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Bacterial Isolation, Optimization and Immobilization for Decolorization and Degradation of the Azo Dye (Basic Red 46).

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ABSTRACT

Dyes are the major toxic substances in all industrial wastewater and are highly carcinogenic to human beings and other soil and water living things. In this study, the most efficient bacterial isolate in decolorization of Basic Red 46 dye, isolate A3, was isolated and identified as *Enterobacter cloacae* sp. under the accession number KP202719. Different parameters such as dye concentration (100 to 4200 mg/L), pH (2, 4, 6, 7, 8 and 10), time (6 to 48hrs), shaking or static and temperature (25, 30, 37, and 45ºC) were optimized in the present study. It was found that *Enterobacter cloacae* sp. A3 decolorized 90% of BR46 dye (2000 mg/L) at pH 6 at 30°C within 18 hrs under shaking condition and the minimum inhibitory concentration for strain A3 was 3600 mg/L. The immobilization of A3 in calcium alginate was more effective in dye decolorization than the using of free cells. The continuous fed batch decolorization study reported that the ability of immobilized strain A3 for the decolorization of repeated added 2000 mg/L of dye reach to 97.7%, 65% and 20.8% after three batch cycles respectively. This immobilized bacterial strain represents eco-friendly biosorbent for dye removal from colored industrial wastewater.

Keywords: Decolorization; Optimization; Basic Red 46 dye; Immobilization; Continuous fed batch; *Enterobacter cloacae* sp.

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INTRODUCTION

Water is not a commercial product but, over the last few decades, increasing globalization, urbanization, and industrialization have causes different environmental pollution [1]. Wastewater containing a number of contaminants one of the important categories of these contaminants are dyes once they enter the water it is no longer good and sometimes difficult to treat as the dyes have an artificial origin and a complex molecular structure which makes them difficult to be biodegraded and more stable [2- 3].

Synthetic dyes are defined as colored substances which extensively used in industries, when applied to products give them a permanent color which is able to resist fading upon exposure to light, sweat, water and many chemicals, including oxidizing agents and microbial attack [3]. More than 10,000 synthetic dyes were developed and used in manufacturing by the end of the 19th century [4].

Moreover, the growth of the worldwide textile industry in these years had seen a proportional increase in the use of such synthetic dyes, and this has been accompanied by a rise in pollution due to wastewater contaminated with dyestuff [5].

Textile industry generates maximum liquid effluent, as a large quantity of water is used in the dyeing processes [6]. As all the dyes do not bind to fabric, their loss in waste water diverges from 2 % of basic dyes to 50 % for reactive dyes, so the discharge of textile industries causing severe contamination of both surface and ground waters [7]. The global discharging of industrial effluents every year is estimated by 2,80,000 tons of textile dyes every year [8]. From all dyestuffs used worldwide azo dyes make up approximately 70% by weight. Which making them the major group of synthetic colorants and the most common synthetic dyes released into the environment [9-11].

Azo dyes accounts for the mainstream (more than 3000 different varieties) of all textile dyestuffs produced because of the ease and cost effectiveness of their synthesis, the variety of colors available compared to natural dyes and their stability [12]. Azo dyes are extensively used in the textile, paper, food, leather, cosmetics, rubber, coloration of products and pharmaceutical industries [13]. Improper discharge of effluents containing azo dyes and their metabolites in aqueous ecosystems is aesthetically unpleasant. Color is the one of the characteristic of an effluent which leads to a reduction in sunlight penetration, which in turn decreases water quality, photosynthetic activity, dissolved oxygen concentration and had acute toxic effects on aquatic flora and fauna, causing severe environmental problems worldwide [14-16]. Azo dyes also have a contrary impact in terms of chemical oxygen demand (COD), biological oxygen demand (BOD) and total organic carbon (TOC) [17]. Many synthetic azo dyes and their metabolites are toxic, mutagenic and carcinogenic [18].

The industrial effluents containing azo dyes and their metabolites are essential to management prior to their final release to the environment. The current existing techniques, such as ozonation, chemical coagulation/ flocculation, chemical precipitation, oxidation processes, reverse osmosis, ion exchange and ultrafiltration etc., for the removal of dyes from dye containing wastewater have serious restrictions such as formation of hazardous by-products, high cost, or intensive energy requirements [19-20].

Microbial or enzymatic decolorization and degradation is an eco-friendly cost-competitive, producing less sludge, yielding end products that are non-toxic or have complete mineralization; and reduce water consumption compared to physicochemical treatment methods [3, 21]. Bioremediation, or the use of microbial techniques to deal with pollution, is a key research area in the environmental sciences. In such approaches microbes adapt themselves to the toxic wastes and new resistant strains develop naturally, which then transform various toxic chemicals into less harmful forms [21]. A number of biotechnological approaches have attracted interest with regard to remove azo dye pollution in a coefficient manner, mainly with the use of microorganisms and often in combination with physicochemical processes [3].

A wide variety of microorganisms are capable of decolorizing of a wide range of dyes including; bacteria, fungi, yeasts, algae and plants. Moreover, these are even capable of completely

mineralizing many azo dyes under certain environmental conditions [22]. One of the main problems concerning the using of microorganisms in the treatment of dyes pollution is the toxic effect of dyes on the microorganisms. Therefore the recent research is focused on new approaches for increasing the cell concentration and protection of it from toxicity. One of the methods applied for maintaining high cell concentration and protection of it is immobilization.

