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Bioremediation of textile Reactive Blue (RB) Azo Dye Residues in Wastewater using Experimental Prototype Bioreactor.

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ABSTRACT

Azo dyes constitute the largest class of synthetic dyes and represent the major chemical in textile industry wastewater. The remediation of textile industry wastewater before reaching the agricultural environment is of significance for the safety of the surrounding environment. However, the recalcitrant nature of these chemicals requires special technologies to remove and/or reduce the risks associated with their discharge. The bioremediation of these toxic synthetic dyes is among the approaches for remediation of textile industry wastewater. This study focuses on the isolation, screening and azo dye removal by bacteria isolated from dumping site of textile wastewater. Fourteen isolates were tested in this study. One of them was the most efficient in decolourization of RB dye. This isolate was identified by 16s DNA sequences to *Pseudomonas aeruginosa* strain OS4 (NCBI accession number: KC762943). Bioremediation of azo dyes was found to occur efficiently under two sequential phases; starting with low oxygen levels (anoxic phase) and completed by aerobic one. Three oxidoreductase enzymes known to take part in this type of biochemical transformation were studies. The Azoreductase enzyme is likely to perform first step in dye bioremediation under anoxic conditions. This enzyme showed high activity being 20 units after six days of incubation. During this time the colour was removed considerably (99%). The two other enzymes, lignin Peroxidase and Polyphenol oxidase were induced in aerobic conditions and gave 26.7 and 20 U. A prototype bioreactor with dual oxygenation levels (anoxic and aerobic) was designed for the bioremediation of RB dye residues. In this bioreactor, the RB dye was almost totally removed and this was associated with high activity of Azoreductase in anoxic phase and Lignin Peroxidase as well as Polyphenol oxidase in aerobic phase.

Keywords: *Pseudomonas aeruginosa* strain OS4, prototype bioreactor, bioremediation, RB azo dye, 16s DNA sequences.

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INTRODUCTION

The first use of dye in ancient Egypt can be traced to the third or fourth dynasty (Nicholson, 2000). Egyptians usually dyed cloths, using either plant dyes or ochreous earth as natural dyes. They were also quite advanced in their understanding of how to attach dyes to fabrics. They came to conclusion that the use of madder (anthraquinone dye) requires a metallic salt to allow it to bind to the fibre and it is believed that Egyptians used the salt alum to accomplish this. The majority of dyes used today are synthetic and their origins synthesized by organic chemists (Gondalia, 2008). The English chemist William Henry Perkin (1838–1907) discovered the dye mauveine while trying to synthesize the antimalarial drug, quinine. Through oxidation and boiling with ethanol he derived a purple solution, which found to be bounded silk more effectively than other natural dyes. Perkin is remembered as the 'father of the dye industry' (Holme, 2006).

The textile mills daily discharge millions of liters of untreated effluents loaded with synthetic dyes wastewater into public wastewater stream that eventually drain into water bodies. Most of them are recalcitrant in nature, especially azo dyes. This can lead to acute toxicity of aquatic ecosystem (Olukanni *et al.*, 2009). This also alters the pH, increases the biochemical oxygen demand (BOD) and chemical oxygen demand (COD), and adversely affects water quality. Without adequate treatment these dyes will remain in the environment for an extended period of time (Olukanni *et al.*, 2006).

The azo dyes are the most extensively studied and the most commercially important. This emanates from the superior properties of this dye class in comparison to the others (Engel *et al.,* 2008). The basic structure includes an azo bond (–N=N–) which is not found naturally in nature consequently making them difficult to degrade. This class of dye is grouped into monoazo, diazo, trisazo and polyazo dyes depending on the number of azo groups (Engel *et al.,* 2008). The stability and their xenobiotic nature of reactive azo dyes make them recalcitrant hence they are not totally degraded by conventional wastewater treatment processes that involve light, chemicals or activated sludge (Grekova-Vasileva and Topalova, 2009).

Jiss Joe *et al.* (2011) showed that glucose enhanced the bioconversion of the azo dye tested by a mixed culture under anoxic and anaerobic conditions. A bacterial consortium was tested in the presence of reactive azo dyes and proved to be effective in breaking the azo dye when supplemented with starch (Rajeswari *et al.*, 2011). Generally, the decolourization of azo dyes occurs under conventional anaerobic and facultative anaerobic by different groups of bacteria (Yan *et al.*, 2012 and Sathyabama *et al.*, 2013).

Recently, the enzymatic approach has attracted much interest with regard to decolorization and degradation of azo dyes in wastewater (Karimi *et al.*, 2012). The oxidoreductive enzymes affect azo dyes generating highly reactive free radicals that undergo complex series of spontaneous cleavage reactions (Grekova-Vasileva and Topalova, 2009).

Azoreductase enzymes are known to break the azo bond (–N=N–) (Leelakriangsak and Borisut, 2012). Several studies investigated bacterial cytoplasmic azoreductases, and suggested that they can be used for bioremediation purpose (Franciscon *et al.*, 2012 and

Pandey and Dubey, 2012). Another enzyme, namely Lignin peroxidase (LiP) catalyzes the oxidation of non-phenolic aromatic compounds. It also catalyse several oxidations in the side chains of lignin and related compounds (Morgenstern *et al.*, 2010). LiP has been used to mineralize a variety of recalcitrant aromatic compounds, polychlorinated biphenyls and dyes (Ghodake *et al.*, 2009). Bacterial LiP was reported to decolourize azo dyes (Anjaneya *et al.*, 2011). Polyphenol oxidase enzyme was used to catalyze the oxidation of phenollic compounds (Jadhav *et al.*, 2010). This enzyme acts as a marker of the oxidative enzymes involved in the degradation of azo dyes (Kalme *et al.*, 2010). Moreover, the involvement of Polyphenol oxidase in the degradation of Direct Green HE4B by *Pseudomonas desmolyticum* NCIM 2112 (Kalme *et al.*, 2010) and Direct orange 39 by a microbial consortium consisting of *Pseudomonas aeruginosa* strain BCH has been reported (Jadhav *et al.*, 2010).
The aim of this work is to asses the removal of RB textile dye in industrial wastewater using bacteria isolated from the industrial discharge site and to test the prototype bioreactor designed special for bioremediation of RB residues in textile industry wastewater.

MATERIALS AND METHODS

Wastewater Samples

Wastewater samples were collected from textile dye industrial site namely, Cairo. The samples were taken from El Mukatem Dyehouse near Cairo region. Wastewaters chemical and physical properties were performed and bacteria capable to remove the dye colour in rather short time were isolated.

