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RESEARCH ARTICLE

Microbial Biodegradation of Reactive Blue (RB) Textile azo dye in Sequential Anoxic/Aerobic Bioreactor

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Abstract

This study aims at setting a prototype model for remediation of wastewaters generated from textile industry using dyes. For this purpose a lab-scale bioreactor was designed to fulfill the bioremediation process. The bioremediation agent used in this bioreactor was a potent *Pseudomonas aeruginosa* strain OS4 isolated from waste disposal site of textile processing plant in Egypt. The unit was operated at flow rate of 50 mL h^{-1} with HRT of 20 h. The textile industry effluent was fed to bioreactor. The sequential anoxic–aerobic treatment of wastewater at dilution rate of 10% of wastewater in water resulted in 99% dye decolourization. The activities of eight enzymes associated with biodegradation process were assessed under anoxic and aerobic conditions to follow the possible mechanism contributing to biodegradation of the dye and dye residues. The activity of Azoreductase enzyme was markedly under anoxic conditions only. The induction of Laccase, Mn Peroxidase and Lignin Peroxidase activities was noted under both anoxic and aerobic conditions. Four other enzymes; Poly phenol oxidase, Tyrosinase, Pyrochatechase and Ascorbate oxidase were activated under aerobic conditions only. The then layer chromatography (TLC) and HPLC analyses were applied to assess the biotransformation of the dye into non-aromatic intermediate compounds. The removal of recalcitrant cyclic RB azo dye from industrial wastewater was also assessed in the same sequential anoxic–aerobic bioreactor using the immobilized bioremediating bacterial cells. The results show that the immobilized cells perform better than the free cells in the prototype model bioreactor tested in this study..

Introduction

Reactive azo dyes are extensively used in the textile, paper, cosmetic and leather industry because of their wide variety of colour shades, high wet fastness profiles, ease of application, brilliant colours, and minimal energy consumption (Axelsson et al., 2006). The excess dye goes to wastewater and the amount could reach up to 50 % of the used dye. Most of these dyes are toxic and/or carcinogenic (Acuner and Dilek, 2004). Disposal of these dyes into the environment causes serious damage, since in addition to their toxic effect they may significantly affect the photosynthetic activity of hydrophytes by reducing light penetration (Aksu et al., 2007). They may be also toxic to some aquatic organisms either directly or due to their breakdown products (Franciscon et al., 2009). Dyes can be removed from wastewater by chemical and physical methods including adsorption, coagulation–flocculation, oxidation and electrochemical methods (Mahne, 2012). However, both the physical and chemical methods have many disadvantages in application, such as high-energy costs and high-sludge production (Sarioglu et al., 2007). Conversely, bioprocessing can overcome these defects because it is cost effective and environmentally benign. Several reports have shown that bacteria can degrade and even completely mineralize many reactive dyes under certain conditions (Kapdan and Erten, 2007; Darwesh et al., 2008; Moawad et al., 2010). The products of intermediate metabolism during the decolourization

process, such as aromatic amines, can be degraded by the hydroxylase and oxygenase enzymes produced by bacteria (Pandey et al., 2007). Several bacterial strains capable of decolorizing a broad-spectrum of dyes have been isolated and characterized (Deng et al., 2008). The process of bacterial degradation of reactive dyes is often initiated under anaerobic conditions by an enzymatic biotransformation step (Carvalho et al., 2008). The resulting products such as aromatic amines are further degraded by multiple-step bioconversion occurring aerobically or anaerobically (Barragan et al., 2007; Xu et al., 2006). Much work is still required to obtain microorganisms capable of degrading a wide range of structurally different dyes and to study their physiological characteristics, in order not only to understand the underlying mechanisms in dye biodegradation, but also for biotechnological application. The optimization of this bioprocess requires special attention. Among these bioprocesses, immobilized cells systems have proven efficient in degradation of toxic chemicals than conventional wastewater treatment systems since high densities of specialized microorganisms are used in immobilized cell systems (HeFang et al., 2004). In this study, a local isolate of *Pseudomonas aeruginosa* strain OS4 was used to Reactive Blue azo dye in textile industry wastewater using lab scale sequential bioreactor consisting of upflow fixed-film column (UFC) operated under anaerobic conditions followed by continuously stirred aerobic (CSA) system.

Materials and Methods

Microorganism

Pseudomonas aeruginosa strain OS4 (NCBI accession number: KC762943) was locally isolated from waste disposal site of textile industry plant near Cairo. This bacterial strain was identified as the most efficient among other isolates in decolourization of wastewater containing RB dye residues (Darwesh et al., 2013).

Inoculums preparation for UFC/CSA bioreactor

Free bacterial system

A loopful of growth from stock culture slope was inoculated into MSM modified medium broth in flasks and incubated at 28-30°C under shaking conditions for 3 days. The bacterial suspension was therefore used to fill 10 % of UFC/CSA bioreactor.

Immobilized bacterial system

Suspension of 3 days growth of *Pseudomonas aeruginosa* strain OS4 was mixed with an equal volume of sterile sodium alginate (4%). The mixture was added as drop-wise into solution of sodium chloride (0.2 mol/L) and calcium chloride (0.5 mol/L) and magnetically stirred at 200 rpm/min till alginate beads were formed.

Operating the prototype sequential UFC/CSA bioreactor

In a previous study, a bioreactor was designed to achieve anoxic/aerobic continuous conditions (Darwesh et al. 2013). The bioreactor containing both UFC and CSA containers was inoculated with 10% bacterial suspension either free or immobilized cells. In the beginning of experiment, the UFC container was incubated at room temperature (28 ±2 °C) to initiate biodecolourization and/or biodegradation of wastewater containing RB azo dye. After complete decolourization, the bioreactor was fed by wastewater containing 300 ppm RB azo dye continuously during 30 days. The CSA was directly fed in continuous mode with the output of UFC during stabilization so as to enrich the cultures capable of degrading the intermediates formed during UFC treatment. The system was operated at room temperature (28 ±2° C) and pH was 6.8- 7.

Analytical methods

Samples collected from UFC and CSA at regular time intervals were centrifuged at 10,000 rpm for 15 min and supernatants were used for further analysis.

