JMBFS

Journal of Microbiology, Biotechnology and Food Sciences

International peer-reviewed scientific online journal

Published by Faculty of Biotechnology and Food Sciences

CHARACTERIZATION OF DYE DEGRADING POTENTIAL OF SUSPENDED AND NANOPARTICLE IMMOBILIZED CELLS OF *PSEUDOMONAS AERUGINOSA* AR-7

Arjuman Surti, Rubina Ansari*

Address(es): Dr. Arjuman Surti,
Department of Microbiology, Sophia College, Bhulabhai Desai Road, Breach Candy, Mumbai 400026, Maharashtra.

*Corresponding author: arjumansurti@gmail.com

doi: 10.15414/jmbfs.2018.8.2.774-780

ARTICLE INFO

Received 27. 3. 2018
Revised 2. 9. 2018
Accepted 7. 9. 2018
Published 1. 10. 2018

Regular article

OPEN ACCESS

ABSTRACT

The commercial use and unrestricted disposal of synthetic dyes in soil and water-bodies, following the industrial revolution, has led to a major threat towards environmental safety. The azo-dye, Remazol Black B (RBB) is one of the most commonly used synthetic reactive dyes in textile industries. In the present study, the decolourization and biodegradation of RBB were investigated using a bacterium isolated from the marine environment, which was later identified as *Pseudomonas aeruginosa* AR-7 by 16S rRNA analysis. *P. aeruginosa* AR-7 showed 99% decolourization at 100mg/L dye concentration when cultured at optimum conditions of incubation i.e., 96h at 37ºC under static conditions using minimal salts medium (pH 7-9) supplemented with 0.1% glucose and yeast extracts. However, the dye degradation ability of the isolate was reduced to 29% on increasing the dye concentration to 500mg/L. In addition, *P. aeruginosa* AR-7 showed decolourization and degradation of RBB in wastewater obtained after dyeing a cotton fabric. In further experiments, the Fe_3O_4 nanoparticles were synthesized using co-precipitation method and were used to immobilize the cells of *P. aeruginosa* AR-7 by adsorption, in order to compare the RBB degrading abilities of the free and coated cells. The prepared nanoparticles (50-150nm) were characterized by FTIR and SEM analysis to study its structural properties. Also, upon magnetization studies using SQUID magnetometer, Fe_3O_4 nanoparticles were shown to have a magnetization of about 63emu/g. Interestingly, the coated cells not only showed better degradation ability of RBB but also produced simpler products such as alkane, carboxylic acids, ketone, etc. on complete degradation. On the other hand, the free cells mainly produced esters as indicated by the comparison of GC-MS results.

Keywords: Biodegradation, Decolourization, *Pseudomonas aeruginosa* AR-7, GC-MS, Remazol Black B, Azo dyes

INTRODUCTION

In an attempt to enhance the appearance of finished goods, chemical dyes have persistently aided the industrial production of textile, cosmetics as well as pharmaceuticals **(Erkurt *et al.*, 2010)**. Azo-dyes are one such group of chemical dyes that contributes to over 70% of the synthetically produced dyes, globally **(Saratale *et al.*, 2011)**. Considering the advantages associated with the use of dyes like diverse colour shades, high wet fastness, profile, ease of application and minimal energy consumption **(Erkurt *et al.*, 2010)**, it has become difficult for industries to contemplate the environmental hazards associated with it. It is estimated that approximately 280,000 tonnes of over 10,000 different dyes are discharged every year worldwide as untreated effluents in the form of wastewater into public drains that eventually empty into rivers **(Hsueh *et al.*, 2005)**.

These dyes are not only toxic to the flora and fauna of water bodies but also a proven mutagen and carcinogen according to various published studies **(Myslak and Bolt, 1998; Vijay *et al.*, 2017)**. The dye bearing effluents are very complex and an inconsistent mixture of pollutants ranging from organic chlorine-based pesticides to heavy metals and are considered to be recalcitrant and non-biodegradable, ensuring its persistent damage to the environment **(Olukanni *et al.*, 2006)**. Textile industries are known to release a huge amount of dyes in the water and soil environments during the dyeing process **(Carliell *et al.*, 1994; Schoeberl *et al.*, 2005)**. When these coloured effluents are discharged into water bodies, it results in reduced dissolved oxygen concentration, thus creating anoxic conditions that are lethal to other organisms. Moreover, the textile dyes and effluent have a toxic effect on the germination rate and biomass of several plant species **(Wang, 1992; Kapsustka and Reporter, 1993)**. In humans, the intoxication of these dyes may develop symptoms like skin irritation, contact dermatitis, permanent blindness, vomiting, gastritis, acute tubular necrosis, hypertension, oedema of the face, neck, pharynx, tongue and larynx **(Jain *et al.*, 2013; Gude *et al.*, 2012)**.

It is for this reason that the treatment of industrial effluent containing synthetic dyes is mandatory prior to its discharge in the environment. The frequently used physical techniques of effluent treatment such as sedimentation, filtration, segregation and chemical techniques such as coagulation/ flocculation, ion exchange, membrane filtration etc., have been in practice for over decades. However, they suffer drawbacks like the excess use of chemicals, sludge disposal, labour intensiveness and high expenditure **(Robinson *et al.*, 2001)**. Most evidently, biological processes of effluent treatment surpass these drawbacks providing challenging alternatives to the existing technologies **(Ekambaram *et al.*, 2016; Bouraie and Din, 2016; Shah, 2014)**.

One of the factors affecting the decolourization of any dye is culture conditions. Maximum decolourization of the dye can be achieved by optimizing conditions such as incubation time, pH, temperature, dye concentration and availability of suitable carbon or nitrogen sources. In free-culture bioreactors, the micro-organisms suffer from substrate inhibition, whereby growth and consequently pollutant degradation is inhibited at high pollutant concentrations **(Jena *et al.*, 2005)**. Therefore, immobilized microbial cells are preferred due to their better operational stability, easier separation from products for possible reuse, and satisfactory efficiency in catalysis as compared to free cells **(Ramakrishna and Prakasham, 1999, Smith *et al.*, 2007)**. Generally, immobilization can be done using entrapment or adsorption method. The adsorption technique is effectively limited by biomass loading, the strength of adhesion, bio-catalytic activity, and operational stability. This is because adsorption is a simple physical process in which the forces involved in cell attachment are so weak that cells that are several micrometres across are not strongly adsorbed and are readily lost from the surface of the adsorbent **(Smith *et al.*, 2007)**. In order to overcome these problems, it is proposed to coat the microbial cells with magnetic iron oxide (Fe_3O_4) nanoparticles by adsorption. The nanoparticles thus formed strongly adsorb on the cell surfaces because of their high specific surface area and high surface energy; thus making it possible to concentrate the dispersed coated cells by application of a magnetic field and to reuse them.