Analysis of collected wastewater samples

Wastewater sample collected from dyeing basin before discharging into the effluent lagoon

One wastewater sample was obtained from dyeing basin before discharging into the effluent lagoon in El Mukatem Dyehouse at Cairo region. The wastewater contained Reactive Blue (RB) azo dye in concentration at 3000 ppm. The chemical structure of the RB dye is shown in Figure (1). Wastewater sample was analyzed for Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD), pH and electric conductivity (EC); employing the standard methods (APHA, 1998). COD was measured using (Hach) spectrophotometer test kit, while, BOD was measured by OXI Top BOD meter (WTW, Comp.). The EC values were determined using digital YSIEC meter (model 35), and the pH was measured by digital Orion pH meter (model 420A). The specific absorption spectrum of wastewater containing Reactive Blue was analyzed by UV vis spectrophotometer (Jenway UV/Visible- 2605 spectrophotometer, England).

Wastewater sample (effluent) collected from the main factory dumping sites

Wastewater effluent was analyzed for physico-chemical and microbiological properties. Wastewater quality assessment was performed using conventional parameters: COD, BOD, Total Soluble Solids (TSS), pH and EC; employing the standard methods (APHA, 1998). Total bacteria and total fungi were enumerated. Serial dilutions of suspension from effluent collected from textile factory dumping site were plated on MSM agar medium and

incubated for 2- 7 days at 30˚C (Darwesh *et al.*, 2008). After incubation period, the different groups of bacteria and fungi were counted.

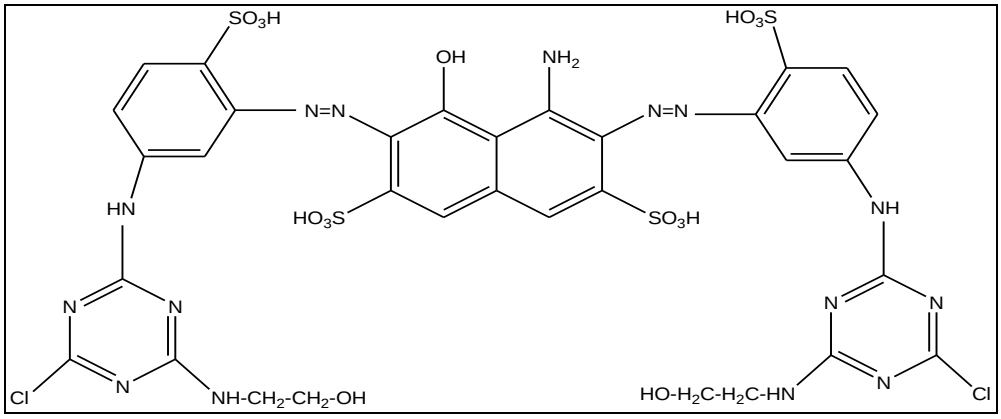

Figure 1: Chemical structure of Reactive Blue azo dye.

Isolation of bacteria from enriched culture and primary identification

The wastewater from dying basin that containing Reactive Blue azo dye was diluted to the dye concentration of (300 ppm). This solution was amended with yeast extract (0.5 g/L) and inoculated by wastewater effluent obtained from the factory dumping site. The mixture was incubated at 30˚C under static conditions for 15 days. The dye colour decolourization of the mixture followed by spectrophometeric analysis. A mixed culture that showed quick and stable decolourization activity was transferred to fresh wastewater sample collected from dying basin and amended with yeast extract as mentioned above. After five successive transfers, the enriched wastewater was plated on MSM agar medium plates supplemented with wastewater containing 300 ppm of Reactive Blue azo dye as the sole carbon and nitrogen sources. The plates were incubated at 30˚C for 4-5 days. Bacterial colonies surrounded with colour free (clear zones) were isolated in pure form. The total 14 isolates capable to decolourize the dye was obtained and stored at 4˚C on MSM agar without dye (Darwesh *et al.*, 2008). The primary identification was done according to Bergey's Manual of Systematic Bacteriology (Holt *et al.*, 1994).

Decolourization of wastewater containing RB dye (300 ppm)

Decolourization of dye is the primary indicator of dye bioremoval from wastewater. The bacterial isolates were tested for their efficiency in dye removal from wastewater. A loopful of bacterial growth from culture slope was inoculated into MSM medium broth and incubated at 30°C under shaking conditions (100 rpm) for 48 h. Two ml of the culture broth was transferred to sterile tubes 20 mL of volume. The tubes were filled to 18 mL working volume by sterile wastewater containing Reactive Blue dye (300 ppm) as the sole carbon and nitrogen source amended with yeast extract (0.5 g/L). The tubes were sealed with screw caps so as to achieve anoxic conditions as described by Darwesh *et al.* (2008). The tubes were incubated under static conditions at 30°C for 6 days. The number of tubes incubated corresponded to the number of samples needed throughout the whole experiment so that each tube is opened only once. After 6 days of incubation under static conditions, the treated wastewater containing bacterial isolates were transferred to 100 mL sterile flasks sealed with cotton plugs under sterile control conditions. Flasks were incubated on shaker

(100 rpm) at 30°C for 6 days. The number of flasks incubated corresponded to the number of samples needed throughout the whole experiment so that each flask is opened only once. The dyes removal was judged by decolourization % of the centrifuged culture every 2 days at wavelength of 595 nm. The un-inoculated control was also incubated to check a biotic decolourization of dye.

Assay of enzymes involved in biotransformation of RB azo dye

Azoreductase enzyme was assayed by the method described by Pandey and Dubey (2012). The reaction mixture contained 400 µl of 50 mM sodium phosphate buffer (pH 7.0), 200 µl of the sample and 200 µl of 100 ppm Reactive Red azo dye as azoreductase enzyme substrate. The reaction was initiated by the addition of 200 µl NADH of 7.09 mg ml^{-1} to give the final concentration 2 mM. The enzyme activity was measured by the decrease in colour intensity at 555 nm at room temperature. Lignin Peroxidase activity was determined by the formation of Purpurogallin at 420 nm in a reaction mixture containing 2.4 ml of 100 mM potassium phosphate pH 6.0, 0.3 ml of 5.33% pyrogallol, 10 mM H_2O_2 according to the method of Ogola *et al.* (2009). Polyphenol oxidase activity was determined in a reaction mixture of 2 ml, containing 0.01% catechol in 0.1 M phosphate buffer (pH 7.4) at 495 nm by the procedure described by Shinde *et al.* (2012).

Molecular identification bacterium efficient in bioremoval of Reactive Blue azo dye from industrial wastewater.

The DNA was extracted from the bacteria by enzymatic lyses using lysozyme (20 mg/ml) and Proteinase K (1 mg/ml). Total genomic DNA was purified using isopropanol extraction as described by Zhang *et al.* (2012). Polymerase chain reaction (PCR) amplification of the 16S rDNA genes was conducted using extracted DNA in the presence of the forward primer 16RW01 (5'- AACTGGAGGAAGGTGGGGAT-3') and the reverse primer 16DG74 (5'- AGGAGGTGATCCAACCGCA -3') [27]. The final 50 µL reaction mixture contained 1× PCR buffer (NEB, England), 1 nmol of dNTPs, 1 pmol of 2 mM $MgSO_4$, 0.25 pmol of forward and reverse primers, 1 unit Taq DNA polymerase (NEB, England) and 10 µL template DNA.