Decolourization assay

Decolourizing activity was expressed in terms of percentage decolourization and was determined by monitoring the decrease in absorbance at 595nm (λ_{max} of Reactive Blue dye) by UV vis spectrophotometer (Jenway UV/Visible- 2605 spectrophotometer, England). The absorption of cell-free supernatant from UFC and CSA containers of bioreactor was measured. The distilled water was used as blank and wastewater containing Reactive Blue azo dye was used as reference for calculating percentage of decolourization (control) as per following equation:

$$Decolourization\ activity\ \% = \frac{\text{Reading of } [C] \text{ decolourization} - \text{Reading of } [S] \text{ decolourization}}{\text{Reading of } [C] \text{ decolourization}} \times 100$$

Where *C*= control, *S*= sample

Spectrophotometric scanning

Aliquots (3mL) of the cell-free supernatant from both UFC and CSA outputs as well as wastewater samples were scanned in the range of 200 to 800 nm to observe shifting of peaks due to transformations of dye after passing through UFC and CSA Bioreactor compartments. The scanning was performed using a UV vis spectrophotometer (Jenway UV/Visible- 2605 spectrophotometer, England).

Estimation of chemical and Biological oxygen demands (COD and BOD)

The COD and BOD of raw wastewater as well as UFC and CSA outputs were determined according to standard methods (APHA, 1998). COD was measured using (Hach) spectrophotometer test kit, while, BOD was measured by OXI Top BOD meter (WTW, Comp.).

Detection of residual dye and biodegradation products using thin layer chromatography (TLC)

At the end of the incubation period (30 days); the cell-free supernatant of outputs from UFC and CSA containers were extracted twice with an equal volume of *n*-butanol to extract the residual dye and biotransformed products of Reactive Blue (Temuujin et al., 2012). The wastewater was also extracted in the same way. The pooled extracts from each treatment were concentrated on a rotary vapor. The concentrated samples were diffused on Silica gel DC-Alufolien-Kieselgel TLC plates using *n*-butanol/acetic acid/distilled water (4:1:5 v/v) as developing solvent. The resolved chromatograms were screened under UV light (254nm).

HPLC analysis of degradation metabolites

The dye and its degradation products were monitored by HPLC to evaluate biodegradation development. The anaerobic and aerobic metabolites of reactive azo dye wastewater were centrifuged and filtered through a 0.45 µm membrane filter (Millipore) to remove bacterial cells. The filtrate was then extracted with equal volume of ethyl acetate after acidification to pH 2-3 with 6N HCl. The extracts were dried over anhydrous Na_2SO_4 and evaporated to dryness in a rotary evaporator. The residue was dissolved in small volume of methanol. Analysis using HPLC was carried out on a Shimadzu model LC-3A chromatograph (Shimadzu Corp., Kyoto, Japan) equipped with Shimadzu model SPD-2A detector and Pegasil ODS (C18 column with 4.6 mm of inside diameter and 150 mm of tall, Senshu Scientific Co. Ltd., Tokyo, Japan). A mobile phase composed of 50% methanol, 0.3% H_3PO_4, and 49.7% water was used with the flow rate of 0.5 ml/min. The eluants were monitored by UV absorption at 275 nm.

Assay of enzymes involved in biotransformation of RB azo dye

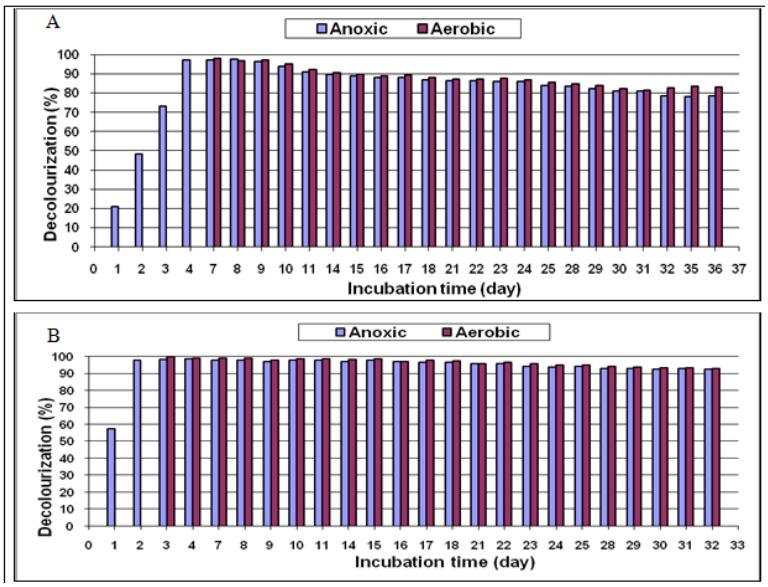
The role of several enzymes in biotransformation of recalcitrant azo dyes was reported. Azoreductase enzyme was assayed by the method described by Pandey and Dubey (2012). The reaction mixture contained 400 µl of 50 mM sodium phosphate buffer (pH 7.0), 200 µl of the sample and 200 µl of 100 ppm Reactive 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). Laccase activity was assayed spectrophotometrically at 469 nm by 10 mM DMP in 100 mM sodium acetate buffer, pH 5 (Zouari-Mechichi et al., 2006). The manganese peroxidase (MnP) activity was assayed by the oxidation of 1 mM $MnCL_2$ in 50 mM sodium malonate, pH 4.5, in the presence of 0.1 mM H_2O_2. Manganic ions, Mn^{+3} form a complex with malonate and the measurement was done at 270 nm (Liu et al., 2009). Tyrosinase activity was determined in a reaction mixture of 2.9 ml, containing 1ml of 0.001 M L-Tyrosine and 0.9 ml of sample in 1 ml of 0.5 M phosphate buffer (pH 6.2) at 280 nm by the procedure of (Conrad et al., 1994). Ascorbate oxidase activity was determined by the decrease in absorbance at 245nm by measuring the oxidation of 0.1 mM L-ascorbate to dehydroascorbate (Hernandez-Romero et al., 2005). A 10 mM L-ascorbic acid stock solution was prepared in 100 ml of 1 mM HCl supplemented with 1 mM EDTA. The 10 mM L-ascorbic acid stock solution was diluted to obtain 0.1 mM L-ascorbic acid with dilution buffer consisting of 0.1 M NaH_2PO_4 and 1 mM EDTA. Then 0.1 ml of sample was added to a cuvette containing 0.9 ml of 0.1 mM L-ascorbic acid. Pyrocatechase (catechol 1,2-dioxygenase) activity was measured spectrophotometrically at 260 nm for the detection of cis, cis-muconic acid (dos-Santos et al., 2009). The reaction mixture contained 100 µl of crude cell-free extract and 2.9 mL of 50 mM Tris–HCl buffer (pH 8.3) containing 5 mM β-mercaptoethanol, 20 µM $FeSO_4$ and 0.1 mM of catechol. Readings were taken at 30s intervals for 5 min at ambient temperature. Control reactions without substrate or extract were performed for each assay. One enzyme unit (U) is defined as the amount of enzyme that catalyzes the formation of 1µmol product per minute at 30°C.