The present study emphasizes the biodegradation potential of *Pseudomonas aeruginosa* AR-7 against Remazol Black B (RBB), also known as Reactive Black 5, which is an azo-dye used extensively in textile industries. Many micro-organisms from different taxonomic groups of bacteria, fungi, actinomycetes and algae have been found to decolourize RBB **(Khan and Malik, 2016; Joe *et al.*, 2011; Shah, 2014)**. However, the biodegradation of these compounds using immobilization of cells with the help of Fe_3O_4 super-paramagnetic nanoparticles

are rare. The major advantage of this method that allows easy separation of the coated cells by an external magnetic field can be exploited for the treatment of RBB containing effluent in industries before its disposal.
Therefore, considering the complications of RBB degradation and its contribution to environmental pollution, the objectives of the present article was to compare the efficiency of free and Fe_3O_4 coated cells for decolourization and degradation of RBB under optimized culture conditions for growth of *P. aeruginosa* AR-7.

MATERIAL AND METHODS

Dyes and chemicals used in the study

The minimal salts medium (MSM) adjusted to pH 7±0.2 was used for carrying out all decolourization and biodegradation studies [composition in g/L: K_2HPO_4 (7.00), KH_2PO_4 (2.00), $MgSO_4$. $7H_2O$ (0.1), NH_2SO_4 (1.00), Sodium citrate (0.5), traces of yeast extract and glucose (0.1% w/v)] **(Shah, 2014)**. RBB (Tetra sodium 4-amino-5-hydroxy-3,6-bis[[4-[[2-(sulphonatooxy)ethyl]sulphonyl]phenyl]azo]naphthalene-2,7-disulphonate) was purchased from Sigma Aldrich (India). All other chemicals used in the study were of analytical grade and purchased from SRL and Hi-media Laboratories, Mumbai, India.

Bacterial isolates used in the study

Two bacterial isolates of Pseudomonas species isolated from the marine environment, in an earlier study on degradation of Polychlorinated Biphenyls (PCB), were used as test isolates in the present study. These isolates were enriched in fresh MSM media and slants were maintained at refrigerated conditions until further use.

RBB decolourization and degradation studies

For decolourization studies, 100ml sterile MSM broth containing 100mg/L RBB was inoculated with 1ml of 18-24h old test isolates (0.1 $O.D_{530nm}$) and incubated at 37ºC for 96h. Following incubation, the cell-free supernatant was collected by centrifuging small aliquots of the inoculated media at 12000rpm for 15min. The decolourization activity was expressed in terms of percentage (%) decolourization using a colorimeter and was determined by monitoring the decrease in absorbance at the maximum wavelength of dye (i.e., 600nm). The un-inoculated culture medium containing 100mg/L of RBB was used as an experimental control and sterile MSM broth without test culture or dye was set up as sterility control. MSM broth inoculated with test isolate and without dye was maintained as blank. All experiments were carried out in triplicates and the mean values were represented. The percentage decolourization was calculated using the following equation;

$$\% \text{ Decolourization (rate)} = \frac{\text{Initial O.D} - \text{Final O.D}}{\text{Initial O.D}} \times 100$$

For biodegradation analysis, after decolourization of broth, the metabolites were extracted thrice with an equal volume of dichloromethane and were identified using Gas Chromatography-Mass Spectrometry (GC-MS). GC-MS of RBB was also performed for comparing the results and the analysis was carried out at IIT, Bombay.

Mode of decolourization

In order to determine the possible mode of decolourization (i.e. physical adsorption v/s biodegradation), the heat killed and the living cells of the bacterium were used for calculating the % decolourization using the same protocol as mentioned above.

Identification of the test isolate

The 16S rRNA fragment was amplified by PCR using universal primers and the pure genomic RNA of the isolate was sequenced at Sai Biosystems Private Limited, India. The 16S rRNA gene sequences thus obtained was converted into the FASTA format and used for the pair-wise alignment. Species identification was carried out using BLAST program available at http://blast.ncbi.nlm.nih.gov/.

Optimization of physicochemical parameters for biodegradation of RBB

The biological decolourization and degradation of RBB depend on various cultural and environmental parameters. For optimization of physicochemical parameters, firstly, the ability of the test organism to utilize RBB dye as a sole source of carbon was determined. It was done by allowing the test isolate to grow in 100ml MSM broth (pH7) supplemented with different concentrations of RBB (5-50mg/L), during its incubation at 37°C for 96h under static conditions. The optimization conditions for biodegradation of RBB were investigated by varying one parameter at a time while keeping the others constant. These varying parameters included the incubation time (24-120h), dye concentration (5-500mg/L), incubation condition i.e., static or shaking (100rpm), pH (3-9) and temperature (4ºC, 30ºC, 37ºC and 55ºC). In addition, the MSM medium was supplemented with different carbon (0.1% glucose, galactose, lactose, sucrose, starch, and dextrin) and nitrogen (0.1% ammonium nitrate, yeast extract, urea, casein, meat extract, peptone, and sodium nitrate) sources to examine their effect on dye decolourization. At the end of incubation period, cell-free supernatant was collected and % decolourization was determined to optimize the culture conditions.

Dye waste-water collection and degradation

With the help of Furkan Dyeing Services, Mumbai, a half meter cotton fabric was dyed using 50g RBB dissolved in 5Ltr of boiling water containing NaCl (rock salt). The cotton fabric was immersed in this solution for 10-12min and allowed to dry. The remaining wastewater, which is generally released directly into the environment, was collected to examine the degradation potential of the test isolate. For this purpose, the dyeing wastewater was filtered and different volumes (i.e., 0.25ml, 0.5ml and 1.5ml) were amended in 3 separate Erlenmeyer flask containing 100ml of MSM broth. These flasks were inoculated with test isolate and incubated at 37ºC until complete decolourization of the media, after which the % decolourization of the cell-free supernatant was determined colorimetrically and metabolic products by GC-MS analysis carried out at IIT, Bombay.

Preparation of Fe_3O_4 magnetic nanoparticles

The Fe_3O_4 magnetic nanoparticles were prepared by the co-precipitation method as described by **Shan *et al.* (2005)**, with few modifications. A solution of 0.2M $FeCl_3.6H_2O$ (150ml) was mixed with equal volume of 0.1M $FeSO_4.7H_2O$ and stirred mechanically using a magnetic stirrer by heating it simultaneously at 60ºC. Later, 25ml of 0.5M NaOH was added dropwise till a black precipitate was formed. After cooling of the above mixture, the magnetic precipitate was separated by centrifuging at 1000rpm for 2min. The supernatant was discarded and the precipitate was mixed with 8ml oleic acid once before centrifuging it again at 1000rpm for 2min and washed three times with acetone. The magnetic precipitate thus formed was modified using 4ml of 0.5M NaOH to form the hydrophilic magnetic nanoparticles, which is an aqueous solution in mono-dispersed form. The Fe_3O_4 nanoparticles thus formed was completely dried at 50ºC and characterized using analytical techniques.

Characterization of Fe_3O_4 nanoparticles

The Fe_3O_4 nanoparticles were characterized using analytical techniques viz., FTIR, SEM and SQUID.

Fourier-transform infrared spectroscopy (FTIR) Analysis

The nanoparticle sample was mixed with potassium bromide (KBr), ground into fine powder and pressed into pellets at 15000psi. The solution sample was measured using a thin-layer (0.5mm) Infra-Red (IR) cell. The IR spectra were collected over the range of 400-4000cm^{-1}. The entire FTIR analysis was carried out at IIT, Bombay.