The PCR amplification included initial denaturation of DNA at 95°C for 5 min, followed by 35 cycles of 95°C for 30 sec, 55°C for 30 sec, and 72°C for 45 sec, the mixture was kept for 10 min at 72°C for complete extension. The amplified PCR product was tested using gel-electrophoresis (agarose gel). The PCR product was purified by QIAquick Gel Extraction Kit (QIAGEN, USA) and run on agarose gel to get the purified 16s DNA fragments for sequencing. Identification was achieved by comparing the contiguous 16S rDNA sequences obtained with the 16S rDNA sequence data from the reference and type strains available in public databases GenBank using the BLAST (National Centre for Biotechnology Information). The sequence of 16s rDNA of Bacterial strain OS4 was deposited in the GenBank of NCBI under accession number KC762943. The sequences were aligned using Jukes Cantor Model. The phylogenetic reconstruction was done using the neighbour-joining (NJ) algorithm, with bootstrap values (Kumar and Sawhney, 2011).

Design and operation of prototype sequential bioreactor for the Reactive Blue azo dye bioremediation

The bioreactor was designed to achieve anoxic/aerobic continuous conditions. The first phase is upflow fixed-film column (UFC) container. Another container (phase) provide continuously stirred aerobic (CSA) container to speed bioremediation and/or biodegradation of the intermediate molecules (Figure 5).

Bacterial strain OS4 was reported as potent bacterium efficient in bioremoval of RB azo dye. This strain was used in small scale (two phases) bioreactor. A loopful of growth from stock culture slope was inoculated into flask contains MSM modified medium broth and incubated at 30°C under shaking conditions (100 rpm) for 3 days. The suspension of the bacteria was added to fill 10 % of each of UFC and CSA bioreactor containers. The bioreactor after then was fed continuously by wastewater from the feed tank (#1 in Figure 5) for a period of 14 days.

RESULTS

Analysis of wastewater samples

Two wastewater samples were collected from textile plant located at El-Mukatem Dyehouse at Cairo. These samples were subjected to chemical, physical and microbiological analysis. The first wastewater sample was obtained from dyeing basin containing RB azo dye before discharging into the effluent lagoon. The analysis of this sample is presented in Table (1). The colour of wastewater was dark blue due to the presence of Reactive Blue azo dye at concentration of 3000 ppm. The wastewater had alkaline pH (10.2) and high value of EC (40.25 ds/m). The chemical oxygen demand (COD) and biological oxygen demand (BOD) of this wastewater sample were very high being 2018 and 954 ppm respectively.

Table 1: Some properties of wastewater collected from dyeing basin before discharging into the effluent lagoon in El-Mukatem Dyehouse

Wastewater properties	Value
Type of dye applied in this process	Reactive Azo dye
Colour of discharged water	Dark blue
Concentration of dye residue (ppm)	3000
pH	10.2
EC (dS/m)	40.25
COD (ppm)	2018
BOD (ppm)	954

The other wastewater sample was collected from general wastewater disposal lagoon. The wastewater sample was analyzed (Table 2) and had high values of pH being 10.7. The EC value was 2.34 ds/m. The wastewater sample had comparatively high values of COD and BOD being 1260 and 400 ppm respectively (Table 2). Total bacterial and fungal counts in this sample were reported Table 3. The total bacterial count in the wastewater was $2.3 * 10^3$ CFU mL^{-1}, whereas the fungal count was only $0.5 * 10^2$ CFU mL^{-1}.

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Table 2: Some properties of effluent wastewater sample collected from the factory dumping site.

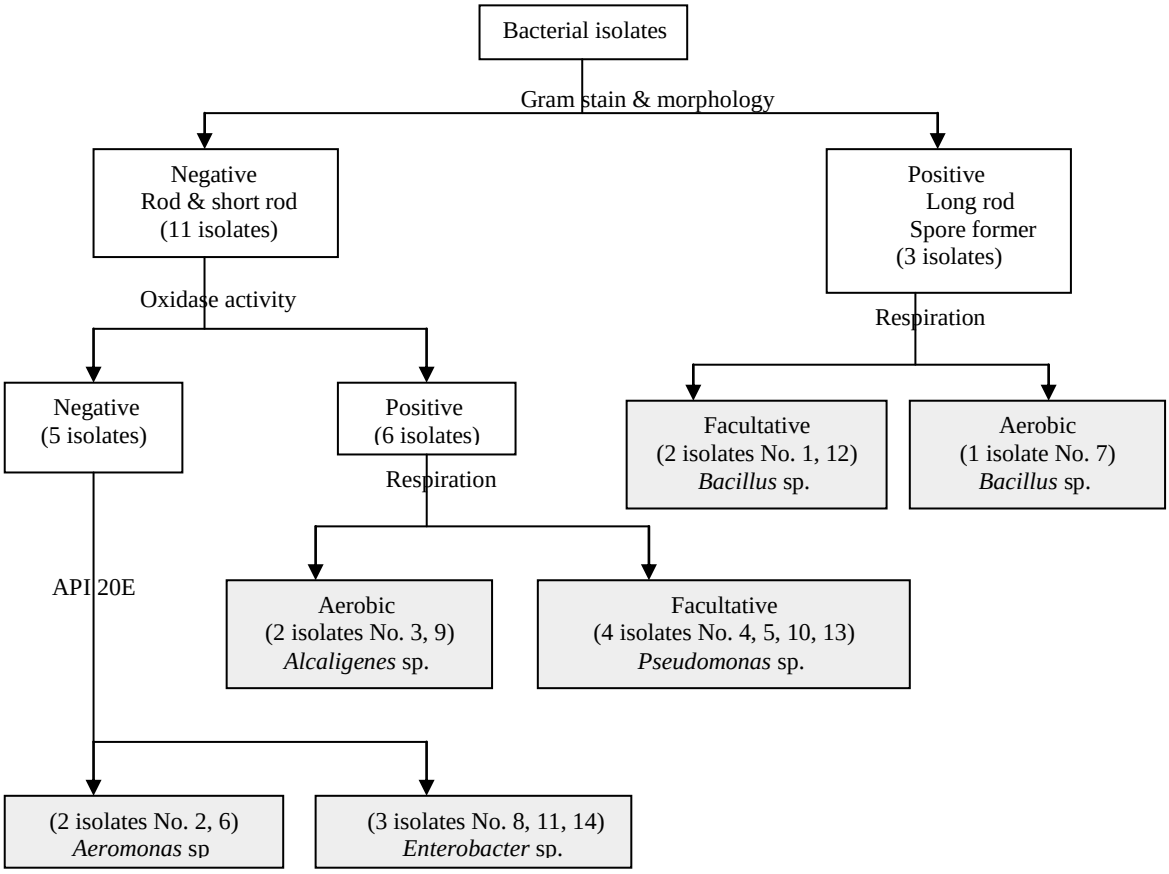
Parameters	Wastewater sample collected from El Mukatem Dyehouse
pH	10.7
EC ds/m	2.34
TSS ppm	2728
COD ppm	1260
BOD ppm	400

Where: TSS= Total soluble solids, BOD= Biological oxygen demand, COD= Chemical oxygen demand.