Results and Discussions

Removal of Reactive Blue azo dye from Textile industry wastewater by *Pseudomonas aeruginosa* strain OS4 bacteria in sequential anoxic/aerobic continuous system.

The textile industry reactive blue azo dye residual wastewater collected from dye basins after dyeing the fabrics was diluted ten times by distilled water and subjected to bioremediation treatment in specific bioreactor with two sequential phases (anoxic then aerobic phases). It is documented that the azo dyes degradation requires anoxic conditions to initiate the biodegradation process (darwesh et al., 2008). In this study; *Pseudomonas aeruginosa* strain OS4 (facultative anaerobe) was applied to the two compartment of the bioreactor (anoxic and aerobic compartment) either as free bacterial cells and/or immobilized cells on sodium alginate. The newly designed bioreactor (Darwesh et al., 2013) was operated in a continuous manor starting with anoxic container providing the appropriate condition for cleavage of the azo bonds then followed by aerobic container for complete aerobic biodegradation of the dye. The results in Figure (1) show that the decolourization of the dye wastewater started from day one after loading the bioreactor with diluted dye wastewater and the bacteria either as free cell suspension or immobilized cells. The decolourization rate reached the maximum level (97.25% in free cell system and 98.25% in immobilized system) after 7 days and 3 days respectively (Fig. 1A & B). The fast decolourization in immobilized system may be due to the protection of bacterial cells from the direct toxic dye effect and /or to the enhancement of the direct contact between cells and the enzymes targeting azo dyes in the bioreactor. These results agree with those obtained by Liu et al. (2012). The decolourization rate of the continuous dye wastewater feeding into the bioreactor was then stabilized at the range of 92 to 97 % in the anoxic phase and 93 to 98 % in the aerobic bioreactor vessel (Fig. 1A & B). The system operated continuously throughout 30 days of experimental period. The results show that the immobilized cells in this bioreactor are more efficient in dye bioremoval under the continuous anoxic/aerobic sequential system. Several authors stated that, for dye removal, the immobilized cells perform better than free cells (Supaka et al., 2004; Xu et al., 2007; Soundararajan et al., 2012).

Fig 1. Continuous system for decolourization of Reactive Blue textile azo dye in experimental bioreactor under sequential partially anoxic and fully aerobic conditions using free (A) and immobilized (B) cells of *Pseudomonas aeruginosa* strain OS4 bacteria.



The Chemical and Biological oxygen demand values are indicators to the total organics loads in the solution. The changes of COD in the dye containing effluents show the degree of dye mineralization as a function of dye removal. The original effluents solution at zero time had COD value of 2018 ppm (Table 1). It was reduced to 1376 and 1540 ppm under anaerobic conditions in immobilized and free cells systems respectively. The wide variation of COD value (27- 37%) could be attributed to recalcitrant nature of certain dye. It is reported that the textile dye effluents are more resistant to oxidation and degradation. The majority of COD was removed in the aerobic phase (97.37%) compared with 31.81% in the anaerobic phase. Similar results were obtained by HeFang et al. (2004) who found that the microorganisms continued to consume the obtained organics until near complete removal of COD value of culture. Similar trends were also found with the changes in BOD values which decreased after treatment of wastewater under anaerobic and aerobic conditions. The BOD removal reached 97.38 and 95.18% with immobilized and free cells systems respectively. The majority of colour was removed in the anaerobic phase, whereas, significant COD and BOD values were removed in the aerobic phase. However, the decolourization and removal of organic loading with free bacterial cells was slower than that with immobilized cells at the same incubation time.

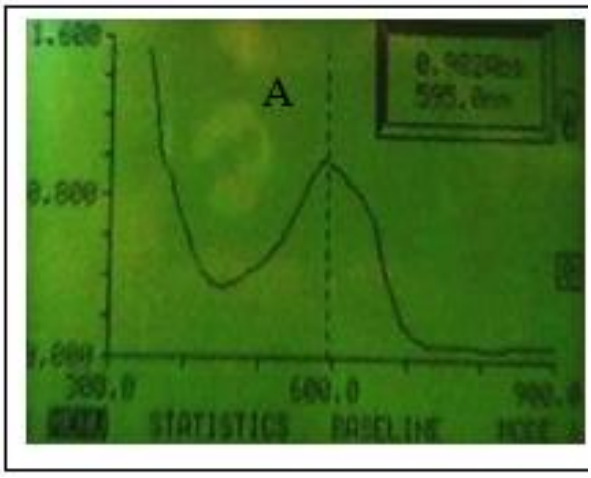
Table 1. Changes in COD and BOD after wastewater treated in UFC and CSA compartments of the bioreactor

Samples from	Bacterial Immobilized cells				Bacterial cell suspensions			
	COD		BOD		COD		BOD	
	ppm	removal %	ppm	removal %	ppm	removal %	ppm	removal %
W.W[a]	2018	————	954	———	2018	———	954	———
UFC[b]	1376	31.81	532	44.23	1540	23.69	700	26.62
CSA[c]	53	97.37	25	97.38	87	95.69	46	95.18

a: wastewater, b and c: anoxic and aerobic containers of bioreactor.

Spectrophotometric analysis of wastewater before and after passing through the bioreactor.