Scanning electron microscopy (SEM) Analysis

SEM analysis of nanoparticles was carried out at ICON Analytical, Mumbai. In order to carry out the analysis, the nanoparticles were dispensed in saline and sonicated for 15min. A drop of this nanoparticles suspension was placed on an aluminum foil and loaded in the instrument for characterization.

Magnetization studies on the Fe_3O_4 Nanoparticles using Quantum design Superconducting quantum interference (SQUID) magnetometer

The SQUID analysis of the nanoparticles was carried out at University of Mumbai, Kalina. For this purpose, the powdered sample of nanoparticles was wrapped in paper, packed in gelatin capsules and properly centered in the straw. The magnetization measurements at room temperature were performed between -2T to 2T (1Tesla = 10kOe) to obtain the M-H curves.

Coating of bacterium with magnetite Fe_3O_4 nanoparticles

The coating of microbial cells with magnetic nanoparticles was carried out as described by **Shan *et al.* (2005)**. A 3ml volume of magnetic suspension (i.e., 0.3g Fe_3O_4 nanoparticles per 20ml saline) was mixed with 20ml of a cell suspension (i.e., 0.5g of cells per 20ml of saline). The microbial cells were coated by adsorbing the magnetic nanoparticles and this immobilization was confirmed by SEM analysis carried out at ICON Analytical, Mumbai. The magnetic property of the immobilized cells was further ensured by concentrating them on the side of

the vessel using a magnet and separating them from the suspension medium by decantation.

Comparative study of decolourization and biodegradation of RBB by free and immobilized cells

For comparative studies, two different systems of free and Fe_3O_4 coated cells of the test isolate were set up and added to sterile MSM media supplemented with 100mg/L of RBB. An un-inoculated control was also set up with the sample to compare the purity of the MSM medium. The flasks were incubated at 37ºC for 96h at static conditions and then decolourization and biodegradation analysis were carried out colorimetrically and by GC-MS analysis respectively as described previously. All experiments were carried out in triplicates and the mean values were represented.

RESULTS

RBB decolourization studies

Table 1 represents the colorimetric analysis of RBB decolourization by test isolates. The decolourization was found to be 100% for isolate 1 and 96% for isolate 2 after 96h incubation. These results suggest that the concentration of RBB in media was reduced significantly by both isolates.

Table 1 Decolourization of RBB by test isolates

Isolate	Absorbance at 600nm		% Decolourization after 96h
	Initial	Final	
1	0.51	0.00	100%
2	0.50	0.02	96%

The results obtained in our study are comparable to those reported by **Wang *et al*. (2009)**, wherein Enterobacter sp. EC3 showed 92.56% decolourization after 108h incubation. Other studies carried out with Bacillus sp. YZU1 and *Pseudomonas entomophila* BS1 showed 95% and 93% decolourization of RBB after 120h of incubation respectively **(Wang *et al*., 2013; Khan and Malik, 2016)**.

Mode of decolourization

Table 2 represents the percentage decolourization of RBB by heat-killed (HK) and live cells of test isolates. When HK bacterial cells were added to the culture medium, only 3.33% and 4.91% decolourization was observed by isolates 1 and 2 respectively after 96h of incubation. In contrast, 98.33% and 96.72% decolourization was achieved in the culture medium inoculated with live cells of test isolate 1 and 2 respectively.

Table 2 Decolourization of RBB by heat-killed and live cells of isolates 1 and 2

Absorbance (600nm)	Isolate 1		Isolate 2	
	HK cells	Live cells	HK cells	Live cells
Initial	0.60	0.60	0.61	0.61
Final	0.58	0.01	0.58	0.02
% Decolourization after 96h	3.33%	98.33%	4.91%	96.72%

Our results mainly suggest the degradation of RBB dye by test isolates which manifests itself in the form of decolourized medium. Hetero-polysaccharide and lipid components of the cell wall contribute to the bio-adsorption capacity of a micro-organism that in turn leads to a strong force of attraction between the azo-dye and the cell wall **(Solís *et al*. 2012)**. Ideally, the bio-adsorption characteristic is prevalent among the fungal isolates due to their large surface area. Very few bacterial species with extra-polysaccharide production, which aids in bio-adsorption, is known **(Binupriya *et al*. 2010)**.

RBB degradation studies

In order to confirm the biodegradation of the dye, GC-MS analysis of 96h old decolourized media was performed to identify the metabolites, and the results obtained were compared with the standard chromatogram of pure RBB. Figure 1 represents the chromatograms of pure RBB dye before and after degradation by test isolates.

The peaks obtained in the GC-MS chromatogram of RBB dye degraded by isolates 1 and 2 were completely different from the standard chromatogram of RBB. They did not show any presence of the standard peaks and also new peaks were observed indicating complete degradation. Different metabolites (listed in table 3) identified by GC-MS analysis confirms that the decolourization of MSM broth was due to degradation of the dye. Moreover, the standard peaks of RBB dye congeners were not observed in the chromatograms of degraded RBB dyes indicating the elimination of virtually all detectable peaks and further confirming complete degradation of RBB at the concentration of 100 mg/L.

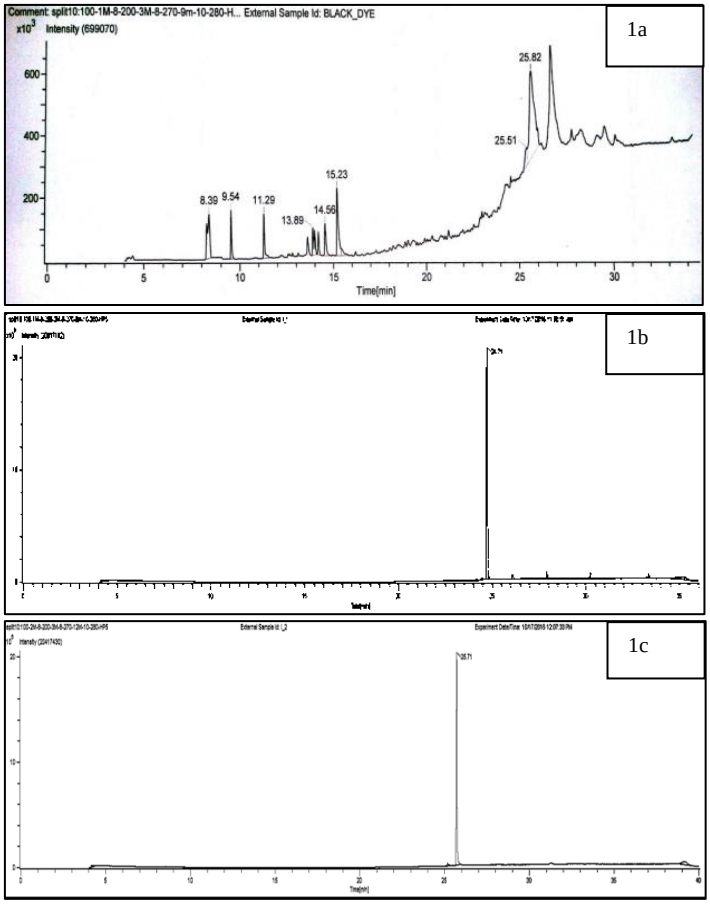

Figure 1 Standard chromatogram of (a) RBB dye (b) RBB dye degraded by isolate 1 (c) RBB dye degraded by isolate 2