Table 3: Enumeration of total bacteria and fungi of El Mukatem Dyehouse factory wastewater.

Groups	Counts (CFU /ml)
Total bacteria	$2.3 * 10^3$
Total fungi	$0.5 * 10^2$

Figure 2 Flowchart for identification of 14 bacteria isolated from textile industry wastewater affected by RB azo dye residues.



Isolation and screening of azo dye decolorizing bacteria

From the prolonged anaerobic enrichment cultures in wastewater sample containing Reactive Blue azo dye, 14 bacterial isolates were isolated on the MSM agar medium amended with 300 ppm RB dye. Figure (2) shows that, among all isolates three were gram

positive long rods and spore formers. These isolates were deferent in respiration system, one isolate No. 12 was aerobic (*Bacillus* sp.) and 2 isolates (No. 1 and 7) were facultative anaerobic (*Bacillus* sp.). Eleven isolates were gram negative short rods. Five of them had negative oxidase activity and therefore they were classified as *Enterobacteriacae* according to Holt *et al.* (1994). *Enterobacteriacae* strains were tested on API 20E kit for further classification. Three of them, isolates number 8, 11 and 14 were classified as *Enterobacter cloacae, Enterobacter cloacae* and *Enterobacter* sp. respectively. Other 2 isolates No. 2 and 6 belonged to *Aeromonas* sp.. Six isolates were oxidase positive and classified as *Pseudomonaseae* (Holt *et al.,* 1994). Four isolates (No. 4, 5, 10 and 13) were facultative anaerobes belonging to *Pseudomonas* sp. and 2 isolates number 3 and 9 were aerobic unless in the presence of nitrate and/or nitrite they become anaerobic (anoxic conditions). These isolates were classified as *Alcaligenes* sp. (Holt *et al.,* 1994).

Each isolate was screened for the ability to decolourize Reactive Blue azo dye in wastewater at the concentration of 300 mg/l in broth medium amended with yeast extract (0.5 gl^{-1}). The results are illustrated in Table (4). The efficiency of bacterial strains/isolates in RB azo dye removal was assessed under anoxic conditions for the first 6 days then under aerobic conditions for another 6 days. Table (4) shows that three out of 14 isolates were efficient in decolourization of RB azo dye and removed substantial amount of dye colour after 6 days of incubation under static (anoxic) conditions. These strains are; *Pseudomonas* sp. No 4, *Aeromonas* sp. No 6 and *Enterobacter* sp. No 14 removed 98.3, 91.7 and 93.4 % respectively. One of them, *Pseudomonas* spp. No. 4 had fast and high capacity to remove 98.2 % after 4 days of incubation. *Pseudomonas* spp. No.4, *Aeromonas* sp. No.6, *Enterobacter* sp. No.8, *Alcaligenes* sp. No.9, *Pseudomonas* sp. No.10 and *Enterobacter* sp. No.14 were very active in decolourization of RB azo dye under aerobic conditions throughout the 6 days of incubation and reached 99, 92.5, 91.7, 91.5, 93.5 and 95.7 % respectively (Table 4). Two isolates; *Aeromonas* sp. No.2 and *Pseudomonas* sp. No.13 were very low in dye removal capacity being 4.9 and 21 % respectively under aerobic conditions after 6 days of incubation following the anoxic incubation period.

Table 4: Decolourization percent of wastewater containing Reactive blue azo dye (300 ppm) by bacterial isolates from El Mukatem Dyehouse.

Strains / Isolates No.	Anoxic conditions			Aerobic conditions		
	Days			Days		
	2	4	6	8	10	12
Bacillus sp. No. 1	0.0	13.7	79.8	84.8	84.8	85.0
Aeromonas sp. No. 2	0.0	0.0	04.0	04.4	04.9	04.9
Alcaligenes sp. No. 3	0.0	32.3	64.5	75.1	75.2	75.2
Pseudomonas sp. No. 4	59.6	98.2	98.3	98.3	98.9	99.0
Pseudomonas sp. No. 5	0.0	29.3	61.6	65.5	65.6	65.6
Aeromonas sp. No. 6	0.0	78.8	91.7	92.2	92.4	92.5
Bacillus sp. No. 7	0.0	26.2	78.8	82.7	82.7	82.7
Enterobacter cloacae No. 8	0.0	37.7	86.5	90.3	90.8	91.7
Alcaligenes sp. No. 9	0.0	40.0	81.4	90.7	91.2	91.5
Pseudomonas sp. No. 10	20.0	79.9	88.6	93.0	93.2	93.5
Enterobacter cloacae No. 11	0.0	59.9	62.5	65.6	65.6	65.8
Bacillus sp. No. 12	0.0	13.2	50.0	54.3	54.3	54.8
Pseudomonas sp. No. 13	0.0	0.0	18.8	20.5	20.9	21.0
Enterobacter sp. No. 14	0.0	74.7	93.4	95.0	95.3	95.7

Three enzymes belong to oxidoreductase enzymes, namely azoreductase, lignin Peroxidase and Polyphenol oxidase were studied and presented in Table (5) for azoreductase enzyme, Table (6) for lignin Peroxidase enzyme and Table (7) for Polyphenol oxidase enzyme. The azoreductase enzyme (Table 5) shows that this enzyme is only induced in the anoxic conditions but the aeration of the medium after additional 6 days resulted in total inhibition of this enzyme. Under anoxic conditions, azoreductase activity (Table 5) was reported after 2 days with strain *Pseudomonas* spp. isolates No. 4 and 10 being 9.3 and 3.9 units respectively. After 4 days all strains/ isolates except *Aeromonas* sp. No. 2, *Pseudomonas* sp. No. 13 and *Enterobacter* sp. No. 14 showed azoreductase activity in the range of 1.0 to 19.2 U. Gradual increase in azoreductase activity was noted with increase the time of incubation. The highest three strains/ isolates in azoreductase activity at the end of anoxic incubation (6 days) were *Pseudomonas* sp. No. 4, *Bacillus* sp. No. 1 and *Aeromonas* sp. No. 6 being 20.0, 9.1 and 8.7 units respectively.