The comparison of UV-visible absorption spectra (200-800 nm) of the wastewater and aqueous solution collected from UFC and CSA containers of the bioreactor indicate complete disappearance of peak at 595 nm corresponding to λmax of dye in the output of UFC container (Fig. 2B). However, a new peak was observed at 320 nm, which was significantly reduced after 30 days of treatment in CSA container (Fig. 2C). These changes could be explained by structural modifications of dye molecule due to azo bond reduction under anaerobic conditions. Many types of bacteria perform this reaction, producing metabolites such as aromatic amines (Seesuriyachan et al., 2007). Similar observations have been reported by Khehra et al. (2006) regarding accumulation of aromatic amines intermediates after decolourization of Acid Red 88 azo dye under anaerobic conditions. However, these amino derivatives/intermediates are reported to be degraded and/or transformed under aerobic conditions.



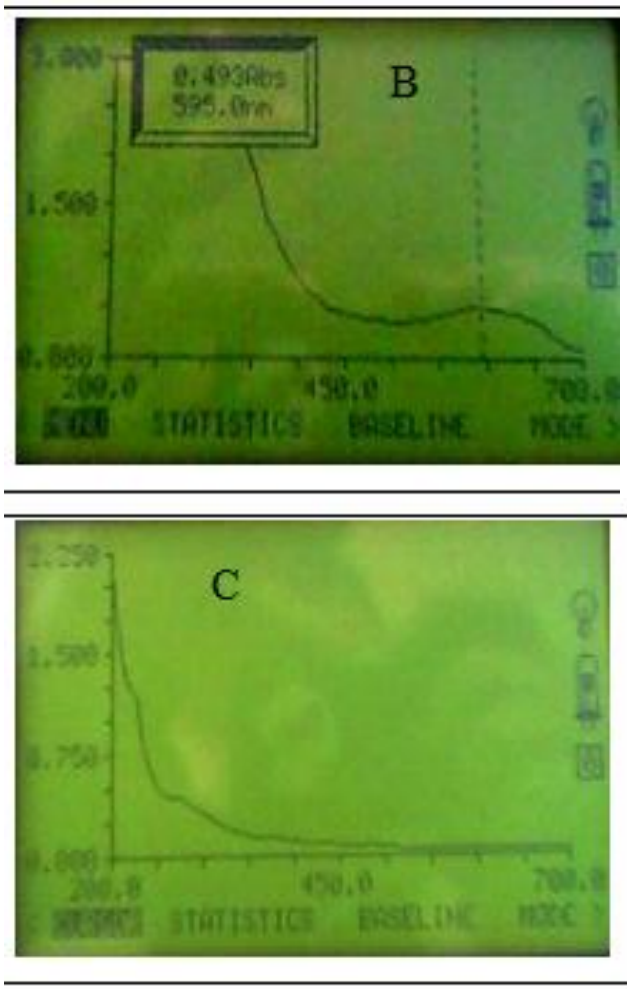
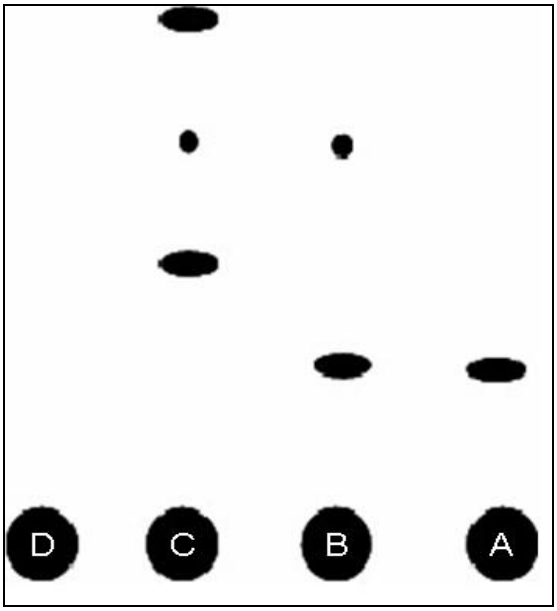


Fig 2. UV-visible scans of real wastewater feed (A), UFC container aqueous solution (B) and CSA container aqueous solution (C).

Thin Layer Chromatography analysis of wastewater and possible intermediates formed in the two compartments of bioreactor.

The chromatogram of *n*-butanol extracted samples of standard Reactive Blue azo dye, real wastewater, and aqueous solutions from UFC and CSA containers showed the presence of single band corresponding to parent dye lane A (Fig. 3). The wastewater sample showed 2 bands (lane B); one of them is similar to parent dye (lane A) in RF (Fig. 3). The solution from UFC gave 3 bands (Fig. 3 lane C). These bands were totally different from those of parent dye. This indicates the formation of certain intermediate compound possibly aromatic amine derivative in UFC container. The chromatogram of CSA solution (Fig. 3D) showed the disappearance of bands totaly. This likely indicates the conversion of aromatic metabolites produced in UFC container into non-aromatic intermediates. Complete mineralization of azo dyes based on substituted benzene rings by anaerobic/aerobic sequential has been reported by Khehra et al. (2006).

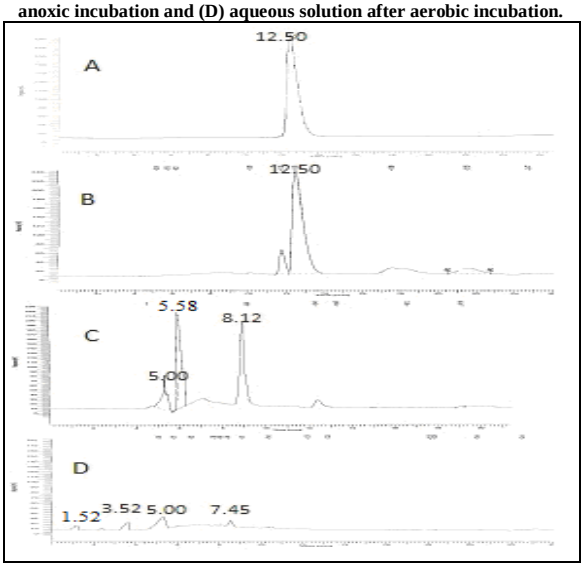
Fig 3. Thin layer chromatogram of standard Reactive Blue azo dye (A), real wastewater (B), UFC container aqueous solution (C) and CSA container aqueous solution (D).