Table 3 Metabolites identified by GC-MS in degraded RBB dye

Retention Time (min)	Products
Degraded products of RBB by isolate 1	
24.18	Didodecyl phathalate
24.48	1-Monolinoleoylglycerol trimethylsilyl ether
24.70	1,2-Benzenedicarboxylic acid, disooctyl ester
35.26	9-Octadecenoic acid (Z), phenylmethyl ester
Degraded products of RBB by isolate 2	
8.5	Heptane, 2,3-dimethyl-
25.18	9,12,15-Octadecatrienoic acid, 2-[(trimethylsily)oxy]-1-[{(trimethysilyl)oxy}methylethyl ester, (ZZZ)
25.71	1,2-Benzenedicarboxylic acid, mono(2-ethylhexyl)ester
31.27	1-Monolinoleoylglycerol trimethylsilyl ether

A similar study by **Ekambaram *et al*. (2016)** reported biodegradation of Remazol reactive dyes by 2 Clostridium species isolated from dyeing effluent collected from Tirupur region, Tamil Nadu, India. In their study, complete decolourization was observed after 72h which was confirmed by the disappearance of the peak in UV spectrum, and peak shifts or disappearance in FTIR spectrum of treated samples. Biodegradation of other azo-dyes has also been reported by single bacterial species of Acinetobacter **(Roy *et al*., 2018)** as well as a bacterial consortium consisting of *Providencia rettgeri* strain HSL1 and Pseudomonas sp. SUK1 **(Lade *et al*., 2015).**

Selection and identification of the isolate

Although, both isolates in our study showed similar ability to decolourize RBB dye, isolate 1 was selected for further studies owing to its comparative competitiveness observed by results represented in tables 1 and 2. Upon 16S rRNA analysis, isolate 1 showed 97-99% homology with five strains of *Pseudomonas aeruginosa*. Isolated pure bacterial species was designated as *Pseudomonas aeruginosa* AR-7 and refined sequences were submitted to the DNA Data Bank of Japan with the accession number LC229067.

Optimization of physicochemical parameters for dye decolourization

The results for optimization of physicochemical parameters of dye degradation are represented in Tables 4 and 5. The optimised culture condition for the growth of *P. aeruginosa* AR-7 to effectively decolourize 100mg/L of RBB was achieved when it was incubated at 37ºC for 96h under static conditions using MSM (pH 7-9) supplemented with 0.1% glucose and yeast extracts.

The results of our study revealed that the decolourization process gradually increases after 12h and reaches a maximum at 96h of incubation at 37ºC by *P. aeruginosa* AR-7 (table 4). It has been reported earlier by **Mali *et al*. (2000)** that decolourization percentage gets accelerated after 15h incubation which indicates that the bacteria requires some time to grow and acclimatize to the environment. Moreover, at optimal temperature, there is an optimal microbial growth and greater production of enzymes. Hence, optimum degradation of dyes is achieved. In our study, dye decolourization process was inhibited at low (4ºC) and high (55ºC) temperature due to inactivation of enzymes or loss of cell viability. It has been previously reported that 37ºC is an optimal temperature for dye decolourization in many bacterial isolates **(Kolekar *et al*., 2008)** as well as in bacterial consortium **(Saratale *et al*., 2010)**. Furthermore, it was observed that the decolourization percentage of the dye decreases as the concentration of dye increases. In our study, *P. aeruginosa* AR-7 was capable of complete decolourization of 100mg/L RBB in 96h which was reduced to only 26.78% as the concentration was increased to 500 mg/L. Similar results are also reported by other authors **(Khan and Malik, 2016; Shah, 2014)**. This observation can be attributed to reasons such as the toxic nature of the dye **(Jadhav *et al*., 2008)** and increased competition for the availability of carbon sources. Dyes are deficient in carbon sources **(Forgacs, 2004)** and hence, it is difficult for the bacteria to utilize it for its survival. Higher the dye concentration, the longer the time required for decolourization. Other factors like the pH also play an important role in the overall biochemical processes and eventually the growth of microorganisms. It is also suggested that pH acts as a rate-limiting factor in decolourization by regulating the transport of dye molecules across the cell membrane **(Lourenco *et al*., 2000)**. Similar to our study, a decrease in decolourization of azo-dyes is reported, at lower pH (3-6) and optimum decolourization at pH 7, by other authors (**Lade *et al*., 2015; Phugare *et al*., 2011).** Our study also reported that the process of decolourization is oxygen sensitive as optimum activity was observed under static conditions. Similar findings are also reported by other authors **(Pearce *et al*., 2003; Moosvi *et al*., 2005)**. It is known that oxygen is a preferred electron acceptor in bacterial system. Hence, the enzymatic reduction of azo-dyes may be inhibited in presence of oxygen. This can be attributed to the competition for NADH utilization by aerobic respiration, which triggers electron transfer, from NADH to oxygen, to form ATP (**Chang *et al*., 2001)**.

Table 4 Optimization of physicochemical parameters for degradation of RBB by *P. aeruginosa* AR-7

Physico-chemical Parameters	Absorbance at 600nm		% Decolourization after 96h	Physico-chemical Parameters	Absorbance at 600nm		% Decolourization after 96h
	Initial	Final			Initial	Final	
Incubation Period (h)				**Temperature (ºC)**			
0	0.51	0.51	0.00%	4	1.11	0.00	00.00%
12	0.51	0.49	3.92%	30	1.11	0.13	88.28%
24	0.51	0.40	21.56%	37	1.11	0.01	99.09%
48	0.51	0.24	52.94%	55	1.11	0.00	00.00%
72	0.51	0.12	76.47%	**Static and shaking (100rpm) condition**			
96	0.51	0.00	100%	Static	0.53	0.00	100%
				Shaker	0.53	0.26	50.94%
Dye concentration (mg/L)				**pH**			
5	0.08	0.00	100%	3	0.58	0.58	00.00%
10	0.16	0.00	100%	4	0.52	0.52	00.00%
20	0.26	0.00	100%	5	0.55	0.18	67.27%
50	0.41	0.00	100%	6	0.53	0.13	75.47%
100	0.51	0.00	100%	7	0.60	0.01	98.33%
200	0.53	0.16	69.81%	8	0.55	0.02	96.36%
500	0.56	0.41	26.78%	9	0.54	0.01	98.14%

Table 5 Effect of carbon and nitrogen sources on RBB decolourization

Nutrients	Absorbance at 600nm		% Decolourization after 96h	Nutrients	Absorbance at 600nm		% Decolourization after 96h
	Initial	Final			Initial	Final	
Carbon source (0.1%)				**Nitrogen source (0.1%)**			
Glucose	0.32	0.03	87.50%	Ammonium nitrate	0.43	0.34	20.93%
Galactose	0.39	0.07	82.05%	Yeast extract	0.48	0.08	83.33%
Lactose	0.38	0.08	78.94%	Urea	0.49	0.09	81.63%
Sucrose	0.40	0.11	72.50%	Casein	0.39	0.10	74.35%
Starch	0.41	0.11	73.17%	Meat extract	0.44	0.08	81.81%
Dextrin	0.50	0.15	62.50%	Peptone	0.40	0.12	70%
Cellulose	0.54	0.24	55.50%	Sodium nitrate	0.51	0.44	13.72%

