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Application of Nanostructured Microbial Enzyme for Bioremediation of Industrial Wastewater

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Abstract: This study focuses on the isolation, screening of bacterial isolates and azo dye removal by bacteria isolated from dumping site of textile industry wastewater as well as using nanotechnology to enhance the bioremediation process. Fourteen isolates were isolated and used for further experiments. One of them was the most efficient in decolourization of Reactive Blue (RB) azo dye. This isolate was identified by morphological, biochemical and 16s rDNA sequences as *Pseudomonas aeruginosa* strain OS4 (NCBI accession number: KC762943). Bioremediation of azo dyes was found to occur efficiently under two sequential phases; starting with anoxic phase and followed by aerobic one. A prototype bioreactor with dual oxygenation levels (anoxic and aerobic) was designed for the bioremediation of RB dye residues. The bioreactor contained 2 compartments; Upflow fixed-film column (UFC) and continuously stirred aerobic (CSA) container. *Pseudomonas aeruginosa* strain OS4 was applied to both compartments as immobilized bacterial cells. The bioreactor operated at flow rate of 50 ml h^{-1} with HRT of 20 h. The CSA was directly fed in continuous mode with the output of UFC. The sequential anoxic–aerobic treatment of wastewater at dilution rate of 10% of wastewater with 90% water resulted in 99% decolourization. The activity of lignin peroxidase enzyme was assessed under anoxic and aerobic conditions. The induction of lignin peroxidase was observed under both anoxic and aerobic conditions. HPLC analysis was used to assess the biotransformation of the dye into non-aromatic intermediate compounds. The Lignin Peroxidase enzyme was partially purified. Magnetic (Fe_3O_4) nanoparticles were prepared by co-precipitation technique. The size and shape of Fe_3O_4 was examined by transmission electron microscopy and the average particles size was 16- 20 nm. The surface charges of Fe_3O_4 magnetic nanoparticles were modified using glutaraldehyde as cross-linker. T hese active groups support the covalant bioconjucation properties of enzyme immobilization. The modified Fe_3O_4 was used in immobilization and stabilization of LiP enzyme. The immobilized LiP was stable until 100°C and pH 12. The immobilized enzyme-regeneration cycle were performed 20 times.

Key words: *Pseudomonas aeruginosa* strain OS4 · Prototype bioreactor · Bioremediation · 16s rDNA sequences · Lignin peroxidase enzyme · Immobilization · Nanotechnology

INTRODUCTION

The first use of dye in ancient Egypt can be traced to the third or fourth dynasty [1]. Reactive azo dyes are extensively used in the textile, paper, cosmetic and leather industry [2]. The textile mills daily discharge millions of litters 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 [3]. 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 [4]. The basic structure of azo dyes includes an azo bond (–N=N–) which is difficult to naturally degrade. This class of dye is grouped into monoazo, diazo, trisazo and polyazo dyes depending on the number of azo groups [5]. The stability and their xenobiotic nature of reactive azo dyes make them recalcitrant hence they are not totally degraded by conventional treatments that involve light, chemicals or activated sludge [6]. A bacterial consortium was tested in the presence of reactive azo dyes and

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proved to be effective in breaking the azo dye when supplemented with starch [7]. Generally, the decolourization of azo dyes occurs under conventional anaerobic and facultative anaerobic by different groups of bacteria [8, 9, 10]. Recently, the enzymatic approach has attracted much interest with regard to decolorization and degradation of azo dyes in wastewater [11, 12]. The oxidoreductive enzymes affect azo dyes generating highly reactive free radicals that undergo complex series of spontaneous cleavage reactions [6]. Lignin peroxidase (LiP) catalyzes the oxidation of non-phenolic aromatic compounds. It also catalyzes several oxidations in the side chains of lignin and related compounds [13]. LiP has been used to mineralize a variety of recalcitrant aromatic compounds, polychlorinated biphenyls and dyes [14]. Bacterial LiP was reported to decolourize azo dyes [12, 15]. The high cost of enzymes represents a hurdle in broad application in some industries. The nanotechnology is among these new approaches for bioremediation and immobilization of enzymes [16]. Superparamagnetic materials become magnetic only in the presence of a magnetic field. Fortunately, magnetic particles with size less than 30 nm show superparamagnetism, meaning that they disperse easily in solution and can be recovered by use of a simple magnet [17]. In this study, a local isolate of *Pseudomonas aeruginosa* strain OS4 was used to degrade Reactive Blue azo dye in textile industry wastewater using lab scale sequential bioreactor. The lignin peroxidase enzyme was covalently attached to modified magnetic nanoparticles. The resulting LiP coated magnetic nanoparticles had long-term stability and high enzymatic activity.

MATERIALS AND METHODS

Isolation of Reactive Blue (RB) Azo Dye Degrading Bacteria: Two wastewater 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. One wastewater sample was obtained from dyeing basin before discharging into the effluent lagoon. The other Wastewater sample (effluent) was collected from the main factory dumping sites. Wastewater samples were analyzed for Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD), pH and electric conductivity (EC); employing the standard methods [18]. 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 azo dye was analyzed by UV vis spectrophotometer (Jenway UV/Visible- 2605 spectrophotometer, England).

The wastewater from dyeing 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 decolourization of the mixture was 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 mineral salt medium (MSM) agar 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 modified MSM agar without dye [8].

Screening of Isolated Bacteria for Their Ability on Reactive Blue Azo Dye Decolourization and Enzyme Production: 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 broth medium 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.* [8]. 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 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. Also the isolated bacteria were evaluated for their ability on lignin peroxidase enzyme production which have important role in biodegradation of textile azo dyes. Lignin Peroxidase (LiP) 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 and 10 mM H_2O_2 according to the method of Ogola *et al.* [19].

Identification of Isolate No. OS4 Using 16s rDNA Analysis: The DNA was extracted from the isolate OS4 bacterium 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.* [20]. 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') [21]. 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 rDNA 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 program (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 [22].

Design and Operation of Prototype Sequential Bioreactor for the Reactive Blue Azo Dye Bioremediation

Design of Prototype Sequential Bioreactor: 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 (Fig. 1).