Assessment of Biodegradation of Reactive Blue azo dye in the experimental bioreactor using HPLC analysis.

The *n*-butanol extracts of anaerobic and aerobic phases of the bioreactor amended with wastewater containing RB azo dye were subjected to analyze by HPLC. Figure (4) shows the results of HPLC analysis of the metabolites resulted from wastewater containing Reactive Blue azo dye treated with *Pseudomonas aeruginosa* strain OS4 in microaerophilic and aerobic bioreactor. The raw wastewater analysis showed one main peak at retention time of 12.50 min (Fig. 4B). This peak was identical to standard dye peak (Fig. 4A). After the completion of microaerophilic incubation, two peaks at retention time 5.58 and 8.12 min respectively were detected (Fig. 4C). The peak areas increased along with a decrease in the concentration of Reactive Blue azo dye peak. It is thus reasonable to assume that the bacterial strain caused cleavage of the azo bond of Reactive Blue azo dye and formed two aromatic amines represented by the 2 peaks (Fig. 4C). Recently, it was reported that partial reduction and complete cleavage of the azo bond could contribute to decolourization of reactive red dye by *Pseudomonas* sp. SUK1 (Kalyani et al., 2009). At the end of aerobic incubation in the bioreactor, the results of HPLC analyses show that the biodegradation metabolites produced during the anaerobic phase were nearly removed in the subsequent aerobic phase (Fig. 4D). This figure also shows that when such metabolites were degraded aerobically forming less aromatic compounds and more polar compounds, since the metabolite peak area decreased and shifted towards a lower retention time (1.52, 3.52, 5.00 and 7.45 min). Degradation in the aerobic phase may result in the formation of oxidized and very polar derivatives (e.g., aldehydes, carboxylic acids) having a lower aromaticity, as suggested by Nortemann et al. (1986) in a study of 6-aminonaphthalene-2-sulfonic acid degradation.

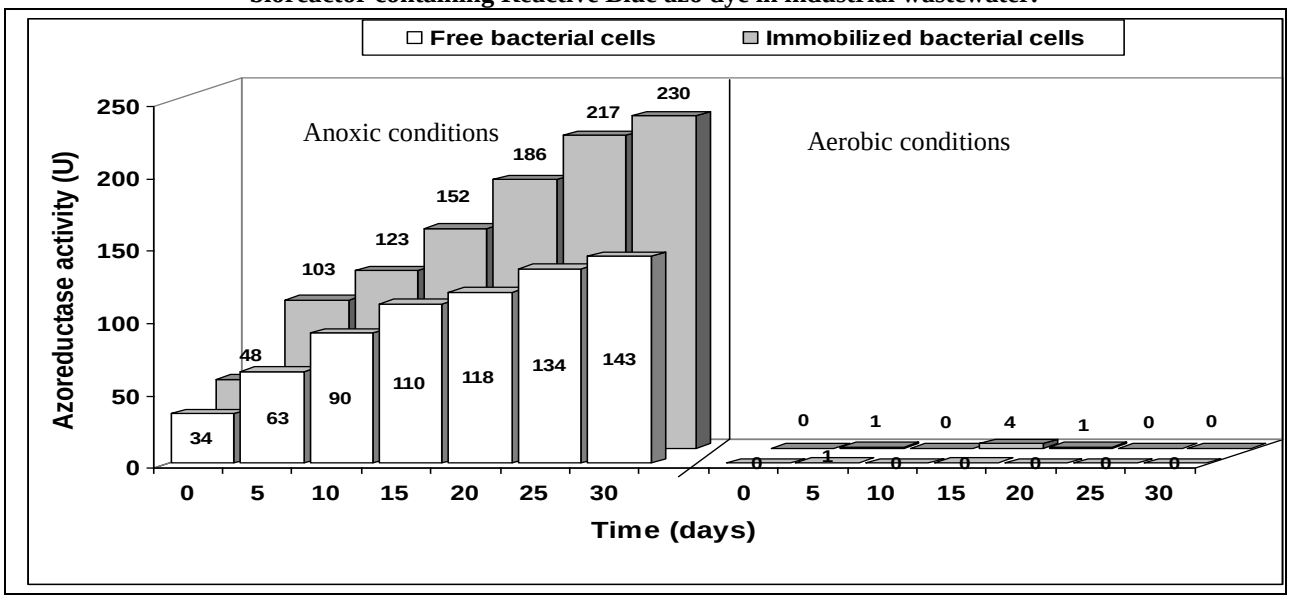
Fig 4. HPLC analysis of metabolites resulting from biodegradation of organic compounds in industrial wastewater containing Reactive Blue azo dye under anaerobic–aerobic conditions: (A) standard Reactive Blue azo dye; (B) industrial wastewater; (C) aqueous solution after anoxic incubation and (D) aqueous solution after aerobic incubation.