In our study, *P. aeruginosa* AR-7 failed to grow in culture medium supplemented with RBB as a sole source of carbon, suggesting its incapability to utilize RBB as a nutrient source for growth and survival. Enzymes that cause reduction of azo groups are termed as azo-reductases. Azo-reductase is the key enzyme expressed in the dye degrading bacteria and can catalyze the reductive cleavage of azo-bonds. Azo-reductase enzymes have been reported in many organisms **(Blumel and Stolz, 2003; Chen *et al*. 2005)**. These enzymes show broad specificity; may be tolerant or sensitive to oxygen, and maybe FMN-dependent or independent. **Khan and Malik, (2016)**, reported FMN and NADH dependent azo-reductase in *P. entomophila* BS1, which was constitutively expressed. In contrast, azo-reductases isolated from several bacteria have been shown to be inducible flavoproteins and are able to use both NADH and NADPH as electron donors **(Russ *et al*., 2000)**. Hence in our study, since *P. aeruginosa* AR-7 was unable to utilize RBB the effect of additional sources of carbon was studied. Metabolism of other carbon sources was expected to produce FMN and NADH, required for the azo-reductase activity, and hence promote degradation of RBB. The results of our study suggested that effective decolourization (more than 55%) could be achieved by *P. aeruginosa* AR-7 by utilizing any of the additional carbon and nitrogen (except nitrates) sources supplemented with the dye indicated in table 5. However, maximum decolourization was achieved in presence of glucose (87.5%) and yeast extract (83.33%).

Dye waste-water degradation

The waste-water obtained after dyeing a cotton fabric was used to test the efficiency of *P. aeruginosa* AR-7 to degrade the dye. Similar to the results observed during optimization of initial dye concentration, a decrease in % decolourization was seen with an increase in the volume of dye waste-water (table 6). More than 66% decolourization was observed after incubation of 96h at 37°C by *P. aeruginosa* AR-7. The GC-MS analysis of metabolites extracted from MSM broth amended with 0.5ml dye waste-water showed complete degradation of dye, as indicated by the comparison between GC-MS chromatogram of pure and degraded dyes. The disappearance of standard peaks (figure 2) and presence of different metabolites (table 7) in degraded dye confirmed the biodegradation of RBB by *P. aeruginosa* AR-7.

Table 6 Percentage decolourization of dye waste-water

Volume of dye waste-water (ml)	Absorbance at 600 nm		% decolourization after 96h
	Initial	Final	
0.25	0.27	0.01	96.29%
0.5	0.47	0.03	93.61%
1.5	0.65	0.22	66.15%

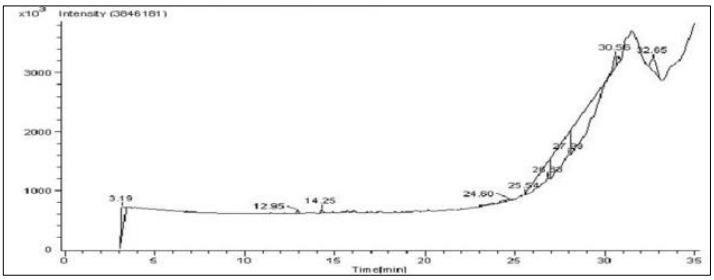

Figure 2 Standard GC-MS chromatogram of dye waste-water

Table 7 Metabolites identified by GC-MS in degraded waste-water dye

Retention Time (min)	Products
3.19	Pentanal
12.95	1,2-Aminotridecane
14.25	Dodecyl fluoride
14.36	1,4-Amino-1-Pentanol
23.03	Oxalic acid, allylpentadecyl ester
23.77	1,6-Methyloctadecane
24.72	2-Trifluoroacetoxypentadecane
30.56	Octadecanoic acid, 2,3-bis[(1-oxotetradecyl)oxy]propyl ester

Preparation and characterization of Fe_3O_4 magnetic nanoparticles

Figure 3 represents the synthesis of Fe_3O_4 magnetic nanoparticles. It was prepared by co-precipitation method which is a facile and convenient way to synthesize iron from a mixture of equal volumes of 0.2M $FeCl_3.6H_2O$ and 0.1M $FeSO_4.7H_2O$. This method helps in size controlled synthesis of nanoparticles. The black precipitate which was obtained is a characteristic of Fe_3O_4 (magnetite). In further steps, the Fe_3O_4 particles were modified using 7M NaOH, and magnetic Fe_3O_4 nanoparticles were modified with oleic acid. The surface- modified Fe_3O_4 nanoparticles were mono-dispersed in an aqueous solution. It is well known that Fe_3O_4 prepared by co-precipitation method have a large number of hydroxyl groups on its surface. These react readily with carboxylic acid head groups of oleic acid molecules. Excess oleic acid is then absorbed to the pre-bound oleic acid layer to form a hydrophobic shell. When the magnetic nanoparticles are put into a NaOH solution, the outer layer of the oleic acid on the Fe_3O_4 surface is transformed into a sodium salt of oleic acid, which modifies the magnetic nanoparticles so that they are mono-dispersed in an aqueous phase as their surfaces become hydrophilic **(Yong *et al*., 2006; Shan *et al*., 2005)**.

The characterization of nanoparticles is essential for understanding and control of its synthesis and applications. It was carried out by FTIR (Figure 4), SEM (Figure 5) and SQUID (Figure 6) analysis.

FTIR spectrum showed intense bending vibration of H-O-H bond at about 1000-1600 cm^{-1}, that is typical of the H_2O molecule. Additionally, the second absorption band between 900-1000cm^{-1} corresponded to bending vibration associated with the O–H bond. The O–H in-plane and out of plane bond appears at 1044.09, 110.78, 1218.35, 1368.97, 1441.00, 1523.19 and 1583.23 cm^{-1} respectively. In the spectrum shown in figure 4, the sample exhibits two intense peaks, in 618.54 and 648.78 cm^{-1} band that is due to the stretching vibration mode associated to the metal-oxygen absorption band (Fe–O bonds in the crystalline lattice of Fe_3O_4). They are characteristically pronounced for all spinel structures and for ferrites in particular. This occurs because, in these regions, the contributions from the stretching vibration bands related to metal in the octahedral and tetrahedral sites of the oxide structure are found. Moreover, the FTIR spectrum shows an absorption band at 1742.51 cm-1, which presents the stretching vibration of the carboxyl group (C=O), associated with the oleic acid molecule, adsorbed on to the surface of the crystallites. Collectively, our results indicated that the magnetite nanoparticles have crystalline structure of inverse spinel type, and FTIR absorption spectroscopy allowed identifying characteristic features of the spinel structure, as well as a presence of certain types of chemical substances adsorbed on the surface of nanoparticles. The SEM imaging of the magnetite nanoparticles showed that the particle size was in the range of 50-150nm (Figure 5). The magnetization study on the magnetic nanoparticles was carried out on Quantum Design SQUID Magnetometer. The M-H plot (figure 6) indicates that the ferrite magnetic nanoparticles have magnetization of about 63emu/g and a near sixteen coercivity.