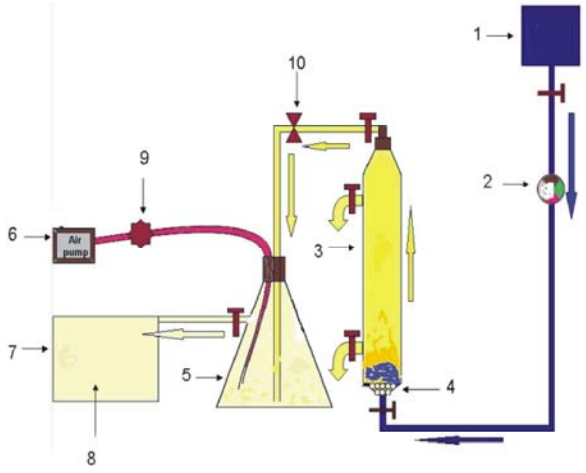


Fig. 1: Schematic diagram of anoxic /aerobic sequential bioreactor. Where: 1, feed tank; 2, controlling dispenser; 3, UFC anoxic container; 4, bioagent *Pseudomonas aeruginosa* strain OS4; 5, CSA aerobic container; 6, air pump; 7, effluent tank; 8, bioremediated wastewater; 9, air filter; 10, six valve;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

Inoculum Preparation for UFC/CSA Bioreactor: *Pseudomonas aeruginosa* strain OS4 (NCBI accession number: KC762943) was reported as potent bacterium efficient in bioremoval of RB azo dye. This strain was used as immobilized cells into small scale (two phases) bioreactor. 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 sterile 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 [23].

Operating the Prototype Sequential UFC/CSA Bioreactor: The bioreactor containing both UFC and CSA containers was inoculated with 5 gm of immobilized *Pseudomonas aeruginosa* strain OS4. At the beginning of experiment, the UFC container was incubated at room temperature (28±2°C) for 2 days to initiate biodecolourization and/or biodegradation of wastewater containing RB azo dye. The system was operated in upflow mode at an average flow rate of 50 ml/h with an average hydraulic retention time (HRT) of 20 h. The

bioreactor was incubated at room temperature (28±2°C) and pH was 6.8- 7.0 in static conditions. 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.

Analysis of Samples Collected from UFC and CSA Bioreactor: Samples collected from UFC and CSA at regular time intervals (every day) 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 of decolourization and was determined by monitoring the decrease in absorbance at 595 nm (λ_{max} of Reactive Blue dye) by UV/vis spectrophotometer (Jenway UV/Visible- 2605 spectrophotometer, England). The absorption of cell-free supernatant of UFC and CSA containers 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 following equation:

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

where C= control, S= sample

Estimation of Chemical Oxygen Demand (COD) and Biological Oxygen Demand (BOD): The COD and BOD of raw wastewater as well as UFC and CSA output were determined according to standard methods [18]. COD was measured using (Hach) spectrophotometer test kit, while, BOD was measured by OXI Top BOD meter (WTW, Comp.).

HPLC Analysis of Degradation Metabolites: The dye and its degradation products were monitored by HPLC to evaluate biodegradation development. 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.

Isolation and Purification of Lignin Peroxidase (LiP) Enzyme: One litter of bioremediation product (UFC and CSA mixed containers) was centrifuged at 3000 rpm for 10 min, the supernatant was pooled and added with ammonium sulphate to 40 % saturation and was left overnight at 4°C. Then centrifuged at 10000 rpm for 10 min, the supernatant was pooled and added with ammonium sulphate to 80 % saturation. The solution was kept overnight at 4°C and centrifuged. The precipitate was dissolved in distilled water and dialyzed against 0.1 M phosphate buffer (pH 6). The previous solution was subjected to gel filtration on Sephadex G-100 column (4×70 cm) previously equilibrated with 0.1 M phosphate buffer (pH 6). The column was eluted in the same buffer at the flow rate of 1 ml/min. The eluted protein fractions were collected (3 ml/min) and peroxidase activity was measured. All the fractions with high enzyme activity were pooled together and subjected to diethyl amino ethyl (DEAE) cellulose column (1.5×30 cm), which were equilibrated with 0.1 M phosphate buffer (pH 6) containing 1 mM $CaCl_2$ and 0.5 mM NaCl. The proteins were determined by the assay of Bradford [24] and bovine serum albumin was used as standard.

Molecular Weight Estimation by Electrophoresis: To determine the relative molecular weight of purified LiP, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a stacking and separating gel according to the method of Laemmli [25] using Mini-gel electrophoresis (BioRad, USA). The molecular weight of the purified LiP was estimated in comparison to standard molecular weight markers (standard protein markers, 21-116 kDa; Sigma, USA). The protein bands were visualized by staining with Coomassie Brilliant Blue G-250 (Sigma, USA) after documentation.

Preparation of Magnetic Nanoparticles (Fe_3O_4) for Used to Enzyme Immobilization: Fe_3O_4 nanoparticles were prepared by the co-precipitation of Fe^{2+} and Fe^{3+} ions (molar ratio 2:1) at 25°C and a concentration of 0.3 M iron ions with ammonia solution (29.6 %) at pH 10, then the hydrothermal treatment at 80°C for 30 min and finally the vacuum drying at 70°C after being washed several times with water and ethanol [26].

Activation and Modification of Magnetic Nanoparticles (MNPs): The synthesized magnetic nanoparticles (~2 g) were dispersed in ethanol and sonicated for about 10 min for getting the complete dispersion. After adding 0.5 ml of carbodiimide solution (25 mg/ml) and 1 ml of

glutaraldehyde solution (10 %) to the above particles and was incubated at room temperature for about 2 h, the particles were then washed with water to remove the excess glutaraldehyde [27].

Immobilization of Lignin Peroxidase on MNPs: Activated MNPs were stored in 0.1 M phosphate buffer, pH 6.0 at 4°C for overnight. After separation of MNPs, 50 ml of phosphate buffer containing peroxidase enzyme (5 U/ ml) was added to activated MNPs and incubation under shaking condition for 24 h. The peroxidase-bound MNPs were then decanted using permanent magnet and washed several times by deionized water.