The immobilized microorganism technology offers a crowd of advantages, such as high metabolic activity, high bio-mass and strong resistance to toxic chemicals. Moreover, immobilized microorganisms can be used several times without significant loss of activity accordingly it is cost effective [23-24]. Among the various developed methods for cell immobilizing the encapsulation in calcium alginate beads was used in this study. Alginates (polymers made of different proportions and sequences of guluronic and mannuronic acids extracted from brown algae) are nontoxic to humans and the environment, easy to handle, the entrapped microorganisms, legally safe for human use, available in large quantities, and economical. From a physiological perspective, a main advantage of alginate is that immobilized cells during the procedure of immobilization do not suffer great changes in physicochemical condition and the gel is transparent and permeable [25].

The aim of this study is to investigate the biodegradation and decolorization of some synthetic azo dyes present in industrial wastewater using biological methods by isolation and identification of microorganisms capable of decolorizing Basic Red 46 azo dye, in addition, the effects of various parameters such as (dye concentration, pH , incubation time, temperature, static and agitated condition incubation) on dye decolorization by the bacterial strains were investigated to know the optimum conditions for their activities. Attempts were made to study their dye degradation potential after immobilization of selected isolates on calcium alginate and their application in continuous culture systems with using immobilized bacteria.

MATERIALS AND METHODS

Collection of Samples

The wastewater and sediment samples were collected from the drain of different locations of some local industrial outlets which contaminate area by dyes in Alexandria, Egypt. The wastewater samples and sediment soil samples were collected using sterilized plastic bottles, allowing enough air space in the bottles for bacteriological analysis.

Reagents and Solutions

Basic Red 46 dye (BR 46) was obtained from local textile factory and was used without further purification. BR 46 was defined as basic azo dye its molecular formula ($C_{18}H_{23}N_6Br$) and measured in wavelength (λ) 530 [26-27]. The chemical structure of this dye is shown in Figure1. All the other chemicals used were of the highest purity available of analytical grade.

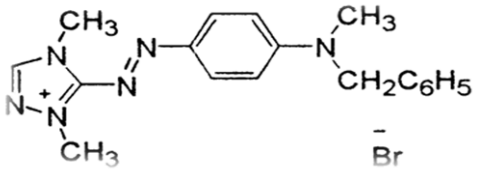

Figure 1. Chemical structure of Basic Red 46 dye.

Isolation and Purification of Decolorizing Bacterial Isolates

Ten gram of each three soil samples or 5 mL of water sample (totally four samples) were inoculated into 100 mL of three different media, LB medium containing (g/L), 10- Peptone, 5- Yeast Extract, 5- Sodium Chloride (pH 7.0) [28], Zhou and Zimmermann ZZ medium containing (g/L), 5-yeast extract, 5-glucose, 0.5-$(NH_4)_2SO_4$, 2.66-KH_2HPO_4, 4.32-Na_2HPO_4, (pH 7.0) [29] and Minimal Salt Medium (MSM) medium had the following composition (g/L): 1.8- K_2PO_4, 1.2- KH_2PO_4, 4.0- NH_4Cl, 0.2-

$MgSO_4$. $7H_2O$, 0.1- NaCl, 0.01- $FeSO_4$.$7H_2O$ (pH 7.0) [30]. The flasks inoculated with 50 mg/L of BR 46, tightly sealed and incubated at 30°C for 3 days under shaking condition (200 rpm). The medium was decolorized, and then the samples were tenfold serially diluted and 100 µL aliquots of each dilution were spread onto LB, ZZ and MSM media agar plates with 50 mg/L of BR 46 [20, 31]. After 24 hrs incubation at 30°C, morphologically different colonies were streaked on agar plates for purification before being inoculated into the liquid medium to check their decolorizing ability. The bacterial isolates with the strongest decolorizing ability, given a prefix of "A" followed by numbers in a series from 1 to 7 (A1, A2, A3, A4, A5, A6 and A7). Then the purified strains were preserved in mixture of LB medium and glycerol at -20°C [32-33].

Molecular Identification of the Bacterial Isolate

A pure colony of the bacterium was grown in LB medium until log phase growth was obtained. The bacteria pallet was obtained with centrifugation. Genomic DNA from the isolate was extracted with the bacterial genomic DNA Isolation Kit (RKT09). Amplification was done using prokaryotes 16S rRNA specific forward Primer: 5´-AGAGTTTGATCMTGGCTCAG-3´ and reverse Primer: 5'-TACGGYACCTTGTTACGACTT-3'. Amplified product was gel purified using Qiagen gel extraction kit and purified products were sequenced [34]. The 16S rRNA sequence (~950 bp) was compared with currently available microorganism sequences in Gen Bank by BLAST (www.ncbi.nlm.nih.gov/blast) sequence analysis to affiliate the bacteria isolate. The Gen Bank accession numbers of isolate A3 is KP202719.

Decolorization of Azo Dye in Liquid Medium

The bacterial isolates (from A1 to A7) that were selected and further examined for their decolorization potential on the BR 46 dye under study. In a 250 mL autoclaved Erlenmeyer flasks containing 50 mL of each sterilized LB and ZZ broth medium mixed with 50 mg/L of BR 46 dye and 4% (v/v) inoculums of each isolate separately (the preculture with OD: 1.4 at 600 nm). The flasks were tightly sealed and were incubated under shaking conditions at 30°C/ 200 rpm for 48 hrs. Aliquots (10 mL) were taken from different flasks and centrifuged at 6000 rpm for 15 min to separate the bacterial cell mass. The culture supernatant was used for spectrophotometric analysis at 530 nm for dye to determine decolorization percentage [20].