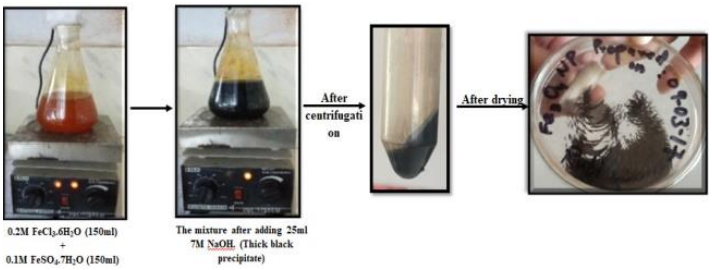

Figure 3 Preparation of magnetic nanoparticles

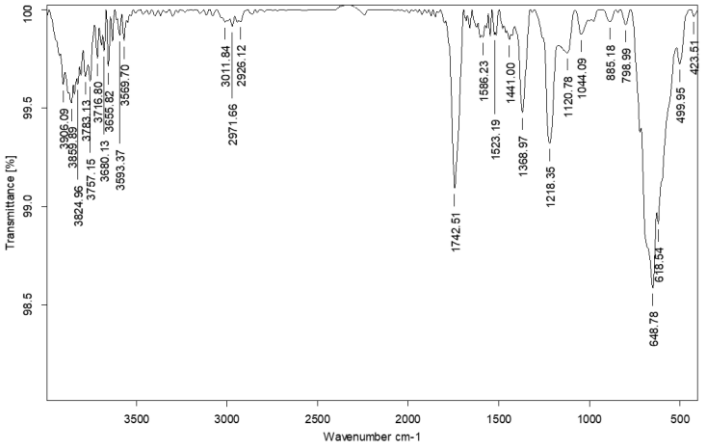

Figure 4 FTIR spectrum of Fe_3O_4 nanoparticles

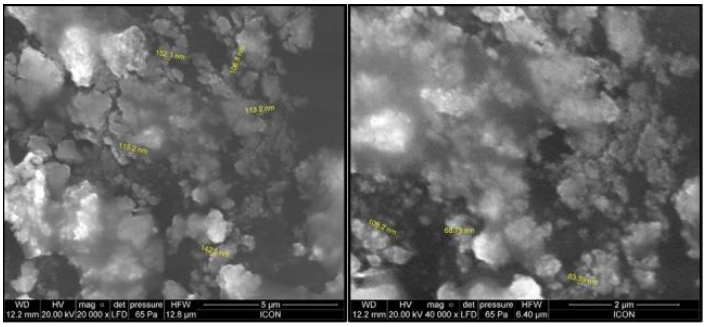

Figure 5 SEM image at high (20,000 and 40,000) Magnetization

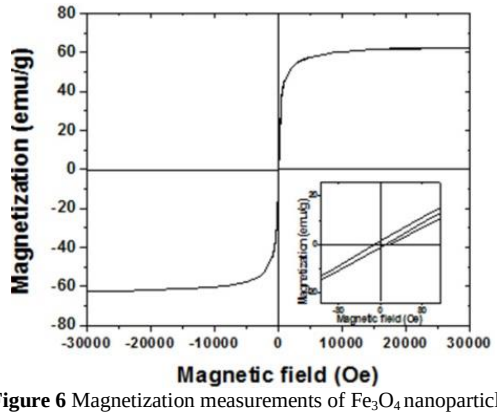

Figure 6 Magnetization measurements of Fe_3O_4 nanoparticles

Coating of bacterium with magnetite Fe_3O_4 nanoparticles

Figure 7 represents the SEM images of free and coated cells of *P. aeruginosa* AR-7. It was observed that nanoparticles had strongly adsorbed on the surface of the cells.

The cells coated with super-paramagnetic magnetite nanoparticles can be immobilized by external application of magnetic field. This is significant since it can help in immobilization as well as magnetic separation of the coated cells from the medium in which they are present.

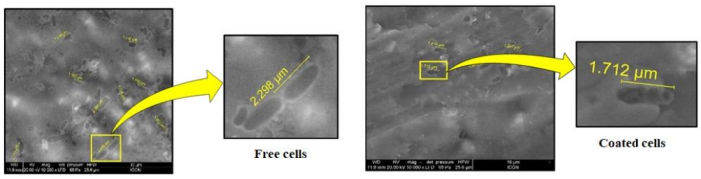

Figure 7 SEM images of free and coated cells of *P. aeruginosa* AR-7

Comparative study of degradation of RBB by free and immobilized cells

The degradation capabilities of coated cells and free cells of *P. aeruginosa* AR-7 were studied. The metabolites extracted from both the samples were subjected to GC-MS analysis in the same way as discussed earlier. Figures 8 and 9 represents the chromatogram of RBB dye degraded by free and coated cells respectively. The peaks of the standard chromatogram of RBB was found to be absent in both samples suggesting its degradation by the bacterium.

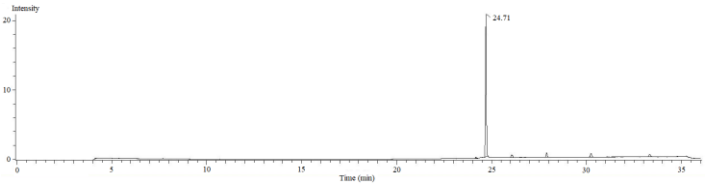

Figure 8 GC-MS analysis of RBB degradation by free cells

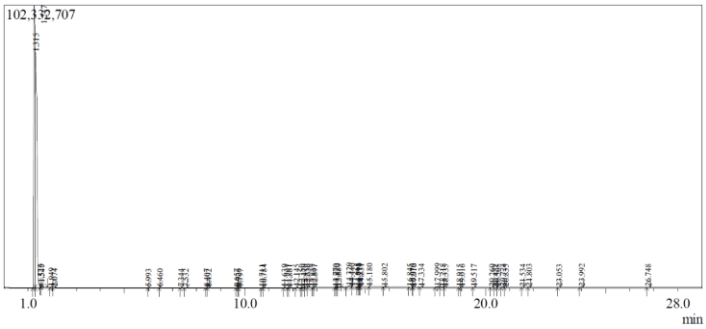

Figure 9 GC-MS analysis of RBB degradation by coated cells

Table 8 Metabolites identified by GC-MS in degraded RBB dye by free and coated cells

Retention time	Products
Degraded products of RBB by free cells	
24.18	Didodecyl phathalate
24.48	1-Monolinoleoylglycerol trimethylsilyl ether
24.70	1,2-Benzenedicarboxylic acid, disooctyl ester
35.26	9-Octadecenoic acid (Z), phenylmethyl ester
Degraded products of RBB by coated cells	
1.24	Ethane, 1,1,2,2-tetrachloro-
1.31	1,3-Butadiene, 1-[(1-methylethyl)thio]-, (E)-
1.52	Toluene
1.54	1,3,5-Cycloheptatriene
1.94	3-Ethyl-2-methyl-1-heptene
7.53	5-Fluoro-2-trifluoromethylbenzoic acid, 2-pentadecyl ester
9.79	Heptadecane
11.63	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester
11.79	Methoxyacetic acid, 4-tridecyl ester
11.86	Octadecane
12.47	Sulfurous acid, pentadecyl 2-propyl ester
12.55	n-Hexadecanoic acid
13.88	Nonadecane
14.71	9,12-Octadecadienoic acid (Z,Z)-, methyl ester
15.80	Octadecanoic acid, 2-oxo-, methyl ester
19.51	Tricyclo[4.3.1.1(3,8)]undecane-1-carboxylic acid
20.42	Tetratetracontane
23.05	9-Tetradecenal, (Z)-
23.99	Tetracosane