Characterization of Magnetic Nanoparticles and Immobilized Enzyme by Transmission Electron Microscopy (TEM): The average particle size, size distribution and morphology of the magnetic nanoparticles and immobilized enzyme were studied using transmission electron microscope JEOL (JEM-1400 TEM). A drop of well dispersed nanoparticle dispersion was placed onto the amorphous carbon-coated 200 mesh carbon grid, followed by drying the sample at ambient temperature, before it was loaded into the microscope [28].

Effect of Temperature and pH on the Enzyme Activity: Effect of temperature on the activity of free and immobilized LiP was estimated after 3 min incubation of 3 ml of the reaction mixture of peroxidase assay at temperature range of 10-100°C. The enzyme activity was then determined by measuring the absorbance of the solution at 420nm. The effect of pH on the activity of the free and immobilized enzyme was investigated in the pH range of 1-12 at 20°C. The highest value of enzyme activity in each set was assigned the value of 100 % activity.

Repeated Use of the Immobilized LiP: The reusability of the immobilized LiP was examined by conducting the activity measurement of the immobilized LiP at 20°C. After each activity measurement, the immobilized LiP was separated magnetically and washed several times. Then, reaction mixtures were added to the immobilized LiP in sequence and the next activity measurement was carried out.

RESULTS AND DISCUSSIONS

Azo dyes are widely used in textile industry. The basic structure of these dyes include an azo bond (–N=N–) that does not 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 treatments [6].

Wastewater Samples (Collection and Analysis): During this study, two wastewater samples were collected from textile plant located at El-Mukatem Dyehouse near 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. In this respect, similar results were obtained by Darwesh *et al.* [8].

The other wastewater sample was collected from general wastewater disposal lagoon. The wastewater sample was analyzed (Table 2). This sample had high values of pH being 10.7 and 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). 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 [29].

Isolation and Screening of Azo Dye Decolourizing Bacteria: From the prolonged static enrichment cultures in wastewater sample containing Reactive Blue azo dye, 14 bacterial isolates were isolated. Each isolate was screened for the ability to decolourize Reactive Blue azo dye in wastewater. Table (3) 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 isolates No. OS4, OS6 and OS14 removed 98.3, 91.7 and 93.4 %, respectively. One of them, isolate No. OS4 had fast and high capacity to remove 98.2 % after 4 days of incubation. Isolates No. OS4, OS6, OS8, OS9, OS10 and OS14 were very active in decolourization of RB azo dye under aerobic conditions throughout the 6 days of

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

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

Parameters	Value
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, EC= Electrical conductivity.

Table 3: Decolourization percent (%) of wastewater containing Reactive blue azo dye (300 ppm) by bacterial isolates from El Mukatem Dyehouse

	Anoxic conditions			Aerobic conditions		
	Days			Days		
Isolates No.	2	4	6	8	10	12
OS1	0.0	13.7	79.8	84.8	84.8	85.0
OS2	0.0	0.0	04.0	04.4	04.9	04.9
OS3	0.0	32.3	64.5	75.1	75.2	75.2
OS4	59.6	98.2	98.3	98.3	98.9	99.0
OS5	0.0	29.3	61.6	65.5	65.6	65.6
OS6	0.0	78.8	91.7	92.2	92.4	92.5
OS7	0.0	26.2	78.8	82.7	82.7	82.7
OS8	0.0	37.7	86.5	90.3	90.8	91.7
OS9	0.0	40.0	81.4	90.7	91.2	91.5
OS10	20.0	79.9	88.6	93.0	93.2	93.5
OS11	0.0	59.9	62.5	65.6	65.6	65.8
OS12	0.0	13.2	50.0	54.3	54.3	54.8
OS13	0.0	0.0	18.8	20.5	20.9	21.0
OS14	0.0	74.7	93.4	95.0	95.3	95.7

incubation and reached 99.0, 92.5, 91.7, 91.5, 93.5 and 95.7 %, respectively (Table 3). Both isolates No. OS2 and OS13 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. The colour removal reached more than 95 % under anoxic conditions. This agrees with Khehra *et al.* [29] who 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 [30]. 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, the oxidoreductase enzyme namely; lignin peroxidase was assessed in relation to RB colour removal.

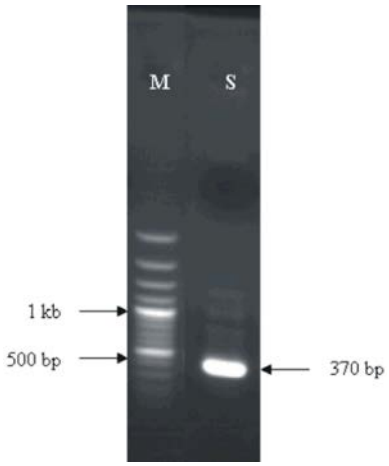
The lignin peroxidase enzyme was induced under both anoxic and aerobic conditions (Table 4). With the exception of isolate No. OS13, all other isolates after 6 days of incubation showed lignin peroxidase activity at the range between 0.8 and 17.1 units. The exposure of microbial growth to the aerobic conditions after 6 days of incubation showed additional lignin peroxidase activity which increases as the time of aerobic incubation increased up to 12 days. No retardation of lignin peroxidase activity was noted with switching from anoxic to aerobic conditions. Isolate No. OS4 was distinguished in higher lignin peroxidase activity as compared to other isolates. The LiP enzyme is considered the super enzyme due to its ability to perform chemical reactions under both anoxic and aerobic conditions. Bholay *et al.* [31], Selvam *et al.* [32] and Zucca *et al.* [33] indicated that the ability of microorganisms to produce LiP enzyme varied within microbial genera and species.