From the above step it was found that the three effective bacterial isolates for their decolorization potentials for BR 46 were (A2, A3 and A5). They were further examined to determine the most effective isolates that were able to make degradation and decolorization for the BR 46 dye at different concentration (100 to 1200 mg/L) with 4% inoculums of uniform cell density (OD: 1.4) at 600 nm for each isolate (A2, A3 and A5) incubated at 30°C/ 200 rpm. Decolorization was measured after 48 hrs at 530 nm by spectrophotometer [20, 35].

Decolorization Assay Via UV-Vis Spectroscopy

Calibration curve was prepared by plotting known concentrations of BR46 dye versus corresponding absorbance values. Decolorizing activity was expressed in terms of percentage decolorization and was determined by monitoring the decrease in absorbance at 530 nm (λ max of BR46) by UV /VIS spectrophotometer (Jenway UV/Visible- 2605 spectrophotometer, England). The distilled water was used as blank, samples containing BR46 dye were used as reference (control) and each experiments culture supernatant was used as sample then applying the following equation for calculating percentage of decolorization [36].

Decolorization % = [Reading of (C) decolorization – Reading of (S) decolorization]/ Reading of (C) decolorization × 100

Where C= control, S= sample.

Effect of Dye Concentration (Minimum Inhibitory Concentration, MIC)

The most powerful isolate that was able to decolorize BR 46 dye was A3 isolate using ZZ medium and various concentrations of dye were tested (100 , 200 , 300 , 500 , 600, 800 , 1000, 1200, 1400 , 1600, 1800, 2000, 2200, 2400, 2600, 2800, 3000, 3200, 3400, 3600, 3800, 4000 and 4200 mg/L). The various concentrations were added to sterilized ZZ broth medium and 4% inoculums of A3. The tubes were tightly sealed and were incubated under shaking conditions at 30°C/ 200 rpm for 48 hrs. After incubation period samples were taken to measure the extent of decolorization of the dye by spectrophotometer. The minimum concentration of dye in the medium inhibiting complete growth was taken as the MIC [20, 35, 37].

Effect of Temperature and Static or Shaking Conditions

The effect of temperature on the decolorization of the BR 46 was monitored in different temperatures (25, 30, 37, and 45°C) [38].This experiment was done using sterilized ZZ broth medium inoculated with isolate A3 (4%) and mixed with 1200 and 2000 mg/L of BR 46 separately. The tubes were tightly sealed and were incubated once under shaking conditions (200 rpm) and also tested at static condition for each temperature. After different time intervals (24 and 48hrs) aliquot 5 mL of the culture media was withdrawn, centrifuged at 6000 rpm for 15 min to separate the bacterial cell mass. The supernatant was measure spectrophotometrically to determine the decolorization percentage [20].

Effect of pH on Decolorization

This was performed by using ZZ broth medium at different pH (2, 4, 6, 7, 8 and 10 adjusted with 1M HCL or 1M NaOH) were inoculated with 4% of the selected bacterial isolate A3 at constant concentration 2000 mg/L of BR 46 and incubated under shaking conditions at 30°C/ 200 rpm. The effect of pH on dye decolorization was checked spectrophotometrically periodically after 24 and 48 hrs incubation [38].

Measurement of Decolorization Extent

The effect of incubation time on the dye decolorization percentage was carried out under optimum conditions obtained from above studies. The ZZ broth medium was cultured with 4% of A3 isolate at optimum temperature (30°C), optimum pH (6), shaking conditions (200 rpm) and amended with 2000 mg/L of BR 46 dye. The incubation time up to 48 hrs and aliquots were taken periodically different time intervals from the culture media (6, 12, 18, 24, 30, 42 and 48 hrs). Samples were withdrawn and supernatant obtained after centrifugation then the dye decolorization was checked spectrophotometrically [29].

Immobilization of Efficient Bacterial Isolate

Cells of isolate A3 were obtained from culture grown in 250 mL of inoculation medium (LB) at 30°C for 24 hrs under shaking conditions at 200 rpm harvested by centrifugation at 10000 rpm for 10 min and washed thoroughly with 0.85 % sterile saline solution. The bacterial cells wet weight was (0.814 g). Then were encapsulated and mixed with 50 mL sodium alginate (4%).This mixture was introduced in an electro static droplet generator. The droplets were collected in 0.2M $CaCl_2$ solution. The beads were left in the $CaCl_2$ solution for about 3 hrs and then thoroughly washed with distilled water three times then it was kept in refrigerator at 4°C to be used for further investigations [36].

Efficient Number of Calcium Alginate Beads for Decolorization

Variable number of beads (1 bead/ mL, 2 beads/ mL, 3 beads/ mL and 4 beads/ mL) were mixed with 2000 mg/L of BR 46 dye and incubated under shaking conditions at 30°C/ 200 rpm for 48 hrs. Aliquots were taken periodically every 24 hrs to calculate the percentage of decolorization. Samples were withdrawn into serum vials and supernatant obtained after centrifugation (10,000 rpm for 10 min) was run through spectrophotometer at λ max for BR 46 dye (530 nm).