Different metabolites identified by GC-MS, in the degraded dye medium are presented in Table 8. Both systems of cells, i.e. free cell system and coated cell system, were found to have the ability to degrade RBB dye at the concentration of 100mg/L. As anticipated, it was observed that the coated cells showed enhanced degradation as compared to free cells. Only four major products were obtained by free cells upon degradation of RBB (table 8) which were found to be complex ether and ester molecules. Phthalate isomers are known to be carcinogenic in nature **(Krishna and Phale, 2008).** These structures are just as difficult to degrade as compared to the original dye, and hence they may persist in the environment causing pollution. In contrast, coated cells were found to produce a mixture of metabolite most of which were alkane, alkene carboxylic acids and ketones which are simple compounds, non-toxic in nature and can be further degraded easily. Although, coated cells system also produced few esters as degraded products, they were identified in very small amounts.

CONCLUSION

Remazol Black B is an azo dye which is extensively used in textile industries. A large amount of this dye is released in the effluent from textile industries, which negatively affects the soil and water environment. Although, free cells of *P. aeruginosa* AR-7 showed considerable biodegradation ability in our study, the magnetite nanoparticle coated cells may offer advantages like the use of continuous bioreactor systems at the industrial sites. This will aid in managing effluent collection and disposal systems. Moreover, it will allow easy separation of coated cells by application of an external magnetic field to prevent loss of bacteria along with the treated effluent, which cannot be avoided with the use of free cells.

Acknowledgment: The current article was edited by Dr. Mobashshera Tariq, E.S.M.U Bioscience editing solutions.

REFERENCES

Binupriya, R., Kumar, S. M., Ku, C. S., & Yun, S. (2010). Sequestration of Reactive Blue 4 by free and immobilized *Bacillus subtilis* cells and its extracellular polysaccharides. *Colloids and Surfaces B: Biointerfaces*, *76*(1), 179-185. https://doi.org/10.1016/j.colsurfb.2009.10.031

Blumel S., & Stolz A. (2003). Cloning and characterisation of the gene coding for the aerobic azoreductase from *Pigmentiphage kullae* K24. *Applied Microbiology and Biotechnology*, *62*(2-3), 186-190. https://doi.org/10.1007/s00253-003-1316-5

Bouraie, M., & Din, W. S. (2016). Biodegradation of Reactive Black 5 by *Aeromonas hydrophila* strain isolated from dye-contaminated textile wastewater. *Sustainable Environment Research*, *26*(5), 209-216. https://doi.org/10.1016/j.serj.2016.04.014.

Carliell, C. M., Barclay, S. J., Naidoo, N., Buckley, C. A., Mulholland, D. A., & Senior, E. (1994). Anaerobic decolorization of reactive dyes in conventional sewage treatment processes. *Water SA*, *20*(4), 341-344.

Chang, J. S., Chou, C., Lin, Y. C., Lin, P. J., Ho, J. Y., & Hu, T. L. (2001). Kinetics characteristics of bacterial azo dye decolorization by *Pseudomonas luteola*. *Water Research*, *35*(12), 2841-2850. https://doi.org/10.1016/s0043-1354(00)00581-9

Chen, H., Hopper, S. L., & Cerniglia, C. E. (2005). Biochemical and molecular characterization of an azoreductase from Staphylococcus aureus, a tetrameric NADPH-dependent flavoprotein. *Microbiology*, *151*(5), 1433-1441. https://doi.org/10.1099/mic.0.27805-0

Ekambaram, S., Perumal, S., & Annamalai, U. (2016). Decolorization and biodegradation of remazol reactive dyes by Clostridium species. *3Biotech*, *6*, 20. https://doi.org/10.1007/s13205-015-0335-0

Erkurt, E. A., Erkurt, H. A., & Unyayar, A. (2010). Decolorization of azo dyes by white rot fungi. In: Atacag Erkurt H. (eds) Biodegradation of Azo Dyes. *The Handbook of Environmental Chemistry* (vol. 9). Springer: Berlin, Heidelberg, 157-167. https://doi.org/10.1007/698_2009_48

Forgacs, E., Cserhati, T., & Oros, G. (2004). Removal of Synthetic Dyes from Wastewaters: A Review. *Environment International*, *30*(7). 953-971. https://doi.org/10.1016/j.envint.2004.02.001

Gude, D., Bansal, D. P., Ambegaonkar, R., & Prajapati, J. (2012). Paraphenylenediamine: Blackening more than just hair. Journal of Research in Medical Sciences : *The Official Journal of Isfahan University of Medical Sciences*, *17*(6), 584-586.

Hsueh, C. L., Huang, Y. H., Wang, C. C., & Chen, S. (2005). Degradation of azo dyes using low iron concentration of fenton and fenton-like system. *Chemosphere*, *58*(10), 1409-1414. https://doi.org/10.1016/j.chemosphere.2004.09.091

Jadhav, S. U., Jadhav, M. U., Kagalkar, A. N., & Govindwar, Sanjay, P (2008). Decolourisation of Brilliant Blue G Dye Mediated by Degradation of the Microbial Consortium of Galactomyces geotrichum and Bacillus sp. *Journal of the Chinese Institute of Chemical Engineers*, *39*(6), 563-570. https://doi.org/10.1016/j.jcice.2008.06.003

Jain, P. K., Sharma, A. K., Agarwal, N., Jain, P. K., Sengar, N. S., Agarwal, N., Siddiqui, M. Z., Pawal, P., Singh, A. K., & Gaba, R. D. (2013). A prospective clinical study of myocarditis in cases of acute ingestion of paraphenylene diamine (hair dye) poisoning in northern India. *Journal of the association of physicians of India*, *61*(9), 633-6, 644.

Jena, H. M., Roy G. K., & Meikap, B. C. (2005). Comparative study of immobilized cell bioreactors for industrial wastewater treatment. WMCI-2005, Ist and 2nd Oct – 2005, NIT, Rourkela.

Joe, J., Kothari, R. K., Raval, C. M., Kothari, C. R., Akbari, V. G., & Singh, S. P. (2011). Decolorization of textile dye remazol black b by *Pseudomonas aeruginosa* CR- 25 isolated from the common effluent treatment plant. *Journal of Bioremediation & Biodegradation*, *2*(2), 118. https://doi.org/10.4172/2155-6199.1000118

Kapustka, L. A., & Reporter, M. (1993). Terrestrial primary producers, in: P. Calow (Ed.), *Handbook of Ecotoxicology*(Vol. 1). Blackwell Scientific Publications, Oxford, 278-297.