Table 5: Changes in Azoreductase activity (U) associated with the growth of bacterial strains/isolates on wastewater containing Reactive blue azo dye (300 ppm)

Strains / Isolates No.	Anoxic conditions			Aerobic conditions		
	Days			Days		
	2	4	6	8	10	12
Bacillus sp. No. 1	0.0	1.1	9.1	0.0	0.0	0.0
Aeromonas sp. No. 2	0.0	0.0	0.0	0.0	0.0	0.0
Alcaligenes sp. No. 3	0.0	1.0	7.1	0.0	0.0	0.0
Pseudomonas sp. No. 4	9.3	19.2	20.0	0.0	0.0	0.0
Pseudomonas sp. No. 5	0.0	3.5	6.0	0.0	0.0	0.0
Aeromonas sp. No. 6	0.0	6.8	8.7	0.0	0.0	0.0
Bacillus sp. No. 7	0.0	3.2	6.7	0.0	0.0	0.0
Enterobacter cloacae No. 8	0.0	3.7	6.8	0.0	0.0	0.0
Alcaligenes sp. No. 9	0.0	2.0	5.5	0.0	0.0	0.0
Pseudomonas sp. No. 10	3.9	4.3	8.0	0.0	0.0	0.0
Enterobacter cloacae No. 11	0.0	5.4	6.5	0.0	0.0	0.0
Bacillus sp. No. 12	0.0	1.1	4.6	0.0	0.0	0.0
Pseudomonas sp. No. 13	0.0	0.0	1.1	0.0	0.0	0.0
Enterobacter sp. No. 14	0.0	0.0	3.0	0.0	0.0	0.0

The Lignin Peroxidase enzyme was induced under both anoxic and aerobic conditions (Table 6). With the exception of strain *Pseudomonas* sp. isolate No. 13, all other strains/isolates after 6 days of incubation showed Lignin Peroxidase activity at the range between 0.8 and 17.1 units. The exposure of microbial growth to the aerobic conditions after 6 days of aerobic incubation showed additional Lignin Peroxidase activity which increases as the time of aerobic incubation reached 12 days (end of the experiment). No retardation of Lignin Peroxidase activity was noted with switching from anoxic to aerobic conditions. Strain *Pseudomonas* sp. No. 4 was distinguished in higher Lignin Peroxidase activity as compared with other strains/isolates in this study. As regards the Polyphenol oxidase enzyme, the results in Table (7) show that on contrary to azoreductase enzyme, no Polyphenol oxidase activity was reported under anoxic conditions. This enzyme started to show activity in the aerobic phase of incubation. The gradual increase in Polyphenol oxidase activity was noted as the incubation time increased under aerobic conditions. Strain *Pseudomonas* sp. No. 4 was also found to be the most efficient strain in the production of this enzyme being 20.0 units of enzyme activity after 12 days.

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Table 6: Changes in Lignin Peroxidase enzyme activity (U) associated with the growth of bacterial strains/isolates on wastewater containing Reactive blue azo dye (300 ppm).

Strains / Isolates No.	Anoxic conditions			Aerobic conditions		
	Days			Days		
	2	4	6	8	10	12
Bacillus sp. No. 1	0.0	0.0	5.0	9.3	10.2	11.6
Aeromonas sp. No. 2	0.0	0.0	0.8	1.1	1.3	1.5
Alcaligenes sp. No. 3	0.7	3.0	6.4	9.5	9.8	10.0
Pseudomonas sp. No. 4	4.3	10.1	17.1	19.3	23.5	26.7
Pseudomonas sp. No. 5	0.0	2.2	6.8	8.3	8.9	9.2
Aeromonas sp. No. 6	0.0	5.3	9.3	10.3	10.6	10.5
Bacillus sp. No. 7	0.0	2.2	5.8	7.0	7.8	8.0
Enterobacter cloacae No. 8	0.0	3.0	6.0	6.8	7.0	7.3
Alcaligenes sp. No. 9	0.0	2.8	5.4	8.0	8.3	8.8
Pseudomonas sp. No. 10	2.5	3.6	5.8	6.5	7.1	7.5
Enterobacter cloacae No. 11	0.0	2.0	3.4	4.9	5.6	5.8
Bacillus sp. No. 12	0.0	0.1	1.3	2.6	3.8	4.8
Pseudomonas sp. No. 13	0.0	0.0	0.0	0.0	0.0	0.0
Enterobacter sp. No. 14	0.0	0.0	2.5	3.1	3.8	4.2

Table 7: Changes in Polyphenol oxidase enzyme activity (U) associated with the growth of bacterial strains/isolates on wastewater containing Reactive blue azo dye (300 ppm).

Strains / Isolates No.	Anoxic conditions			Aerobic conditions		
	Days			Days		
	2	4	6	8	10	12
Bacillus sp. No. 1	0.0	0.0	0.0	5.4	7.3	10.9
Aeromonas sp. No. 2	0.0	0.0	0.0	0.0	2.1	2.8
Alcaligenes sp. No. 3	0.0	0.0	0.0	2.5	5.0	7.3
Pseudomonas sp. No. 4	0.0	0.0	0.0	8.5	12.3	20.0
Pseudomonas sp. No. 5	0.0	0.0	0.00	8.1	9.8	10.8
Aeromonas sp. No. 6	0.0	0.0	0.0	7.3	9.5	10.8
Bacillus sp. No. 7	0.0	0.0	0.0	5.6	6.9	8.1
Enterobacter cloacae No. 8	0.0	0.0	0.0	6.3	7.0	7.9
Alcaligenes sp. No. 9	0.0	0.0	0.0	6.5	7.6	8.8
Pseudomonas sp. No. 10	0.0	0.0	0.0	7.2	9.2	11.8
Enterobacter cloacae No. 11	0.0	0.0	0.0	4.1	5.3	6.1
Bacillus sp. No. 12	0.0	0.0	0.0	3.5	4.8	5.5
Pseudomonas sp. No. 13	0.0	0.0	0.0	1.3	2.8	3.6
Enterobacter sp. No. 14	0.0	0.0	0.0	6.7	8.6	11.8

The data of azo dye bioremoval by isolated bacteria (Table 4) and the changes in the activity of the three enzymes suggests the possible microbial biodegradation of the azo dye. Five strains/isolates namely *Pseudomonas* No 4, *Aeromonas* No 6, *Enterobacter* No 8, *Pseudomonas* No 10 and *Enterobacter* No.14 have removed more than 92 % of RB azo dye. Strain *Pseudomonas* sp. isolate No 4 performed the best in this relation. This strain was the highest among all other strains in the production of azoreductase, lignin Peroxidase and Polyphenol oxidase as well (Tables 5, 6, 7). From these data, strain *Pseudomonas* sp. No. 4 was characterized as potential bioremediation bacteria to be used in the removal and biodegradation of RB azo dye. This isolate, therefore, was subjected to further molecular identification using 16s rDNA analysis.