The isolation of different microorganisms from the sample indicates the natural adaptation of microorganisms to survive in the presence of toxic dyes. The difference in their rate of decolourization may be due to the loss of ecological interaction, which they might be sharing with each other under natural conditions [34]. One isolate (OS4) was identified as the most efficient in decolourization of wastewater containing RB dye residues as well as production of assessed enzymes. This isolate accordingly, was subjected to further molecular identification using 16s rDNA analysis.

Identification of Isolate No. OS4 Using 16s rDNA: The primary identification of bacterial isolate No. OS4 was performed using standard procedures according to Bergey's Manual of Systematic Bacteriology and classified as *Pseudomonas* sp. This Identification was confirmed by 16S rDNA analysis. The PCR amplified DNA fragment (370 Pb) was obtained on agarose gel electrophoreses (Fig. 2). The fragment sequence of *Pseudomonas* sp. OS4 strain was compared with available 16S rDNA gene sequences from organisms in

Table 4: Lignin peroxidase enzyme activity (U) associated with the growth of bacterial strains/isolates on wastewater containing Reactive blue azo dye (300 ppm).

	Anoxic conditions			Aerobic conditions		
	Days			Days		
Isolates No.	2	4	6	8	10	12
OS1	0.0	0.0	5.0	9.3	10.2	11.6
OS2	0.0	0.0	0.8	1.1	1.3	1.5
OS3	0.7	3.0	6.4	9.5	9.8	10.0
OS4	4.3	10.1	17.1	19.3	23.5	26.7
OS5	0.0	2.2	6.8	8.3	8.9	9.2
OS6	0.0	5.3	9.3	10.3	10.6	10.5
OS7	0.0	2.2	5.8	7.0	7.8	8.0
OS8	0.0	3.0	6.0	6.8	7.0	7.3
OS9	0.0	2.8	5.4	8.0	8.3	8.8
OS10	2.5	3.6	5.8	6.5	7.1	7.5
OS11	0.0	2.0	3.4	4.9	5.6	5.8
OS12	0.0	0.1	1.3	2.6	3.8	4.8
OS13	0.0	0.0	0.0	0.0	0.0	0.0
OS14	0.0	0.0	2.5	3.1	3.8	4.2



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

Fig. 2: Agarose gel electrophoresis of PCR product 16s rDNA analysis of *Pseudomonas* sp. OS4.

the GenBank databases. The 16S rDNA sequence 370 Pb of *Pseudomonas* sp. No. OS4 was illustrated in Fig. (3). From these data, the OS4 strain was identified as *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 system for treatment of these recalcitrant azo dyes. The bioreactor was designed to achieve anoxic/aerobic continuous conditions. In this study, the facultative anaerobe *Pseudomonas aeruginosa* strain OS4 was applied to both compartments of the bioreactor (anoxic and aerobic compartment) as immobilized cells on sodium alginate. The bioreactor was fed by wastewater containing 300 ppm RB azo dye continuously for 30 consecutive 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 continuously feeding of bioreactor by wastewater containing RB azo dye was found to be proper to handle the textile industry effluents flow.

Samples collected from UFC and CSA at regular intervals were centrifuged at 10,000 rpm for 15 min and supernatants were used for further analysis. Some analyses were conducted on these samples to evaluate the bioremediation processes. The decolourization test was the main one to assess the bioremediation of textile wastewater containing azo dye as stated by Liu *et al.* [35].

Bioremediation of Wastewater Containing Reactive Blue Azo Dye by *Pseudomonas aeruginosa* Strain OS4 in Sequential Anoxic/Aerobic Bioreactor: In this study, *Pseudomonas aeruginosa* strain OS4 (facultative anaerobe) was applied to both compartments of the bioreactor (anoxic and aerobic compartments) as immobilized cells on sodium alginate. The system was operated continuously throughout 30 days of the experimental period (after the Preliminary stage). The results in Fig. (4) show the decolourization of the dye wastewater started from day one after loading the bioreactor with diluted dye wastewater and the immobilized bacteria. The decolourization rate reached the maximum level of 98.25 % after 3 days (Fig. 4). 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. 4). The fast decolourization 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 enzymes targeting azo dyes in the bioreactor. These results agree with those obtained by Liu *et al.* [35].

The Chemical and biological oxygen demand values are indicators to the total organic load in the solution. The changes of COD in the dye containing effluents show the degree of dye mineralization as a function of dye removal [36]. The original effluent solution at zero time had COD value of 2018 ppm (Table 5). Such value was reduced to 1376 ppm under anoxic conditions. The majority of COD was removed in the aerobic phase

Fig. 3: Partial sequence of 16S rDNA from *Pseudomonas* OS4.

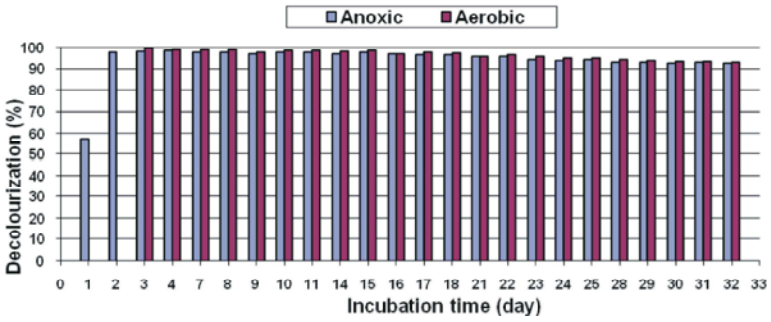


Fig. 4: Continuous system for decolourization of Reactive Blue textile azo dye in experimental bioreactor under sequential partially anoxic and fully aerobic conditions using immobilized cells of *Pseudomonas aeruginosa* strain OS4.

Table 5: Changes in COD and BOD after wastewater treated in UFC and CSA compartments of the bioreactor

	Bacterial Immobilized cells			
	COD		BOD	
Samples from	ppm	removal %	ppm	removal %
W.W[a]	2018	———	954	———
UFC[b]	1376	31.81	532	44.23
CSA[c]	53	65.56	25	53.15
Total removal	97.37		97.38	

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

(97.37 %) compared with 31.81% in the anoxic phase. HeFang *et al.* [36] found that the microorganisms continued to consume the obtained organics until near complete removal of COD value in the culture media. 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 %. From the previous results, it is clear that the majority of colour was removed in the anaerobic phase, whereas, significant COD and BOD values were removed in the aerobic phase. These results agree with those previously obtained by Darwesh *et al.* [8].

