81 and (B) HPLC profile of DR81 treated by *Aspergillus clavatus*, showing dye metabolites after decolorization

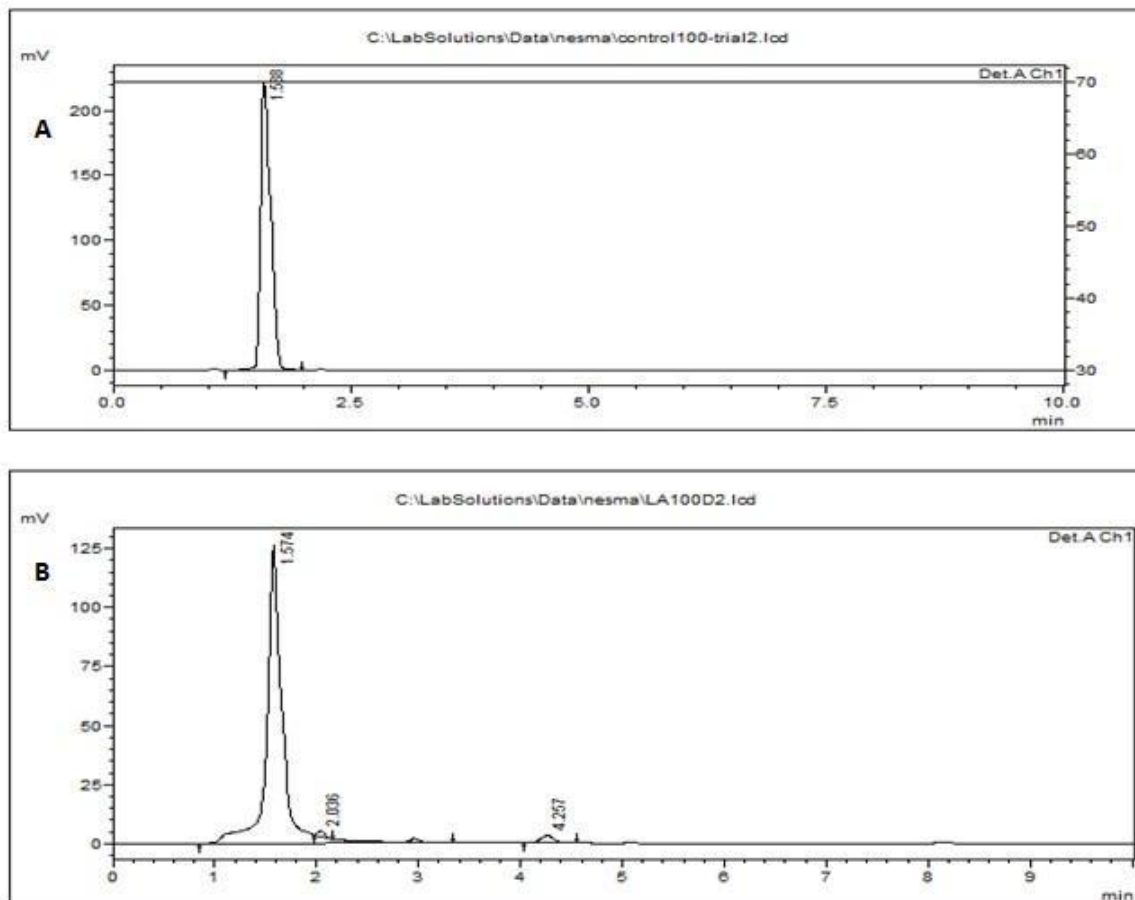


Fig. (6i) DR81 degradation after 2 days by *Bacillus pumilus* when supplemented with glucose or yeast extract



a) At zero time, b) supplemented with glucose, c) supplemented with yeast extract

Fig. (6ii) DR81 degradation after 2 days by *Aspergillus clavatus* when supplemented with glucose or yeast extract



a) At zero time, b), supplemented with glucose c) supplemented with yeast extract

Fig.7 Decolorization percent of DR81 (100 mg/L) in industrial wastewater of low cell density of (A) *Bacillus pumilus*, and (B) *Aspergillus clavatus*