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Continuous Fed Batch Process for Dye Decolorization

The experiment was done by using the efficient number of beads (2 beads/ mL) in ZZ broth medium mixed with 2000 mg/L of BR 46 dye. That flask was tightly sealed and incubated at 30°C/ 200 rpm for a time period (48 hrs) under shaking conditions. 1 mL was taken periodically different time intervals from the culture media (3, 6, 12, 18, 24, 30, 36, 42 and 48 hrs). Samples were withdrawn into serum vials and supernatant obtained after centrifugation (10,000 rpm for 10 min) was measure spectrophotometrically to determine the decolorization percentage.

After the first cycle (2 days), the same concentration of RB 46 dye (2000 mg/L) was added to the alginate beads containing encapsulated cells in a second cycle and incubated for 48 hrs at the same conditions, also aliquots were taken periodically at different time intervals from the culture media (3, 6, 18, 24, 30, 36, 42 and 48 hrs). Samples were measured the decolorization percentage by spectrophotometer. The third repeated batch cycles were performed at the same condition.

RESULTS AND DISCUSSION

Isolation, Purification and Identification of Bacterial Isolate

Dye waste effluent samples were collected from the drain of different locations of some local industrial outlets which contaminate area by dyes in Alexandria, Egypt. About 50 isolates were obtained from primary screening and 7 isolates from secondary screening which named from A1 to A7. The three effective isolates A2, A3 and A5 having decolorization ability of Basic Red 46 dye from 100 to 1200 mg/L concentration with 39%, 99% and 20% of decolorization for A2, A3 and A5 respectively after 48 hrs of incubation (Figure 2), and by testing onto different media LB, ZZ and MSM broth and agar plates it was found that the ZZ medium is the best in the decolorization efficiency according to decolorization area shown on the agar plate. So the isolate A3 and the ZZ medium were selected for further experiment.

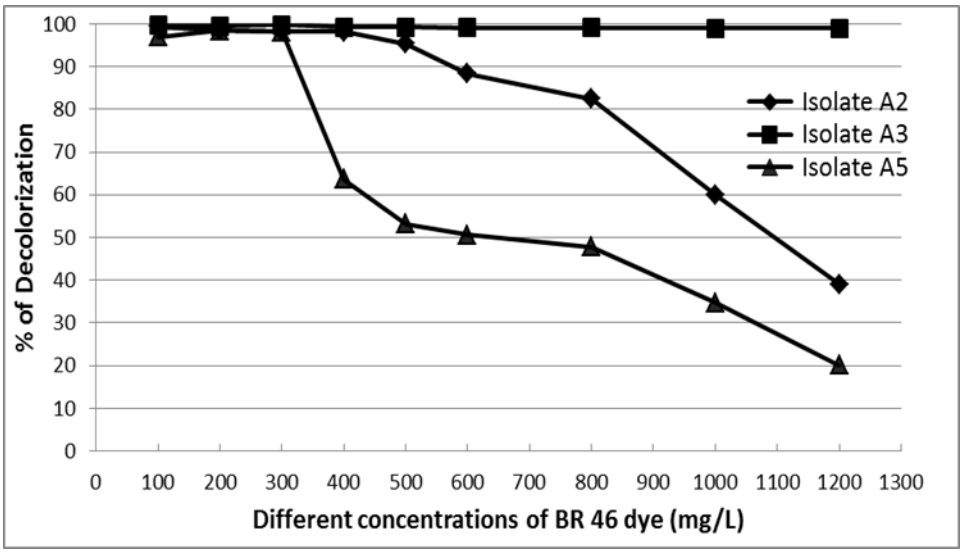

Figure 2. Effect of BR 46 dye concentration on decolorization performance of different isolates.

16S ribosomal RNA gene sequence was performed for identification of the isolate A3. 16S rRNA sequence (~960 bp) was analyzed using BLASTn and multiple sequence alignment was performed using CLUSTAL W. Based on the phenotypic characteristics and phylogenetic analysis, Phylogenetic tree was constructed using BioEdit [39]. Result revealed that the isolate A3 identified as *Enterobacter cloacae* sp. which was found to be 99% identical to *Enterobacter cloacae* sp. strain U27616 (KF516278) among selected taxonomic group. The isolate A3 was deposited in the Gen Bank database under accession number KP202719 (Figure 3).