Assessment of Biodegradation of Reactive Blue Azo Dye in the Experimental Bioreactor Using HPLC Analysis: The *n*-butanol extracts of the anoxic and aerobic phases of the bioreactor amended with wastewater containing RB azo dye were subjected to analyze by HPLC. Figure (5) shows the results of HPLC analysis of the metabolites that 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. 5 B). This peak was identical to standard azo dye peak (Fig. 5 A). After the completion of anoxic incubation, two peaks at retention time 5.58 and 8.12 min respectively were detected (Fig. 5 C). 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. 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 [37]. The peak areas increased simultaneously 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. 5 C). 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. 5 D). This figure also shows that when such metabolites were degraded aerobically; less aromatic compounds were formed and more polar compounds were detected, since the metabolite peak area decreased and shifted towards a lower retention time

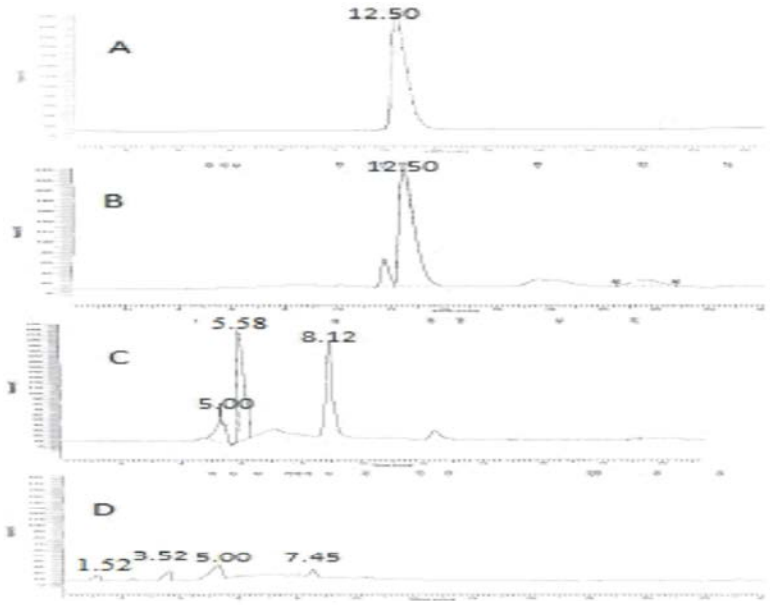


Fig. 5: HPLC analysis of metabolites resulting from biodegradation of organic compounds in industrial wastewater containing Reactive Blue azo dye under anaerobic/aerobic conditions: (A) standard Reactive Blue azo dye; (B) industrial wastewater; (C) aqueous solution after anoxic incubation and (D) aqueous solution after aerobic incubation

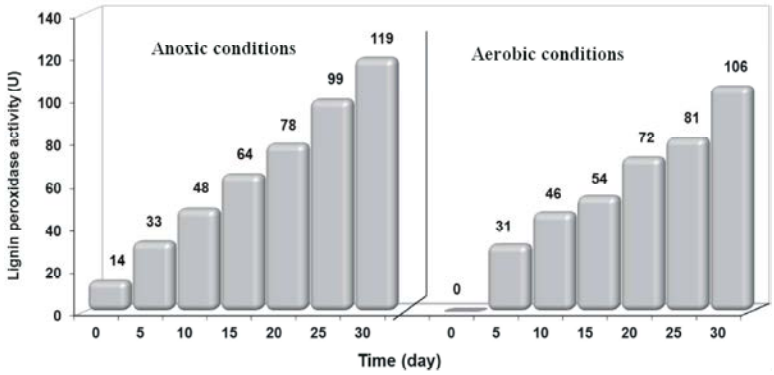


Fig. 6: Lignin Peroxidase enzyme activity of immobilized *Pseudomonas aeruginosa* strain OS4 in a bioreactor containing Reactive Blue azo dye wastewater

(1.52, 3.52, 5.00 and 7.45 min). This confirms the reports indicating that the degradation in the aerobic phase may result in the formation of oxidized and very polar derivatives (*e.g.*, aldehydes, carboxylic acids) having a lower aromatic nature [38].

Lignin Peroxidase Activity Associated with the Biodegradation of RB Azo Dye: The lignin peroxidase enzyme plays a major role in the transformation of aromatic phenolic compounds. In this experiment LiP enzyme was studied throughout the bioremediation process over 30 days, in order to understand the role of this enzyme in the detoxification of textile industrial dyes in wastewater before releasing into the surrounding environment. The LiP enzyme plays a major role in the transformation of aromatic phenolic compounds. The changes in the enzymatic activities of this enzyme in the sequential anoxic/ aerobic bioreactor amended with wastewater containing Reactive Blue azo dye in the presence of *Pseudomonas aeruginosa* strain OS4 in immobilized form show steady increase in the activity of the above mentioned enzyme throughout the 30 days operating time of the bioreactor. The activity of this oxidoreductase enzyme was present in Fig. (6). This enzyme was induced in both anoxic and aerobic conditions of the bioreactor. The highest value of lignin

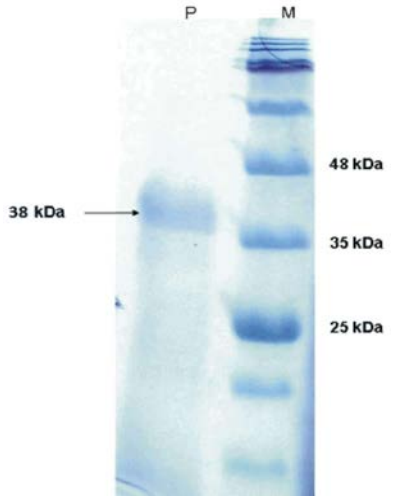


Fig. 7: Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of purified LiP enzyme from *Pseudomonas aeruginosa* strain OS4.

peroxidase activity was reached after 30 days of incubation being 119 U. In aerobic CSA bioreactor container, the highest enzyme activity was reached after 30 days of incubation immobilized system being 106 U (Fig. 6). It is imperative that the oxidoreductase enzyme plays 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.* [38] who reported high oxidoreductase enzymes activity during the biotreatment process suggesting the role of these enzymes in the decolourization and degradation process.