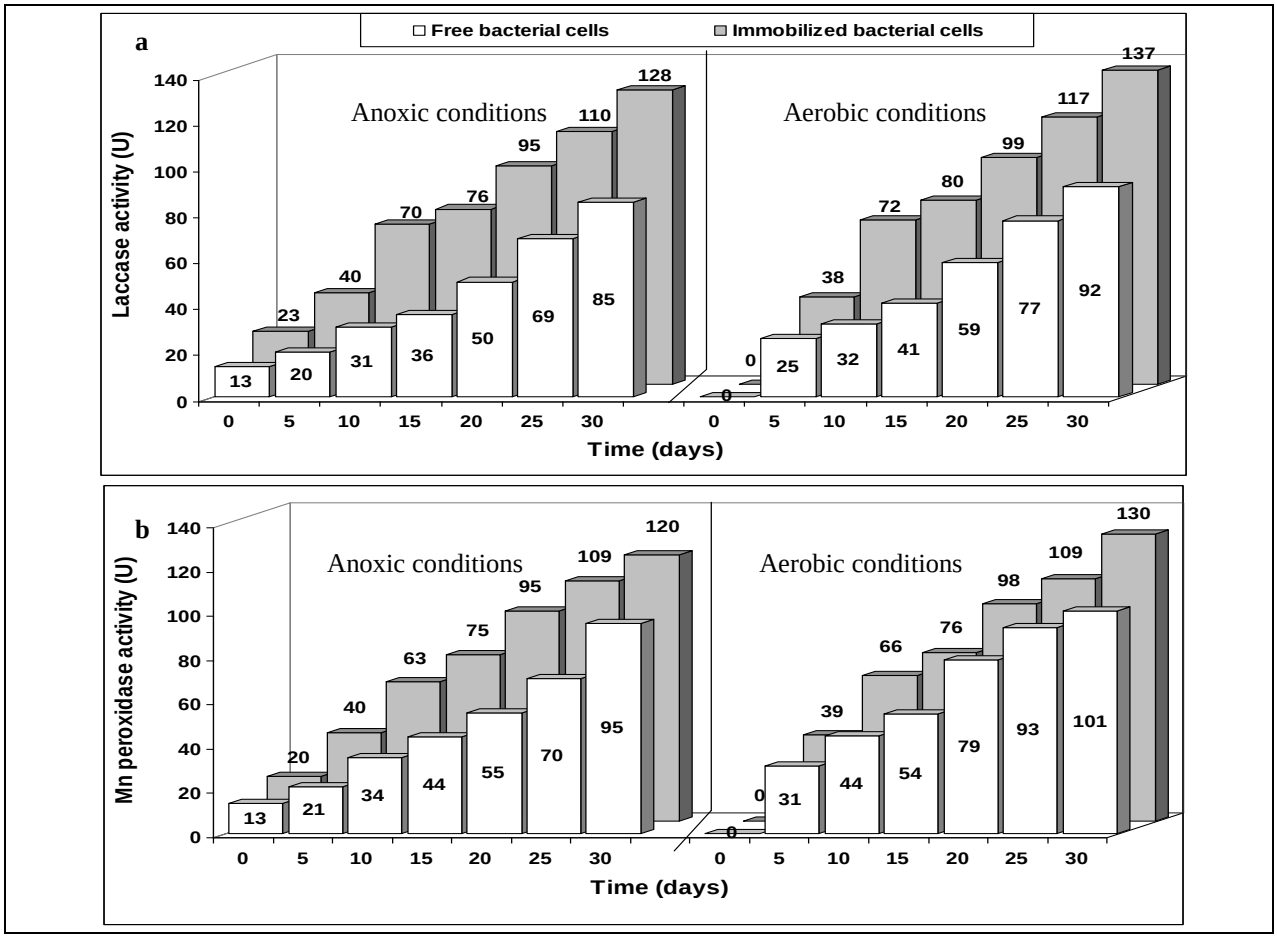
Enzymes activities associated with the biodegradation of RB azo dye in real textile wastewater

The evidence of biodegradation of the reactive blue azo dye by *Pseudomonas aeruginosa* strain OS4 was presented in the previous section. The results of HPLC and the TLC in addition to the decrease of organic load in the media are proves that the dye has been biodegraded either completely or to more simple less toxic compounds. This is in agreement with the results of other scientists (Dave and Dave, 2012; Tripathi and Srivastava, 2012). In this experiment eight specific enzymes were studied throughout the bioremediation duration (30 days), in order to understand the role of each of these enzymes in the detoxification of textile industrial dyes in wastewater before releasing into the surrounding environment. The presence of azo bond in the structure of reactive blue azo dye (Bergsten-Torralba et al., 2009) indicates that the first step of biodegradation will require Azoreductase enzyme to cleave the azo bond of the dye. The induction of this particular enzyme requires specific conditions. It was stated that the Azoreductase work in the anoxic conditions, where the oxygen is only available in combined form and the dissolved oxygen in the media is near to zero mg/L (Greenop et al., 2001). The used bioreactor for the azo dye biodegradation (patent 439/2009; Darwesh et al., 2013) fulfils these requirements by establishing the anoxic conditions in the first compartment of the upflow fixed film column (UFC). The retention time of the fed wastewater containing Reactive Blue azo dye in the UFC was set at the rate of 20 hr. Samples were taken every 5 days from the UFC compartment to determine the Azoreductase activity. This system was performed once by using *Pseudomonas aeruginosa* strain OS4 free cells and the other by immobilized cells of the same strain. The results at Figure (5) show that Azoreductase activity increased steadily and gradually from the start of the experiment until 30 days of continues system operation. The increase had similar trend in both free cells and immobilized systems. However, the increase was much higher in the immobilized *Pseudomonas* being 230 U as compared with free cells being 143 U. The improved performance of *Pseudomonas* in the immobilized system could be due to the protection of bacterial cells and/or enzyme from the toxic azo dye effect. Rezaee et al. (2008) stated that the immobilization of bacterial cells could protect the bacteria against the harsh conditions. Therefore, the immobilization of bacterial cells is receiving increased interest in the bioremediation of industrial wastewater. The Azoreductase activity in the other compartment of the bioreactor (continuously stirred aerobic -CSA-container) was totally retarded due to the presence of high dissolved oxygen in the system. It is clear that the anoxic condition established for both free and immobilized cells is crucial for induction of blue azo dye biodegradation by *Pseudomonas aeruginosa* strain OS4. Similar results were obtained by previous authors (Tripathi and Srivastava, 2011; Illanjiam and Arunachalam, 2012).

Fig 5. Azoreductase activity of *Pseudomonas aeruginosa* strain OS4 free or immobilized bacterial cells in a bioreactor containing Reactive Blue azo dye in industrial wastewater.