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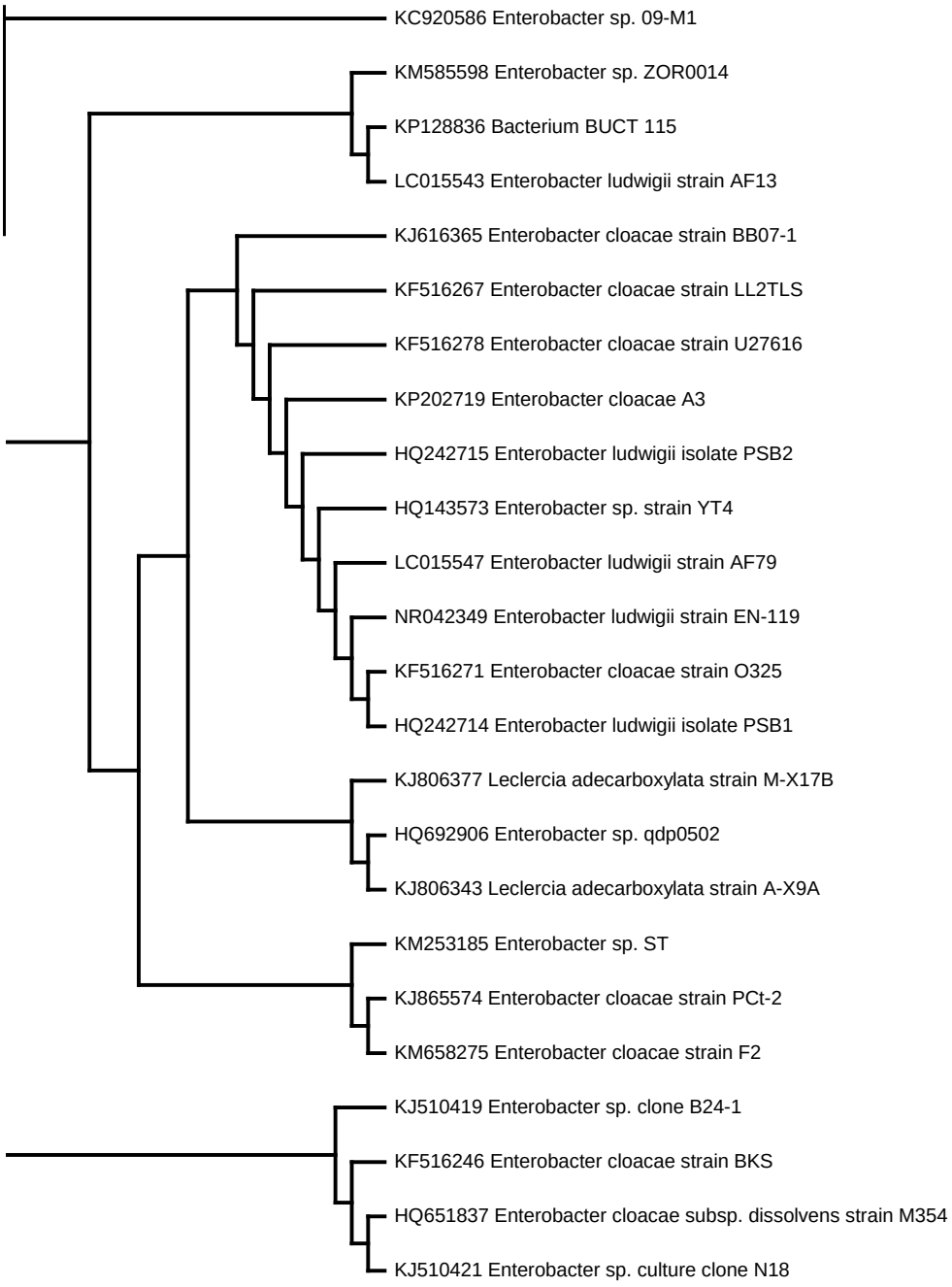

Figure 3. Phylogenetic tree showing the relationships among selected isolate.

Effect of Dye Concentration

The effect of initial dye concentration for the BR 46 dye was investigated in the concentration range (100 to 4200 mg/L). The results in Figure 4 reveals that the decolorization capacity of dyes by using *Enterobacter cloacae* sp. A3 reach to more than 99% up to 1200 mg/L but it has a high efficiency for dye decolorization which reach to 93.7% of decolorization at 2000 mg/L after 48 hrs. The percentage of decolorization still at the same range until 3400 mg/L but at 3600 mg/L decreased to 67.85% then the percentage of decolorization decrease continuously by increasing the dye concentration to reach 11.37% at 4200 mg/L, this result indicated that strain A3 showed high decolorizing performance even in high initial dye stuff concentrations. The result obtained pointed out that the exact MIC of the *Enterobacter cloacae* sp. A3 was 3600 mg/L.

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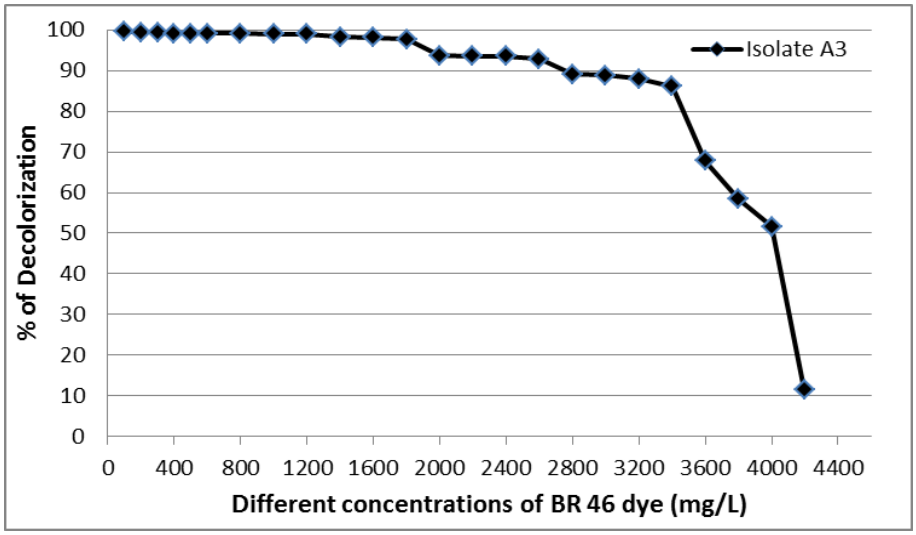

Figure 4. Effect of BR 46 dye concentration on decolorization performance of *Enterobacter cloacae* sp. A3.