Isolation and Purification of Lignin Peroxidase (LiP): LiP enzyme was partially purified from bioremediation products of textile wastewater containing Reactive Blue azo dye by UFC/CSA prototype bioreactor. The molecular weight and purity of the LiP enzyme was assessed by running through electrophoreses SDS-PAGE. After Coomassie blue staining, a single band was detected (Fig. 7). The purified LiP enzyme had molecular mass of approximately 38 kDa compared with protein marker (Fig. 7). This is likely to be near to complete purification of LiP enzyme. The molecular weight of the tested enzyme is different from the same enzyme isolated from other microorganisms. For example, the molecular weight of LiP from *Cunninghamella elegans* and *Phanerochaete sordida* YK-624 was 50 kDa [39]. LiP enzyme from *Hexagona tenuis* MTCC 1119 had molecular weight of 48 kDa [40] and from *Trametes versicolor* IBL-04 was 30 kDa [41].

The high cost of enzymes represents a hurdle in broad application in some industries. Consequently, seeking new techniques to improve enzyme applicability and reduce their cost is of great importance. Enzyme immobilization is one of the most powerful tools in this direction. The stability here is both in terms of residual activity during storage (storage stability) as well as the amount of enzyme that remains immobilized during chemical reaction (operational stability). The immobilized enzyme must be robust enough to withstand multiple recoveries so that it can be reused many times, making the immobilization cost effective. The nanotechnology is among these new approaches for bioremediation [16].

Physical Properties of Magnetic Nanoparticles: Magnetic (Fe_3O_4) nanoparticles were prepared by co-precipitation technique [26]. These nanoparticles changed in colour, size, shape, magnetic properties and surface area charge compared to the raw $FeCL_3$ chemical. The colour of prepared magnetic nanoparticles was black. The Fe_3O_4 nanoparticles were separated and washed several times using permanent magnet. The size and shape of Fe_3O_4 was reported by transmission electron microscopy (TEM) and the average particles size was estimated to be 16- 20 nm (Fig. 8). The magnetic properties of Fe_3O_4 were reported as superparamagnetic material [42]. The advantages of these nanoparticles for industrial enzyme immobilization were reported by Wahajuddin and Arora [43].

Immobilization and Characterization of Immobilized LiP: One of the main objectives of this study is to enhance the stability and efficiency of azo dye degrading enzyme to be stable under various harsh conditions. Covalant bioconjugation experiments were performed to immobilize the LiP enzyme on magnetic nanoparticles. Glutaraldehyde was used as a cross-linking and modifying agent for covalent coupling of LiP enzyme to magnetic nanoparticles. Before the beginning of immobilization, the surface of magnetic nanoparticles was modified using glutaraldehyde cross-linker to synthesise aldehyde as an active group with a positive charge. The modified nanoparticles was used to immobilize the LiP enzyme. Some approach was used by Mahdizadeh *et al.* [44] for preparation of Fe_3O_4 magnetic nanoparticles and used it for immobilization of Glucose Oxidase and application of immobilized enzyme for Water Deoxygenation. This technique facilitated the economic use of several enzymes. After immobilization, the activity of immobilized enzyme was assessed to judge the effect of immobilization