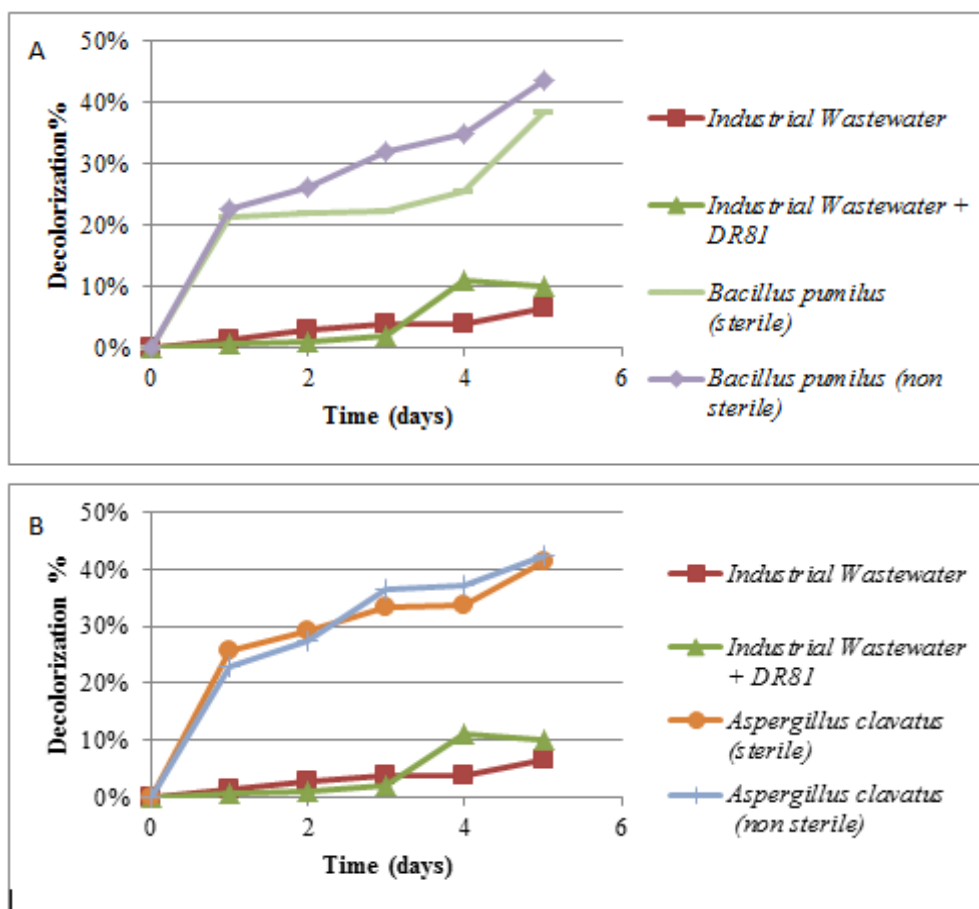
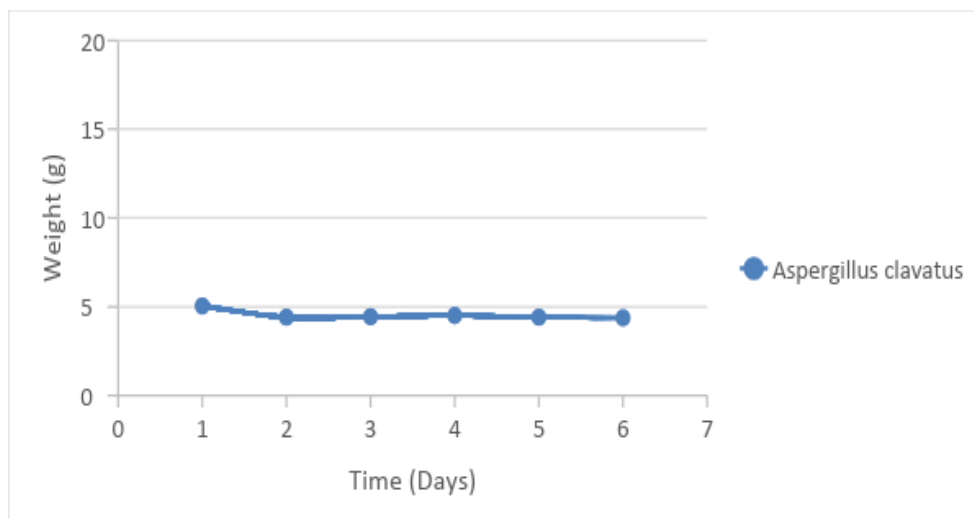


Fig.8 Fungal growth of *Aspergillus clavatus* during decolorization of DR81 in industrial wastewater



The objective of this study was evaluating the potential isolates that were screened out in solid/broth media for DR81 decolorization because of their consumption demand and extensively used azo dye in the textile industry (Lade *et al.*, 2015; Kadam *et al.*, 2011).