It is evident that the removal of dye molecules was more efficient at low concentration. Wang et al., (2009) [40] was found that the Reactive Red 180 was decolorized by *Citrobacter sp*. CK3 with the concentration of (200 mg/L), resulting in approximately 95% decolorization within 36 hrs, and could tolerate up to 1000 mg/L of dye also with increase of the initial dye concentration, the decolorization extent over the same time interval decreased. It was reported that dye decolorization can be strongly inhibited when high concentration of dye stuff was used to examine the poisonous effect of the dye on the degrading microorganisms [41-42].

The Effect of Temperature and Static or Shaking Conditions

The effect of temperature and static or shaking conditions on the decolorization efficiency of strain A3 (*Enterobacter cloacae* sp.) was tested by using different incubation temperature (25, 30, 37, 45°C) at static and shaking (200 rpm) condition with initial dye concentration 1200 and 2000 mg/L. As shown in Table 1 the decolorization efficiency reach to its maximum percentage 99.2% and 92.7% at 30°C incubation temperature and shaking condition at initial dye concentration 1200 and 2000 mg/L respectively while it is 60.9% and 54% at 30°C incubation temperature and static condition at initial dye concentration 1200 and 2000 mg/L respectively. From this experiment we conclude that the optimum temperature is 30°C and the shaking conditions were necessary for bacteria decolorization, therefore, shaking conditions and temperature 30°C were adopted to investigate bacterial decolorization in the following experiments. Dhanve et al., (2009) [43] pronounced that a novel bacterial species identified as *Exiguobacterium* sp. RD3 degraded the diazo dye reactive yellow 84A (50 mg/L) within 48 hrs at static condition, at 30°C and pH 7. Wang et al., (2009) [40] described that the Citrobacter sp. CK3 showed strong decolorizing activity from 27°C to 37°C. Decolorizing activity was significantly suppressed at high temperature, which might be due to the loss of cell viability or deactivation of the enzymes responsible for decolorization [44-45].

Table 1: Effect of temperature and static or shaking condition on dye decolorization by *Enterobacter cloacae* sp. A3.

Different Temp	1200 mg/L		2000 mg/L	
	Static	Shaking	Static	Shaking
25˚C	52.3	82.3	15.2	20.6
30˚C	60.9	99.2*	54	92.7*
37˚C	69.8	81.1	13.6	21.7
45˚C	26.8	34.9	24.2	30.4

*The best value of decolorization percentage

Jaiswal et al., (2014) [46] reported that the effective decolorization of malachite green and indigo carmine by *Bacillus* sp. was observed after 96 hrs of incubation period and it decolorized 95.12 % of malachite green at pH 9.5 and 66.66 % of indigo carmine at pH 6 within 96 hrs of incubation under shaking conditions. While Shah et al., (2013a) [20] found that the dye decolorization of azo dyes Reactive Red was studied under static and shaking conditions with an initial dye concentration of 0.5 g/L by using *Bacillus* ETL-1982 strain. It was observed that under static conditions, the dye decolorization efficiency was 85-95% within 24 hrs as compared to 35% decolorization under shaking conditions.

Effect of Different pH on Decolorization

The effect of pH on decolorization was investigated in the pH ranges of 2, 4, 6, 7, 8, 10 at two times of incubation 24 hrs and 48 hrs. It was found that change in pH significantly affect the decolorization rate. The *Enterobacter cloacae* sp. A3 showed high decolorization efficiency 93.3 % and 91.3 % at pH 6 and 7 respectively after 48 hrs of incubation by using 2000 mg/L of dye, while pH 2, 4, 8 and 10 were found to be inhibitory for the decolorization of dye. Bacterial cultures generally exhibit maximum decolorization at pH 6 and pH 7 (Figure 5). Rajeswari et al., (2014) [47] was found that the maximum decolorization of RSV-1 dye was found to be 94.37 % and 91.99 % at pH 7 and 9 respectively after 36 hrs of incubation. It has been indicated that neutral pH would be more favorable for decolorization of the azo dye. In the case of live bacterial cells, the optimum pH for color removal is often at a neutral pH value or a slightly alkaline pH value. Sheth and Dave (2009) [48] found that about 91% decolorization of 300 mg/L Reactive Red BS dye within 5.5 hrs was decolorized by *Pseudomonas aeruginosa*. Chen et al., (1999) [31] reported that the optimal pH and temperature for the decolorization of RED RBN (1000 mg/L concentration) by the strain *Proteus mirabilis* were pH (6.5-7.5) and 30-35°C. The strain could decolorize the dye within 20 hrs of incubation.