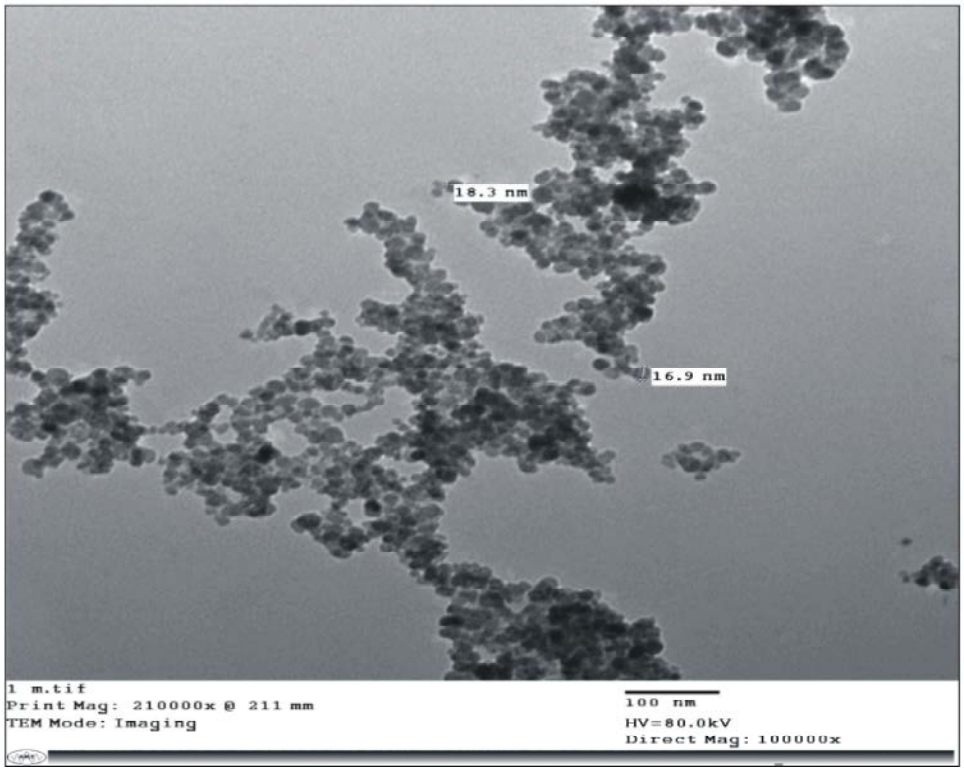


Fig. 8: Transmission electron micrograph of Fe_3O_4 nanoparticles

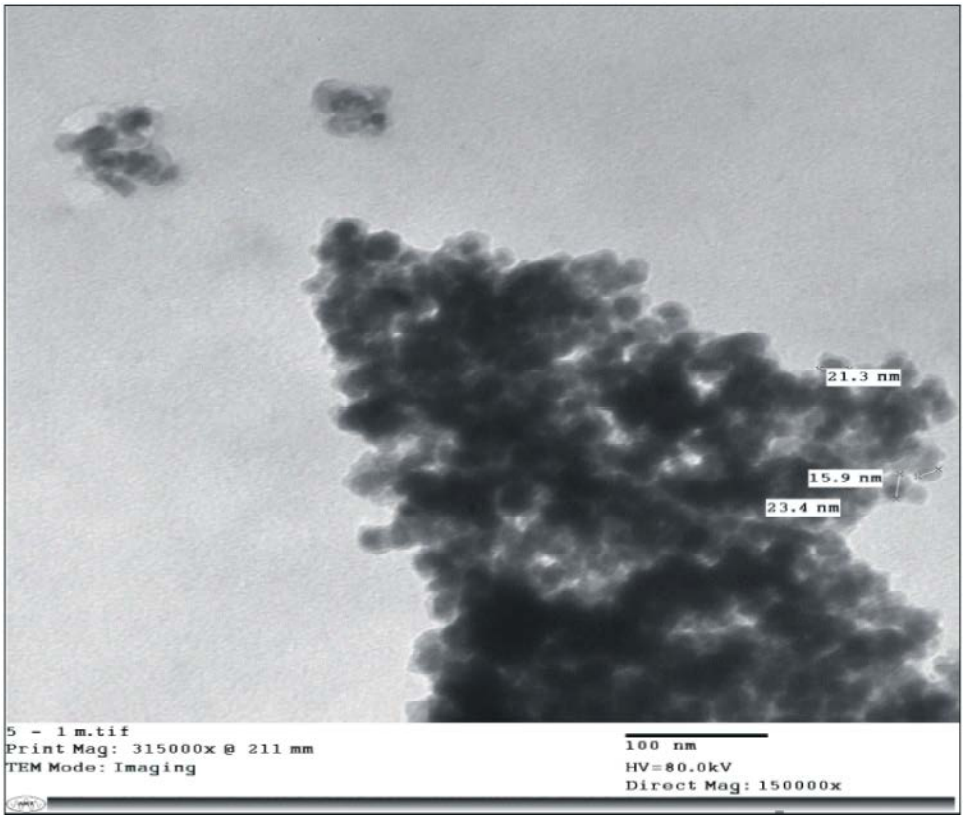


Fig. 9: Transmission electron micrograph of immobilized enzyme onto Fe_3O_4 nanoparticles

process on the enzyme performance. In this study, the activity of LiP enzyme wasn't affected by immobilization process. It is likely that the conjugation between enzyme and magnetic nanoparticles was out of the enzyme active site [35].

To check the immobilization success, transmission electron microscopy (TEM) was applied. Figure (9) illustrates the TEM micrographs of immobilized LiP on Fe_3O_4 nanoparticles. The average p articles size was 16.5- 24 nm. Clear increase of immobilized particle over the free magnetic nanoparticles was noted (Fig. 9). Also, the TEM analysis showed the formation of film surrounding the magnetic nanoparticles indicating the enzyme attachment on magnetic nanoparticles surfaces. These results show the success of LiP enzyme immobilization on magnetic nanoparticles. Similar results were obtained by Ranjbakhsh *et al.* [45] who reported enhanced lipase stability and catalytic activity by covalently immobilization on surface of silica-coated modified magnetic nanoparticles.

One of the main objectives of this study is to enhance the efficiency of azo dye degrading enzyme for environmental bioremediation purposes. For the success of this technology, the immobilized enzyme such as LiP should be stable under various harsh conditions. The main advantage of immobilized enzyme is to enable enzymes to perform efficiently under various conditions of temperature, pH, pressure, storage time, etc [16].

Effect of Temperature and pH on the Enzyme Activity: Effect of temperature on the activity of free and immobilized LiP enzyme was estimated using pyrogallol as specific substrate for the enzyme. Figure (10) shows that the maximum activity of the free LiP enzyme was obtained at 20°C, while the maximum activity of the immobilized LiP was observed in the range between 20 and 40°C. This show that the performances of the enzyme undergo marked change when the enzyme is in the immobilized form. These results supports those results obtained for Glucose oxidase and Lactate dehydrogenase enzymes immobilized on polymeric supports [27]. The activity of free LiP enzyme decreased gradually between 30 and 50°C then sharply at 60°C. The activity of LiP enzyme in free state was inactivated totally at 70°C till the end of the experiment. The activity of immobilized LiP enzyme started to decrease after the reaction medium temperature exceeded 40°C and the decrease was gradual till the end of the experiment. The immobilized enzyme tolerated the temperature range between 60 and 100°C. The relative activity remained high even at 100°C being 50 %. This clearly shows that the enzyme immobilization protected the enzyme from the adverse effect of elevated temperature. The tolerance of immobilized enzyme to elevated temperature may be due to multipoint attachment of the LiP to the magnetic nanoparticles which probably provides protection for the enzyme [46].