The oxidoreductase enzymes namely: laccase, Lignin peroxidase and Mn peroxidase play major role in the transformation of aromatic phenolic compounds. The changes in the enzymatic activities of these enzymes in the sequential anoxic/ aerobic bioreactor amended with wastewater containing Reactive Blue azo dye in the presence of *Pseudomonas aeruginosa* strain OS4 in free or immobilized form show steady increase in the activity of the above mentioned enzymes throughout the operating time of the bioreactor (30 days). The activities of these oxidoreductase enzymes were much high in the case of immobilized *Pseudomonas* cells as compared with free cells (Fig. 6). These enzymes performed perfectly well in both anoxic and aerobic conditions of the bioreactor. The highest values of laccase, Lignin peroxidase and Mn peroxidase activities were reached after 30 days of incubation being 128, 119 and 120 U respectively in the case of *Pseudomonas* immobilized cells. The highest activities of the same enzymes in free cells system were 85, 98 and 80 U respectively after 30 days. In aerobic CSA bioreactor container, the highest three enzymes activities were reached after 30 days of incubation immobilized system being 137, 106 and 130 U respectively. Under the same conditions, the free cells of *Pseudomonas* strain reached the highest enzyme activities values at the end of incubation (30 days); however the laccase, Lignin peroxidase and Mn peroxidase activity values were less than those of the immobilized system and gave 92, 85 and 101 enzyme activity units respectively (Fig. 6). It is likely that the oxidoreductase enzymes play significant role in azo dye biodegradation due to their adaptation to oxygen status of the media. These findings are in agreement with the results of Franciscon et al. (2012) who stated that oxidoreductase enzymes activity was observed during the biotreatment process suggesting the role of these enzymes in the decolourization and degradation process.



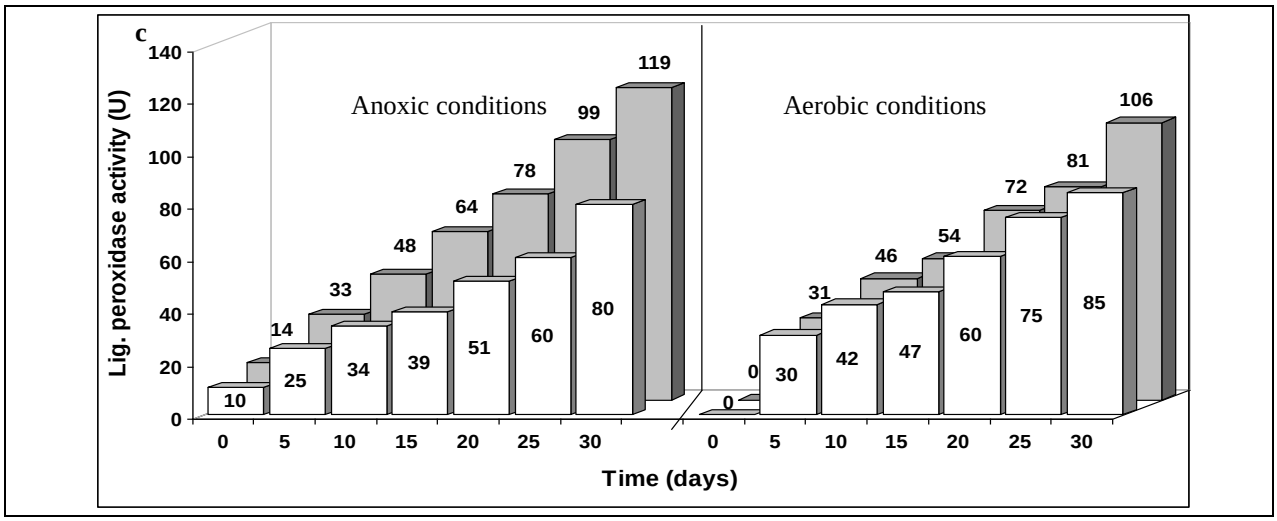
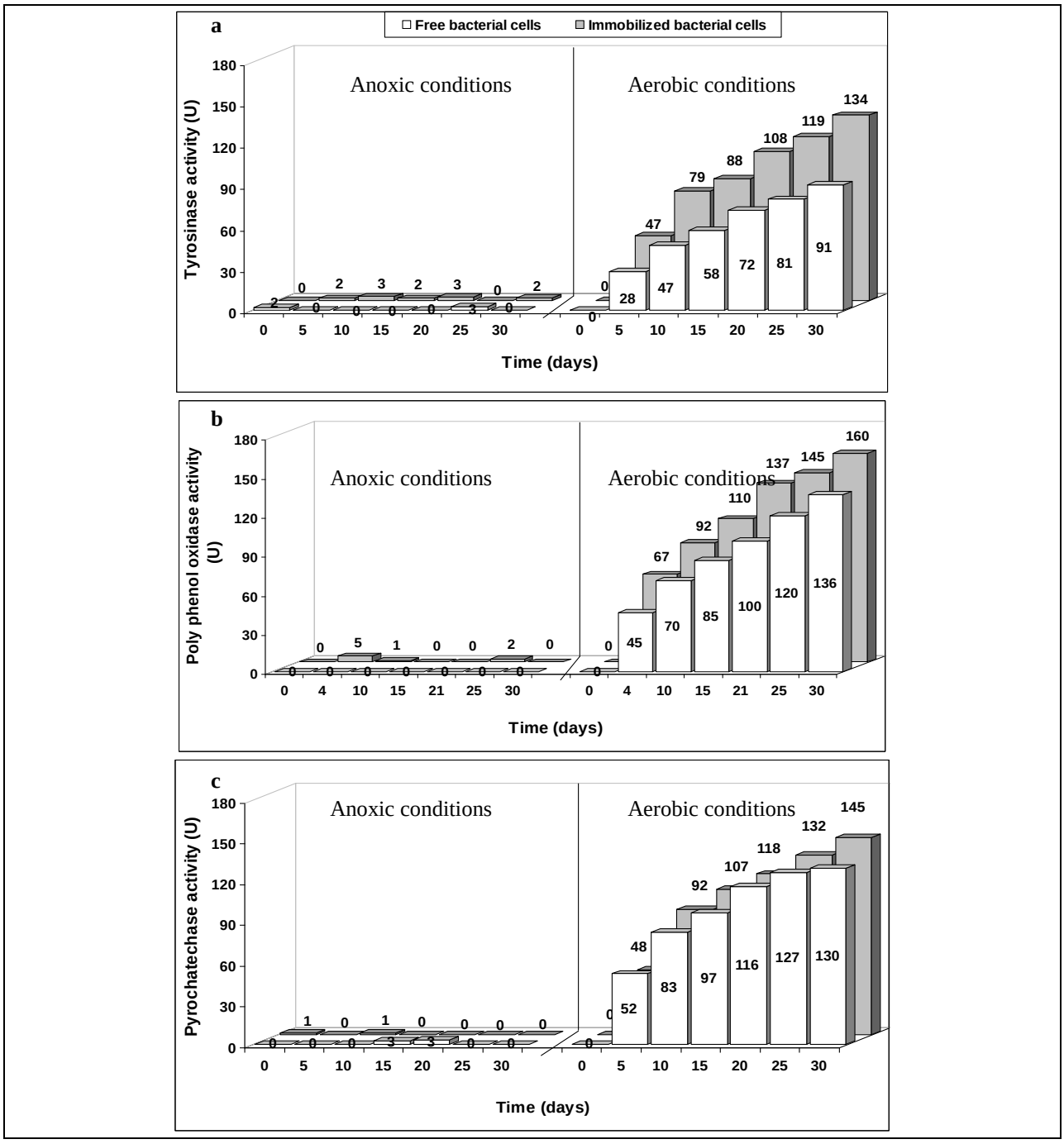