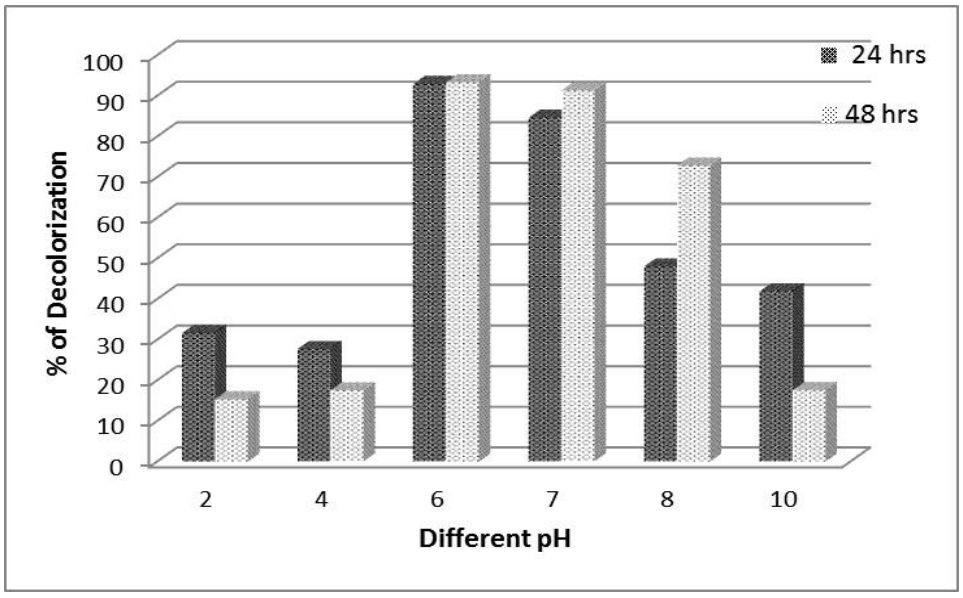

Figure 5. Effect of pH on BR 46 dye decolorization by *Enterobacter cloacae* sp. A3.

Wang et al., (2009) [40] was reported that the best decolorization was achieved at pH 6 and 7, with 96% decolorization in 48 hrs. This could be due to the fact that the optimum pH for the growth of Citrobacter sp. CK3 was neutral or slightly acidic. Similar decolorization extent was observed at pH 8 after 72 hrs and at pH 10 in 120 hrs. pH has a major effect on the efficiency of dye decolorization, and the optimal pH for color removal is often between 6 and 10 [49-51].

Effect of Contact Time with Different Initial Dye Concentration

The results of variation of adsorption of BR 46 dye with contact time are shown in Figure 6. The decolorization of BR 46 dye that resulting at initial concentration of 1600 and 2000 mg/L were

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studied at different time of incubation from 6 to 48 hrs. It was observed from Figure 6 the dye decolorization was increased with time increased, and reaches the contact equilibrium at 18 hrs which measure 97.7 % and 90 % of decolorization at 1600 and 2000 mg/L respectively and increased slowly to 99.4% and 93.2% at 1600 and 2000 mg/L respectively after 48 hrs. The result suggests that, at the initial stage on the external surface of the adsorbent the adsorption takes place rapidly followed by a slower internal diffusion process, which may be the rate determining step [52-53]. In addition, the fast adsorption at the initial stage also may be due to the fact that a large number of surface sites are available for adsorption but after a delay of time, the remaining surface sites are difficult to be occupied [54]. The same result achieved by Aljebori et al., (2012) [55] who found that the adsorption of Maxilon Blue GRL dye that derived at initial concentration of 10 mg/L at different contact time (0-120 min.) the dye adsorption and uptake was increased with time increased, and reached the contact equilibrium at 60 minutes.

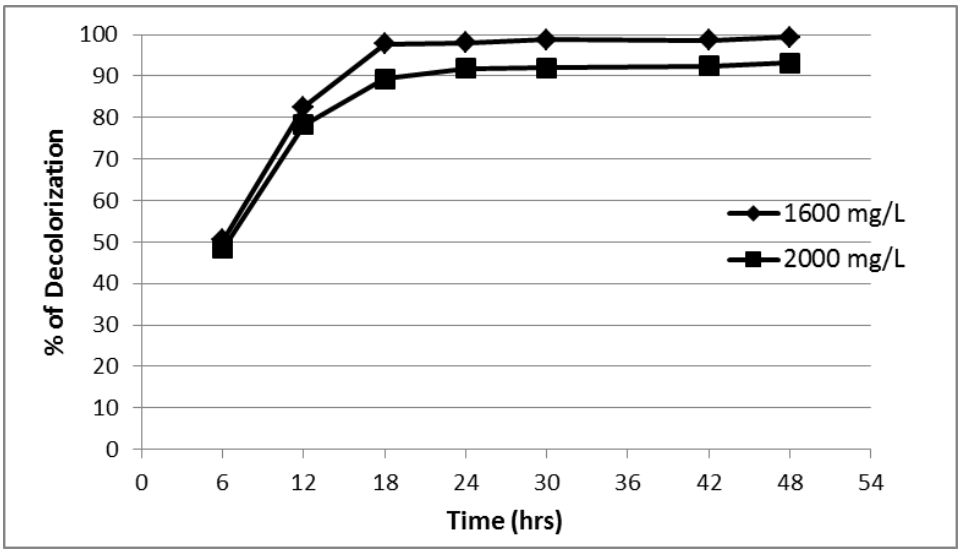

Figure 6. Effect of contact time on decolorization of BR 46 dye by *Enterobacter cloacae* sp. A3.