The pH has a pronounced effect on enzyme activity. It plays an important role in ionization state of the amino acids in the active site and maintaining the proper structure of an enzyme [47]. The pH has a pronounced effect on enzyme activity. The effect of pH on the LiP activity either free or immobilized was investigated at different pH from 1 to 12 (Fig. 11). The optimum pH for LiP enzyme in free state was 6. The deviation of pH either by increasing and decreasing resulted in sharp decline of enzyme activity. Total LiP enzyme inactivation was recorded less than pH 3 and over pH 10 (Fig. 11). The enzyme immobilization on magnetic nanoparticles changes the performance of the enzyme activity dramatically. While the optimum pH of the reaction media for maximum Peroxidase activity remained at pH 6 as was found with the enzyme in free state (Fig. 11), the range of the pH at which the enzyme performance in a very satisfactory manner was extremely extended. None of the tested pH value induced total inactivation of the enzyme. It can also be concluded from Fig. (11) that the pH range of 5-10 kept the enzyme activity close to the maximum. This may be due to the role of immobilization in making the enzyme active sites more exposed to solvent than that in the folded- free LiP form. Hence the proton transfer to the amino acid residues at the active site becomes less hindered [46]. Also, Ashtari *et al.* [47] reported that the basic nature of the un-reacted amino groups on the surface, favorable conditions of active sites at solid-liquid interface could be attained at lower pH. This, once again, suggests that the immobilization play significant role in the protection of the immobilized enzyme from adverse cultural conditions particularly temperature and pH (Fig. 10 and 11).

Repeated Use of the Immobilized LiP: Reusability of the immobilized enzyme represents also a critical aspect in industrial applications [47]. In this study the same immobilized LiP enzyme on magnetic nanoparticles was repeatedly used in 20 consequent reaction cycles. After each cycle the magnetic nanoparticles loaded with the enzyme were precipitated using the permanent magnet and the supernatant containing the reaction mixture was decanted. The enzyme magnetic nanoparticle complex was washed with phosphate buffer twice and a new enzymatic

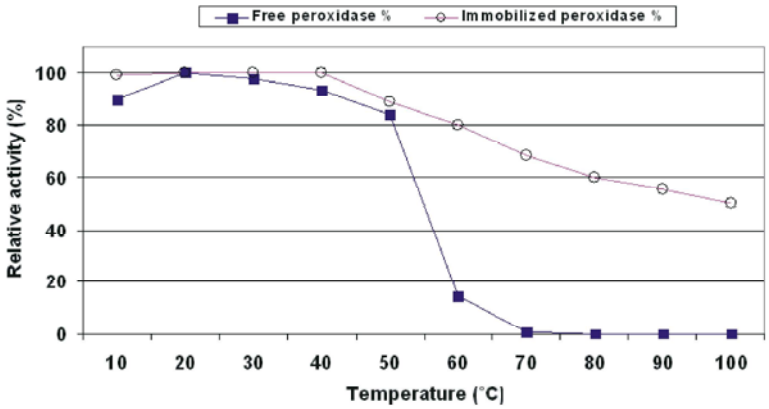


Fig. 10: Performance of LiP enzyme in free and immobilized states in relation to reaction temperature changes

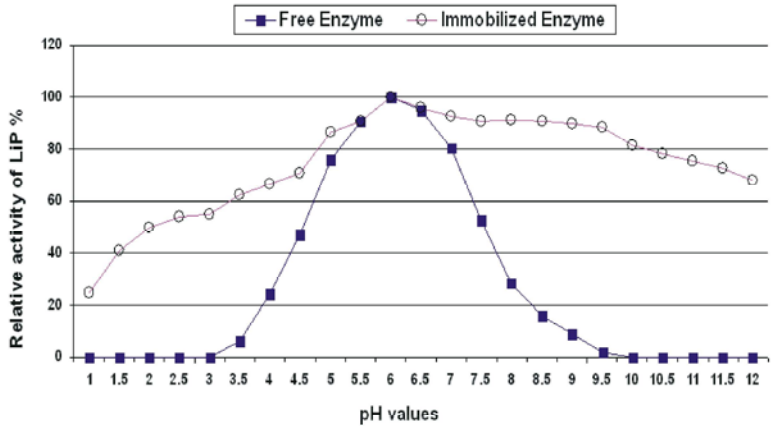


Fig. 11: Performance of LiP enzyme in free and immobilized states in relation to reaction pH changes

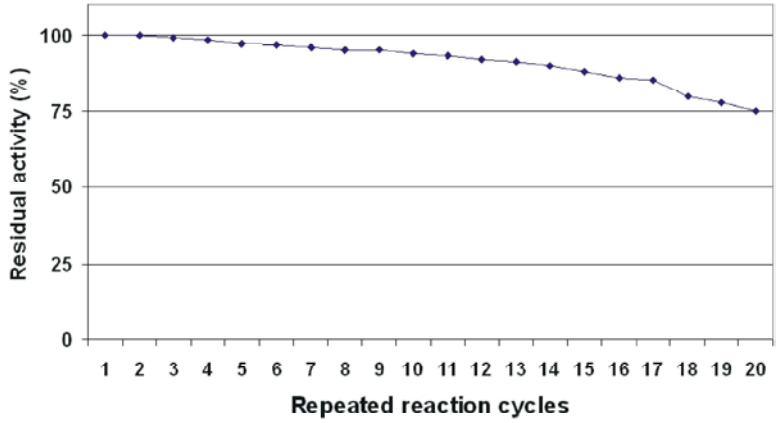


Fig. 12: Residual activities of the immobilized LiP after 20 repeated uses at 25°C in phosphate buffer (0.1 M, pH 6)

reaction cycle was started. Twenty cycles were performed to test the capacity of enzyme LiP nanoparticle performance in relation to the oxidation of pyrogallol. The results in Fig. (12) reveal that the immobilization prevented significant loss in enzyme activity throughout the 20 reaction cycles. Also, the results show that the peroxidase immobilized on magnetic nanoparticles lost only 5% of its activity after 10 repeated reaction cycles, whereas, the enzyme LiP nanoparticle complex preserved the Peroxidase activity at 75 % efficiency after 20 reaction cycles. This is economically very promising to enhance the enzyme application in industrial processes. However, additional research and development work is needed to optimize the repeated use of the LiP nanoparticle with up

scaling the reaction to suit the industrial applications. This significant effect of the immobilization on the enzyme activity prolonged performance may be attributed to the better multipoint attachment of enzyme on the proper nanoparticles [45, 47, 48].

CONCLUSIONS

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.

It is expected that the nanoparticle immobilization of enzymes will have wide range of technological uses in various industrial and environmental applications.

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