Fig 6. Oxidoreductase enzymes (a: Laccase, b: Mn Peroxidase, c: Lignin Peroxidase) activity of *Pseudomonas aeruginosa* strain OS4 free or immobilized bacterial cells in a bioreactor containing Reactive Blue azo dye wastewater.

The oxidative enzymes are known to oxidize various aromatic and non-aromatic compounds. The activity of these enzymes is crucial to complete the biodegradation of residual radicals resulting from the primary partial degradation of azo dye by Azoreductase and oxidoreductase enzymes. Four specific oxidase enzymes known to take part in the degradation of aromatic and phenolic compounds by catalyzing the oxygenation of wide range of substrates was studied (Fig. 7). The results show that all oxidase enzymes in this study namely; Tyrosinase, Poly phenol oxidase, Pyrochatechase and Ascorbate oxidase didn't express any activity in the anoxic compartment of the bioreactor containing Reactive Blue azo dye in the presence of either free or immobilized *Pseudomonas* cells (Fig. 7). In the bioreactor sector where continuous stirring of air takes place and the aerobic conditions prevail all previously mentioned oxidative enzymes progressively increased as the time of system operation increased until the end of incubation (30 days). The enzyme activity expressed, as international units, reached 134, 160, 145 and 146 for Tyrosinase, Poly phenol oxidase, Pyrochatechase and Ascorbate oxidase respectively in immobilized *Pseudomonas* cells system. In free cells system, the activities of the same enzymes were much lower being 91, 136, 130 and 136 units respectively. The gradual nature of oxidase enzyme activities increase followed the same trend observed with the azoreductase and oxidoreductase enzymes. This is likely to be due to continues feeding of fresh substrate into the bioreactor and/or to the increased induction of specific enzymes in both cell free and immobilized systems. These findings agree with Kumar et al. (2009) and Leelakriangsak and Borisut (2012). The results clearly show that the enzyme activities of immobilized *Pseudomonas* cells in the bioreactor performed better (20% more) as compared with the cell free cells system.


a
Free bacterial cells
Immobilized bacterial cells
Anoxic conditions
Aerobic conditions
Tyrosinase activity (U)
Time (days)
b
Anoxic conditions
Aerobic conditions
Poly phenol oxidase activity (U)
Time (days)
c
Anoxic conditions
Aerobic conditions
Pyrochatechase activity (U)
Time (days)

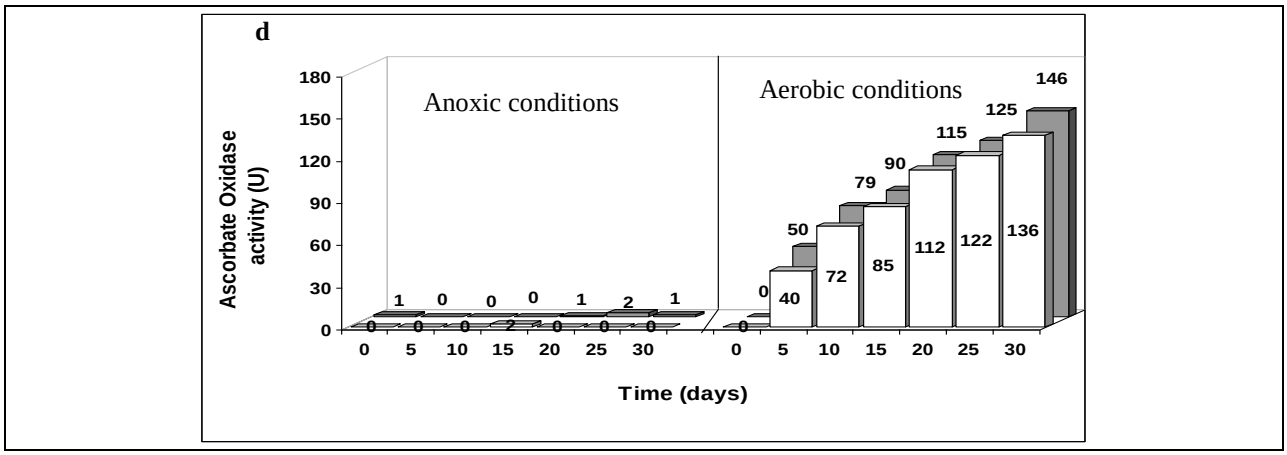


Fig 7. Oxidase enzymes (a: Tyrosinase, b: Poly phenol oxidase, c: Pyrochatechase, d: Ascorbate oxidase) activity of *Pseudomonas aeruginosa* strain OS4 free or immobilized bacterial cells in a bioreactor containing Reactive Blue azo dye wastewater.

Conclusion

The particular strain of *Pseudomonas aeruginosa* strain OS4 used in this study proved to be effective in the biodegradation of the RB residue in textile industry wastewater. It is documented that *Pseudomonas* bacteria enzymatic machinery is diverse and capable to degrade wide variety of aromatic and aliphatic chemicals in nature. The results obtained in this study show the active role of this bacterium in biodegradation of recalcitrant Reactive Blue azo dye. Therefore, we believe that the performance of this immobilized *Pseudomonas aeruginosa* strain OS4 in the anoxic/ aerobic bioreactor can be further developed for in situ bioremediation of excess azo dye released for textile industry.

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