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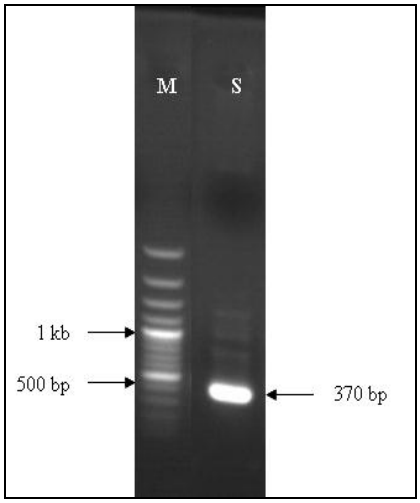
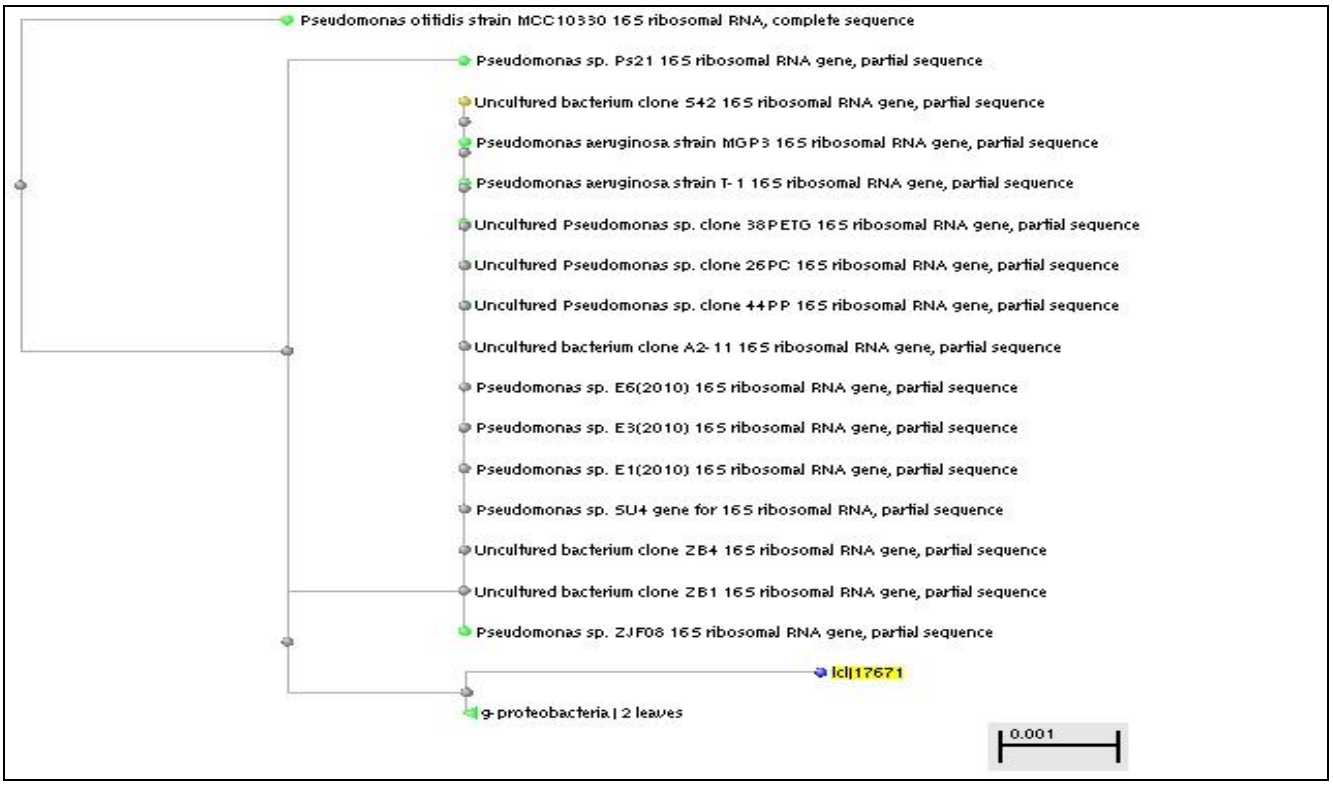


Where: M= DNA marker, S= PCR product of sample

Figure 3: Agarose gel electrophoresis of PCR product 16s rDNA analysis of *Pseudomonas* OS4.



Where: Query ID= lcll17671

Figure 4: phylogenetic tree constructed from the 16S rDNA sequence of *Pseudomonas* isolate OS4 and their related strains in GenBank.

Identification of *Pseudomonas* Isolate OS4 efficient in bioremoval of Reactive Blue azo dye using 16s DNA analysis

The primary identification of this bacterial isolate No 4 (Figure 2) was performed using standard procedures according to Bergey's Manual of Systematic Bacteriology (Holt *et al.,* 1994) and classified as *Pseudomonas* sp. OS4. This Identification confirmed by 16S rDNA analysis. The PCR amplified DNA fragment (370 Pb) was obtained on agarose gel

electrophoreses (Figure 3). The fragment sequence of *Pseudomonas* sp. OS4 strain was compared with available 16S rDNA gene sequences from organisms in the GenBank databases. The *Pseudomonas* spp. OS4 strain according to molecular identification was phylogenetically positioned in genus *Pseudomonas* (Figure 4). The nucleotide alignment and distance matrix showed high similarity value (99 %) with species: *Pseudomonas aeruginosa*. From these data, the OS4 strain was identified as *Pseudomonas aeruginosa* strain OS4 (NCBI accession number: KC762943).

Design and operation of prototype sequential bioreactor for the Reactive Blue azo dye bioremediation

Figure (5) illustrates the details of the bioreactor that has been submitted to the patent bureau at the Egyptian Academy of Scientific Research and Technology (Patent, 2009). The bioreactor was designed to achieve anoxic/aerobic continuous conditions. The first part of the bioreactor is upflow fixed-film column (UFC) container. Another part provides continuously stirred aerobic (CSA) container to speed bioremediation and/or biodegradation of the intermediate molecules (Figure 5). The UFC container was built from glass column of 70 cm height and 4 cm internal diameter (Figure 5). The glass column was filled with 3 days old *Pseudomonas aeruginosa* strain OS4 suspension starter to the height of 7cm. The UFC was fed with industrial wastewater containing RB azo dye diluted 10 times with H_2O. The system was operated in upflow mode at an average flow rate of 50 mL/h with an average hydraulic retention time (HRT) of 20h. The bioreactor was incubated at room temperature (28 ±2 °C) for 4 days in static conditions. The bioreactor after then was fed continuously by wastewater from the feed tank (#1 in Figure 5) for a period of 14 days.
The CSA container of the bioreactor was designed as bottle shape vessel having inner dimensions of 13.3 cm diameter and height of 19 cm with working volume of 2.0L (Figure 5). The contents of the reactor were agitated and aerated by small air pump. The CSA was directly fed in continuous mode with the output of UFC to enhance the biodegradation of the intermediates formed during UFC treatment. After the stabilization period, the CSA was operated in continues for 14 days. The system was operated at room temperature (28 ±2° C) at pH 6.8-7.