Influence of Beads Number on the Decolorization of BR 46 Dye

The effect of beads number on the decolorization was studied to know the efficient number of beads create greatest decolorization. Different beads number (1, 2, 3, 4 beads/ mL) were verified by adding to 2000 mg/L of BR 46 dye and applying the optimum condition (pH 6, temp. 30°C, and shaking condition at 200 rpm). The efficiency of immobilized *Enterobacter cloacae* sp. A3 beads in decolorization increased slightly with increasing the number of beads 95.2%, 97.1%, 97.2% and 97.6% of decolorization by using (1, 2, 3, 4 beads/ mL) respectively after 24 hrs of incubation and 97.46%, 98.53%, 99.16%, 99.7% of decolorization by using (1, 2, 3, 4 beads/ mL) respectively after 48hrs of incubation as shown in Table 2. It was found there is not great different in decolorization from 2beads/ mL so it will be used in the next experiment.

Table 2: Effect of beads number on the decolorization of BR 46 dye.

Number of beads	% of Decolorization	
	24 hrs	48 hrs
1/mL	95.2	97.46
2/mL	97.1	98.53
3/mL	97.2	99.16
4/mL	97.6	99.7

The decolorization percentage remarkably increased after immobilization of strain A3 with Ca alginate. The decolorization percentage reach to (99.7%) when the cells were immobilized and (93.2%) when the cells used free at the same condition. This might suggest that by immobilization the

toxic effect of the recalcitrant dyes on the growth rate of the bacteria was canceled out. It might also be attributed to the increase in microbial activity inside the immobilized beads [56].

The Fed Batch Decolorization by *Enterobacter cloacae* sp. A3

The fed batch decolorization study was carried out to check the ability of strain A3 for the decolorization of repeated added dye concentration (2000 mg/L) each cycle at optimum condition for decolorization (pH 6, temp. 30°C, and shaking condition at 200 rpm). The immobilized *Enterobacter cloacae* sp. A3 can decolorized the first two repeated addition of BR 46 dye (each 48 hrs) with high efficiency reach to 97.7% in the first batch cycle and 65% in the second cycle but after the addition of third BR46 dye concentration (2000 mg/L) the decolorization rate decreased to 20.8% as shown in Figure 7. In addition the percentage of decolorization increased in each batch cycle with increasing the time of incubation. Immobilized microbial cells create chances in a wide range of sectors including environmental pollution control. Compared with suspended microorganism technology, cell immobilization shows many advantages, such as resistance to toxic chemicals by protection of bacterial cells from the direct toxic dye effect [24].

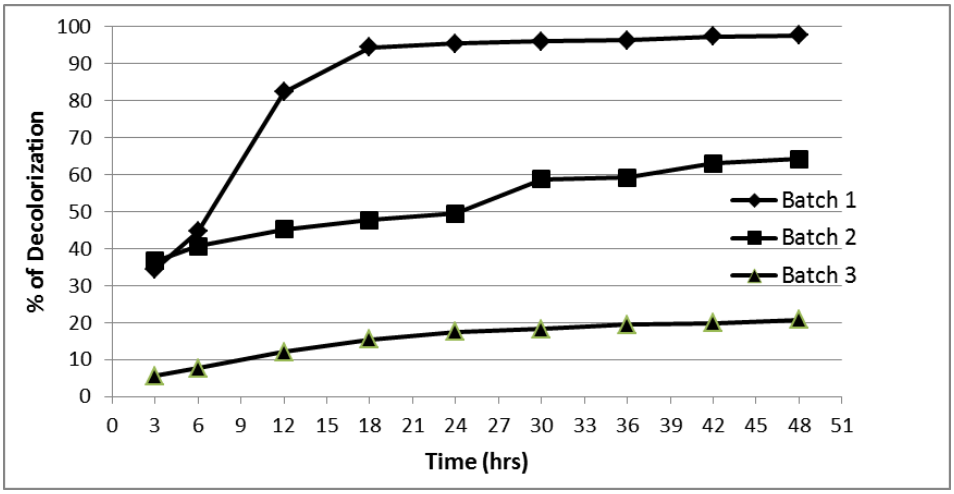

Figure 7. Basic Red 46 decolorization by *Enterobacter cloacae* sp. A3 in continuous fed batch process.

The same result was reported by Shah et al., (2013b) [57] who study the decolorization efficiency of *Pseudomonas spp.* ETL-1982 to decolorized methyl orange dye by repeated addition of dye (120 mg/L) up to five cycles and give variable decolorization rate (9-91%).In first cycle 91.85% decolorization occurred, 82.85% decolorization in second cycles & the percent decolorization goes on decreasing (up to 9% at 5th cycle) as the number of cycle increases.

CONCLUSION

In this study, a decolorizing bacterial strain, *Enterobacter cloacae* sp. A3, was isolated from local industrial outlets contaminated by dyes in Alexandria, Egypt. Strain A3 showed high decolorizing activity, and it could tolerate high concentrations (up to 3600 mg/L) of Basic Red 46. The optimum pH and temperature for maximum decolorization ability was found to be 6-7 and 30°C under shaking condition. The immobilization of strain A3 in calcium alginate was more effective in dye decolorization than the using of free cells. The continuous fed batch decolorization study reported that the ability of immobilized *Enterobacter cloacae* sp. A3 for the decolorization of repeated added 2000 mg/L of dye reach to 97.7% in the first batch cycle and 65% in the second cycle but after the addition of third BR46 dye the decolorization rate decreased to 20.8%. This bacterial strain could be efficiently used for the treatment of real textile effluent containing high concentration of dyes.

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