Khan, S., & Malik A. (2016). Degradation of Reactive Black 5 dye by a newly isolated bacterium *Pseudomonas entomophila* BS1. *Canadian Journal of Microbiology*, *62*(3), 220-232. https://doi.org/10.1139/cjm-2015-0552

Kolekar, Y.M., Powar, S.P., Gawai, K.R., Lokhande, P.D., Shouche, Y.S., & Kodam, K.M. (2008). Decolorization and degradation of Disperse Blue 79 and Acid Orange 10, by Bacillus fusiformis KMK5 isolated from the textile dye contaminated soil. *Bioresource Technology*, *99*(18), 8999-9003. https://doi.org/10.1016/j.biortech.2008.04.073

Lade, H., Kadam, A., Paul, D., & Govindwar, S. (2015). Biodegradation and detoxification of textile azo dyes by bacterial consortium under sequential microaerophilic/aerobic processes. *EXCLI Journal*, *14*, 158-174. https://doi.org/10.17179/excli2014-642

Lourenco, N. D., Novais, J. M, & Pinheiro, H. M. Reactive textile dye colour removal in a sequencing batch reactor. *Water Science and Technology*, *42*(5-6), 321-328.

Mali, P. L., Mahajan, M. M., Patil, D. P., & Kulkarni, M. V. (2000). Biodecolorization of members of triphenylmethane and azo groups of dyes. *Journal of Scientific and industrial research*, *59*, 221-224.

Moosvi, S., Keharia, H., & Madamawar, D. (2005). Decolorization of textile dye reactive Violet 5 by a newly isolated bacterial consortium RVM11. *World Journal of Microbiology and Biotechnology*, *21*(5), 667-672. https://doi.org/10.1007/s11274-004-3612-3

Myslak, Z. W., & Bolt, H. M. (1998). Occupational exposure to azo dyes and risk of bladder cancer. *Zentralblatt für Arbeitsmedizin*, *38*, 310-21.

Olukanni, O. D., Osuntoki, A. A., & Gbenle, G. O. (2006). Textile effluent biodegradation potentials of textile effluent-adapted and non-adapted bacteria. *Africal Journal of Biotechnology*, *5*(20), 1980-1984.

Pearce, C. I., Lloyd, J. R., & Guthrie, J. T. (2003). The removal of color from textile wastewater using whole bacterial cells: a review. *Dyes and Pigments*, *58*(3), 179-196. https://doi.org/10.1016/s0143-7208(03)00064-0

Phugare, S. S., Kalyani, D. C., Patiol, A. V., & Jadhav, J. P. (2011). Textile dye degradation by bacterial consortium and subsequent toxicological analysis of dye and dye metabolites using cytotoxicity, genotoxicity and oxidative stress studies. *Journal of Hazardous Materials*, *186*(1), 713-723. https://doi.org/10.1016/j.jhazmat.2010.11.049

Ramkrishna, S. V., & Prakasham, R. S. (1999). Microbial fermentation with immobilized cells. *Current Science*, *77*, 87-100.

Robinson T., McMullan G., Marchant R., & Nigam P. (2001). Remediation of dyes in textile effluent: a critical review on current treatment technologies with a proposed alternative. *Bioresource technology*, *77*(3), 247-255. https://doi.org/10.1016/s0960-8524(00)00080-8

Roy, U., Das, P., Bhowal, A., & Datta, S. (2018). Biodegradation of azo dye using the isolated novel bacterial species: Acinetobacter sp. In: Ghosh S. (eds) *Utilization and Management of Bioresources*. Springer, Singapore, 187-193. https://doi.org/10.1007/978-981-10-5349-8_18

Russ, R., Rau, J., & Stolz, A. (2000). The function of cytoplasmic flavin reductases in the reduction of azo dyes by bacteria. *Applied and Environmental Microbiology*, *66*(4), 1429-1434. https://doi.org/10.1128/aem.66.4.1429-1434.2000

Saratale, R. G., Saratale, G. D., Chang, J. S., & Govindwar, S. P. (2011). Bacterial decolorization and degradation of azo dyes: A review. *Journal of the Taiwan Institute of Chemical Engineers*, *42*(1), 138-157. https://doi.org/10.1016/j.jtice.2010.06.006

Saratale, R.G., Saratale, G.D., Changand, J.S., & Govindwar, S.P. (2010). Decolorization and degradation of reactive dyes and dye wastewater by a developed bacterial consortium. *Biodegradation*, *21*(6), 999-1015. https://doi.org/10.1007/s10532-010-9360-1

Schoeberl, P., Brik, M., Braun, R. & Fuchs, W. (2005). Treatment and recycling of textile wastewater-case study and development of a recycling concept. *Desalination*, *171*(2), 173-183.

Shah, M. P. (2014). Exploring the strength of *Pseudomonas Putida* ETL-7 in microbial degradation and decolorization of Remazol Black-B. *International Journal of Environmental Bioremediation & Biodegradation*, *2*(1), 12-17. https://doi.org/10.12691/ijebb-2-1-3

Shan, G., Xing, J., Zhang, H., & Liu, H. (2005). Biodesulfurization of dibenzothiophene by microbial cells coated with magnetite nanoparticles. *Applied and Environmental Microbiology*, *71*(8), 4497-4502. http://doi.org/10.1128/AEM.71.8.4497-4502.2005

Smith, M. L., Gourdon, D., Little, W. C., Kubow, K. E., Eguiluz, R. A., Luna-Morris, S., & Vogel, V. (2007). Force-induced unfolding of fibronectin in the extracellular matrix of living cells. *PLoS Biology*, *5*(10), e268. http://doi.org/10.1371/journal.pbio.0050268

Solís, M., Solís, A., Pérez, H. I., Manjarrez, N., & Flores, M. (2012). Microbial decolorization of azo dyes: A review. *Process Biochemistry*, *47*(12), 1723-1748. https://doi.org/10.1016/j.procbio.2012.08.014

Krishna, V. C., & Phale, P. S. (2008). Bacterial degradation of phthalate isomers and their esters. *Indian Journal of Microbiology*, *48*(1), 19-34. http://doi.org/10.1007/s12088-008-0003-8

Vijay, U., Gupta, S., Mathur, P., & Bhatnagar, P. (2017). Evaluating geno-toxicity of treated wastewater from health centers with special reference to their mutagenicity. *EC Microbiology*, *12*(2), 83-96.

Wang, H., Zheng, X. W., Su, J. Q., Tian, Y., Xiong, X. J., & Zheng, T. L. (2009). Biological decolorization of the reactive dyes Reactive Black 5 by a novel isolated bacterial strain Enterobacter sp. EC3. *Journal of Hazard Materials*, *171*(1-3), 654-659. https://doi.org/10.1016/j.jhazmat.2009.06.050

Wang, W. (1992). Toxicity assessment of pretreated industrial effluent using higher plant. *Water Environment Research*, *64*(2): 186-187.

Wang, Z. W., Liang, J.S., & Liang, Y. (2013). Decolorization of Reactive Black 5 by a newly isolated bacterium Bacillus sp. YZU1. *International Biodeterioration & Biodegradation*, *76*, 41-48. https://doi.org/10.1016/j.ibiod.2012.06.023.

Yong, S., Zhao, T. S. & Guo, Z. L. (2006). Lattice Boltzmann method for incompressible flows with large pressure gradients. *Physical Review E*, *73*(2), 026704. https://doi.org/10.1103/physreve.73.026704