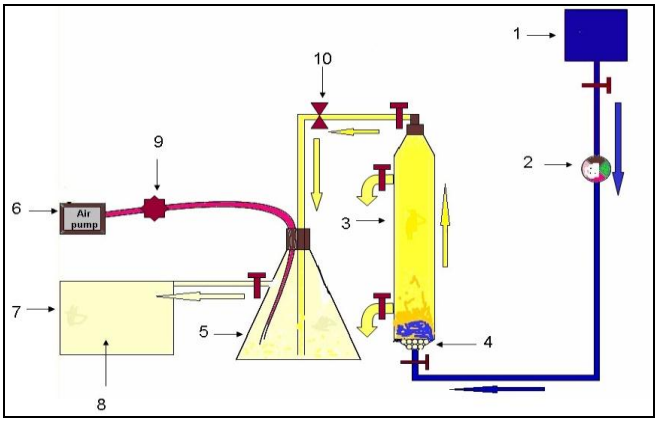


Figure 5: Schematic diagram of anoxic /aerobic sequential bioreactor (patent application 439/ 2009). Where: 1, feed tank; 2, controlling dispenser; 3, UFC anoxic container; 4, bioagent *Pseudomonas aeruginosa* strain OS4; 5, CSA aerobic container; 6, air pump; 7, effluent tank; 8, bioremediated wastewater; 9, air filter; 10, six valve ; direction of wastewater flow.

Figure (6a) shows that Azoreductase enzyme showed steady gradual increase through the operation time (14 days) in the anoxic container of the bioreactor (UFC) and reached the maximum values of 75 units of enzyme. In the aerated bioreactor container (CSA) no azoreductase activity has been detected. This is likely to be due to the inactivation of the enzyme by increased oxygen level due to continuous stirring.

The induction of oxidoreductase enzyme (Lignin Peroxidase) was reported under both anoxic and aerobic conditions (Figure 6b). The activity of this enzyme reached 30 and 38 international units under anoxic and aerobic conditions respectively after 14 of incubation.

The Polyphenol oxidase enzyme activity (Figure 6c) was induced only in aerated container of the bioreactor (CSA) and totally retarded in UFC container. The changes of this oxidase enzyme followed the same pattern of the gradual increase throughout the experimental period and reached the maximum value at 14 day being 60 units.

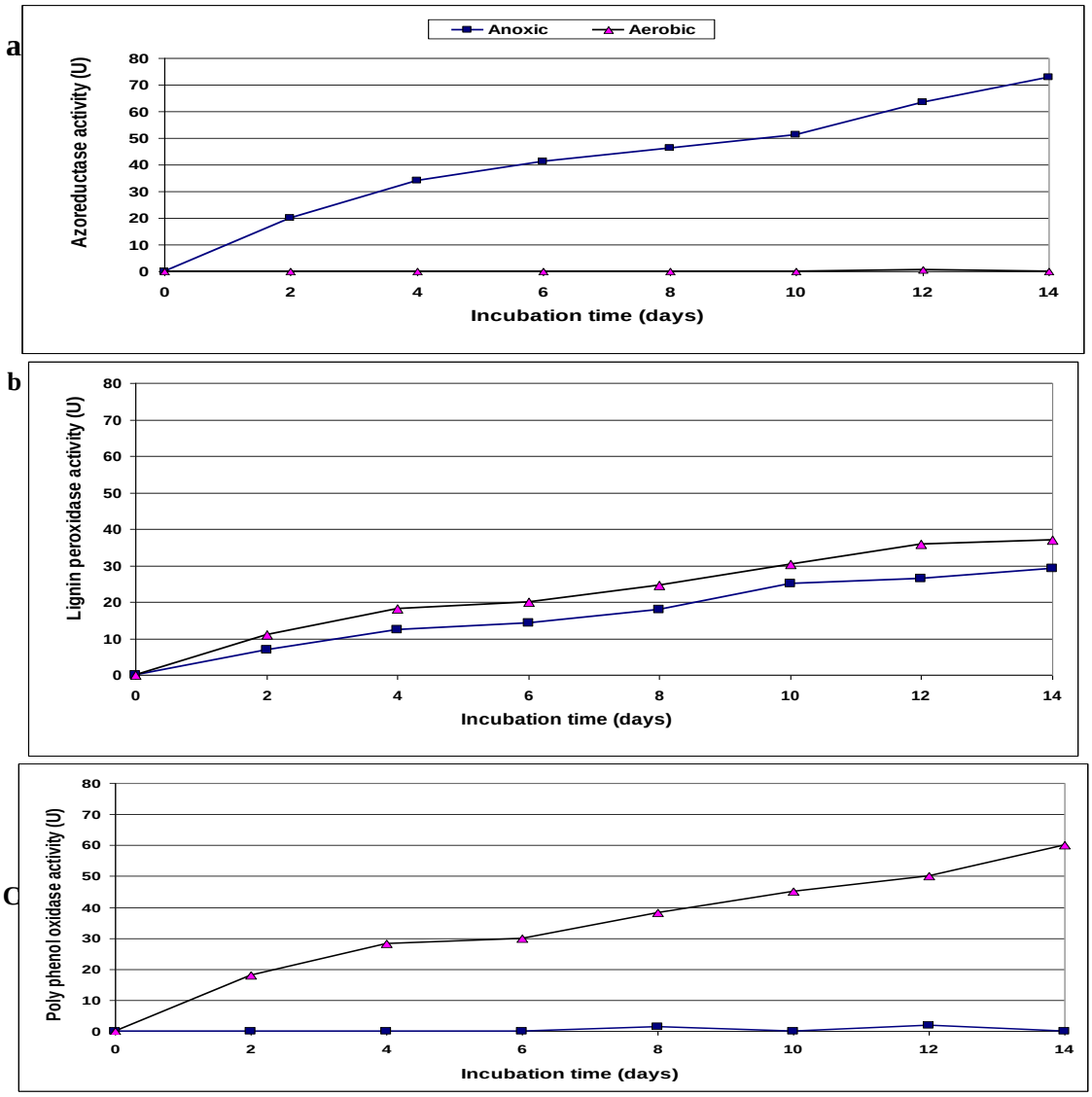


Figure 6: Changes in Azoreductase, Lignin Peroxidase and Polyphenol oxidase enzymes activity in UFC/ CSA bioreactor amended with industrial wastewater containing Reactive Blue azo dye residues and *Pseudomonas aeruginosa* strain OS4 as bioremediation agent.