The bacterial culture showed the low extent of decolorization (17%), while fungal culture showed higher decolorization (95%). Many bacteria need the availability of a supplementary carbon source, and therefore they do not use azo dyes as a sole of carbon and energy source (Sahasrabudhe *et al.*, 2014; Stolz, 2001), this is the reason for the low extent of bacterial decolorization. On the other hands, fungi can produce the lignin-modifying enzymes to decolorize and/or mineralize azo dyes (Unyayar *et al.*, 2005) and can also act by biosorption which defined as binding of solutes to the biomass. Also, the processes do not involve metabolic energy or transport (Mehta and Saini 2017; Bishnoi and Garima 2005; Tobin *et al.*, 1994).

HPLC chromatographic analysis of biodegradation metabolites of DR81 by

Bacillus pumilus showed no new peak indicating that decolorization was due to its degradation into unidentified intermediates, while by *Aspergillus clavatus* it showed new metabolites peaks with different retention times (2.036 and 4.257 min.).

As mentioned before, azo dyes are a lack in carbon source so, decolorization is difficult when to use it as a sole of carbon source (Palanivelan *et al.*, 2014; Chang *et al.*, 2004). Erum and Ahmed (2010) reported that excellent correlation between the decolorization speed and additional nutrient concentration reached up to 92.9% in a short time. Supplemental nutrients could change decolorization ability of azo dye. Therefore, in the present study effect of carbon and nitrogen sources on the extent of decolorization was demonstrated. Palanivelan *et al.*, (2014); Jang *et al.*, (2007) observed that addition of 1% yeast extract (as a nitrogen source) or glucose (as a carbon source) in the medium increased the color removal which also reported in the present study. After two days of incubation, decolorization of DR81 by *Bacillus pumilus* and *Aspergillus clavatus* without any supplement was 8% and 73%,

respectively. While it was 91% and 98% when yeast extract was supplemented as a nitrogen source for *Bacillus pumilus* and *Aspergillus clavatus*, respectively. Moreover, after two days of incubation, the percentage of DR81 decolorization with glucose was found to be 20% for *Bacillus pumilus* and that for *Aspergillus clavatus* was 98%.

From the above data, it is observed that *Aspergillus clavatus* was more efficient in decolorizing DR81 than *Bacillus pumilus*. Yeast extract and glucose had the same effect with fungal isolate while yeast extract was more efficient in the decolorizing of DR81 than glucose with bacterial isolate. The use of organic nitrogen source yeast extract gave better decolorization and it counted as a fundamental supplement to regain NADH in the medium, that act as an electron donor for dye reduction by bacteria (Palanivelan *et al.*, 2014; Hu 1994).

Most studies used the bacterial mixture or fungal mixture separately. Junnarkar *et al.*, (2006) mentioned that decolorization of DR81 by a bacterial consortium was reached 84-95% while, Rathnan *et al.*, (2013) mentioned that fungal dye decolorization was ranged from 85% to 100%. In this study, two potential isolates were used as the bacterial/fungal mixture (*Bacillus pumilus* with *Aspergillus clavatus*).

Pure individual cultures of *Bacillus pumilus* and *Aspergillus clavatus* gave dye decolorization percent of 17.5% and 94.9%, respectively while their mixed culture gave decolorization percent of 87.7%. These results indicated that bacterial/fungal mixed culture had dye decolorization effect better than decolorization effect of pure bacterial culture.

Immobilized cells of *Bacillus pumilus* had much better decolorization effect on the DR81 compared to its free cells, and its best

decolorization effect was with kaolin (71%). This result indicates that the dye decolorization by immobilized bacterial cells is better than free cells. Chen *et al.*, (2017); Kattimani *et al.*, (2009); Rogalski *et al.*, (1995) observed similar findings, concerning immobilization, who reported that it is a technique to enhance cells productivity and concentration.

Besides, the immobilization enhances enzymes stabilities, which are much less exposed to inhibitors than the free system. But surprisingly, *Aspergillus clavatus* free cells had the highest extent of dye decolorization (94.9%) compared to their immobilized cells (73.77%). As mentioned before, the reason was due to the fungi can produce the lignin-modifying enzymes to decolorize and/or mineralize azo dyes without needing any support to enhance their action, and can also act by biosorption.

Fungal survival and growth is dependent on the ability of obtaining energy from the metabolism of substrate. The rough conditions that could be found in the industrial wastewater, could limit the survival of organisms. So it is important to combine a high efficiency treatment (Spina *et al.*, 2012).

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