DISCUSSION

Azo dyes are widely used in textile industry. The basic structure of these dyes include an azo bond (–N=N–) that doesn't exist in nature. The stability and the xenobiotic nature of the reactive azo dyes make them recalcitrant hence they are not totally degraded by conventional wastewater treatment processes that involve light, chemicals or activated sludge (Grekova-Vasileva and Topalova, 2009). The remediation of textile industry wastewater before releasing into the surrounding environment is of significance particularly for the safety of the agricultural ecosystem nearby the textile industries. The recalcitrant nature of these chemicals requires special technologies to remove and/or reduce the risks associated with their discharge into the environment without treatment. Therefore, the bioremediation of these toxic dyes is among the approaches for remediation of textile industry wastewater.

Two wastewater samples were collected from textile company located at El-Mukatem Dyehouse near Cairo. The first wastewater sample was obtained from dyeing basin receiving RB azo dye before discharging into the effluent lagoon. This wastewater had alkaline pH and high value of EC. The COD and BOD of this wastewater sample were very high. The other wastewater sample was collected from the factory general wastewater disposal lagoon. This wastewater had high values of pH and had comparatively high values of COD and BOD. Similar results were obtained by Darwesh *et al.* (2008). The COD and BOD of the lagoon wastewater sample were much lower than those of wastewater collected from dye basin. This may be due to stabilization of the effluent constituents in the open lagoon by vigorous microbial activities which take part in the biodegradation of the organic load in the lagoon. The lower values of EC in the wastewater from lagoon may be due to the dilution effect of excessive water used in washing fabrics throughout the industrial process. The count of bacteria in the wastewater was $2.3 * 10^3$ CFU/ml^{-1}, whereas the fungal count was lower ($0.5 * 10^2$ CFU/ml^{-1}). This low count of fungi may be due to the high pH of the wastewater and the possible toxic effect of the dye residues reaching the lagoon after dying the fabrics and discharging the wastewater into the main lagoon disposal site. Similar results were reported by Selvam *et al.* (2012).

From the prolonged anaerobic enrichment cultures in wastewater sample containing Reactive Blue azo dye, 14 bacterial isolates were obtained. These isolates were screened for the ability to decolourize RB azo dye in wastewater. The efficiency of the bacterial isolates in RB azo dye removal was assessed under anoxic conditions for 6 days then under aerobic conditions for another 6 days. The colour removal capacity reached above 95 % under anoxic conditions. Khehra et al. (2006) reported that, the main colour removal phase in the dye operation system was the anaerobic phase. The contribution of aerobic phase to colour removal at the early stages of azo dye microbial interaction was negligible. This is in agreement with the results obtained by Isik and Sponza (2003). The decolourization of RB azo dye under anoxic and/or aerobic conditions may be attributed to either physicochemical effect and/or to microbial biodegradation. It is known that any microbial biodegradation activity is due to the activity of certain enzymes. In this study, three oxidoreductase enzymes, namely azoreductase, lignin Peroxidase and Polyphenol oxidase were assessed in relation to RB colour removal. The azoreductase enzyme was only induced in the anoxic conditions and the aeration of the medium after 6 days resulted in total inhibition of this enzyme. These results agree with those obtained by other authors (Franciscon *et al.*, 2012

and Isik and Sponza 2004). The azoreductase enzyme activity show wide variations depending on the strains used and the sampling time under anoxic conditions. Similar finding were reported by Leelakriangsak and Borisut (2012) and Mohan *et al.* (2012).

The Lignin Peroxidase enzyme was induced under both anoxic and aerobic conditions. The activity of this enzyme in the first phase of growth (anoxic phase) showed gradual increase with the incubation time. The exposure of microbial growth to the aerobic conditions showed additional Lig. Peroxidase activity increased as the time of aerobic incubation reached till the end of the experiment. No retardation of Lignin Peroxidase activity was noted with switching from anoxic to aerobic conditions. Similar results were obtained by several authors (Selvam et al., 2012; Bholay et al., 2012 and Zucca et al., 2012).

As regards the Polyphenol oxidase enzyme on contrary to azoreductase enzyme, no Polyphenol oxidase activity was reported in the growth medium under anoxic conditions until the end of 6 days of incubation. This enzyme started to show activity in the aerobic phase of incubation. The gradual increase in Polyphenol oxidase activity was noted as the incubation time increased under aerobic conditions. Franciscon *et al.* (2012) reported that the Polyphenol oxidase activity under aerated conditions increased and remained increased for the entire 168 h aeration period suggesting that Polyphenol oxidase could be involved in further biodegradation of azo dye metabolites.

One isolate was identified as the most efficient in decolourization of wastewater containing RB dye residues as well as production of assessed enzymes. This isolate was identified by classical identification schemes and 16s DNA sequences to *Pseudomonas aeruginosa* strain OS4 (NCBI accession number: KC762943).

The information generated from bench scale laboratory experiments on RB azo dye decolourization and enzymes involved in the dye transformations was used to design small scale prototype bioreactor as a step forward to establish bioremediation technology for treatment of recalcitrant azo dyes. The bioreactor design that has been submitted, as part of bioremediation procedure for azo dye residues treatment, to the patent bureau at the Egyptian Academy of Scientific Research and Technology (Patent, 2009). The bioreactor was designed to achieve anoxic/aerobic continuous conditions. Bacterial strain *Pseudomonas aeruginosa* strain OS4 was used in this small scale (two phases) bioreactor to bioremediate RB azo dye in industrial wastewater. The bioremediation power of any organism is usually associated with complex activity of specific enzymes consortium. Three enzymes namely; Azoreductase, lignin peroxidase and Polyphenol oxidase known to play role in the biodegradation of heterocyclic compounds such as textile synthetic azo dyes were assessed in the bioreactor. These enzymes belong to three enzyme types namely; reductase, oxidoreductase and oxidase enzymes. These activities reported to be involved in initiation and biotransformation of RB azo dye and the intermediate compounds of its initial degradation. The changes in enzyme activities were monitored in both bioreactor containers by sampling of effluents every 2 days intervals for the duration of 14 days. Azoreductase enzyme showed steady gradual increase throughout the operation time (14 days) in the anoxic container of the bioreactor (UFC). In the aerated bioreactor container (CSA) no azoreductase activity has been detected. This confirms the results obtained in bench scale flask experiments in this work. This is likely to be due to the inactivation of the enzyme by increased oxygen level due to continuous stirring. Franciscon *et al.* (2012) Stated that

azoreductase enzyme are inhibited by oxygen. The induction of Lignin Peroxidase was reported under both anoxic and aerobic conditions. The Polyphenol oxidase enzyme activity was induced only in aerated container of the bioreactor (CSA) and totally retarded in UFC container.

CONCLUSION

It is evident that the bioremediation agent used in this study (*Pseudomonas aeruginosa* strain OS4) harbor strong enzyme machinery necessary to remediate the recalcitrant azo bonds in the RB dye and therefore, this agent can be used for building sound bioremediation system to control the environmental pollution by recalcitrant azo dyes (particularly RB dye) before discharging into the terrestrial and/or aquatic environment.

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