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Bioremediation of Textile Reactive Blue Azo Dye Residues using Nanobiotechnology Approaches.

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ABSTRACT

Microbial cells contain wide assortment of specific enzymes capable to biodegrade various organic chemicals, Lignin peroxidase (LiP) plays an important role in biodegradation and mineralization of recalcitrant aromatic compounds, polychlorinated biphenyls and dyes. This study focuses on bioremediation of Reactive Blue azo dye residues generated from textile dye basins. Immobilization and stabilization of lignin peroxidase enzyme form potential bioremediation bacterial strain was among the nanobiotechnological approaches tested in this study. The lignin peroxidase enzyme isolated from *Pseudomonas aeruginosa* strain OS4 was partially purified. The enzyme purity was checked using SDS/PAGE. The results showed single band on gel having the molecular weight of approximately 38 kDa. Magnetic (Fe_3O_4) nanoparticles were prepared by co-precipitation technique. The size and shape of Fe_3O_4 was examined by TEM 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 to modify the nanoparticles surface for conjugation with the enzyme. The active groups support the covalent bioconjucation properties necessary for enzyme immobilization. The modified Fe_3O_4 nanoparticls were used for immobilization and stabilization of LiP enzyme as well as whole bacterial cells. The immobilized enzyme on magnetic nanoparticles performed much better than free enzyme in decolourization of the dye residues. The repeated use of the enzyme linked to magnetic nanoparticle is very promising for environmental application.

Keywords: Magnetic (Fe_3O_4) nanoparticles, Immobilized LiP enzyme, Nanotechnology, Bioremediation, TEM.

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INTRODUCTION

Lignin peroxidase (LiP) catalyses several oxidations in the side chains of lignin and related compounds. This enzyme contributes to mineralization of variety of recalcitrant aromatic compounds, polychlorinated biphenyls and dyes [1, 2]. Franciscon *et al.* [3] described the transformations of six industrial azo and phthalocyanine dyes by lignolytic peroxidases from *Bjerkandera adusta*. Several studies reported that LiP enzyme from bacteria can play role in the decolourization of azo dyes [2,4,5]. Dawkar *et al.* [6] and Ghodake *et al.* [7] performed studies concerning the purification and characterization of the lignin peroxidase from the *Bacillus* sp. strain VUS and from *Acenetobacter calcoaceticus* NCIM 2890. They observed that purified enzyme has a greater ability to degrade various azo dyes. The high cost of enzymes represents a hurdle in broad industrial applications. 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 [8]. Co-precipitation of Fe^{2+}/Fe^{3+} ions under alkaline conditions has been the method of choice for the synthesis of iron oxide nanoparticles applied in bioconjugation [9]. This synthetic method often produces particles averaging less than 10 nm in diameter with broad size distribution [9]. 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 [10]. Studies with magnetic nanoparticles have been mostly dedicated for improvement of enzyme activity and loading, rather than to enzyme stabilization. Magnetic separation has been successfully used to concentrate biological samples for purification, isolation, and subsequent quantitative assays [11]. For example, bio-functionalized magnetic nanoparticles were recently used to capture and detect bacteria at very low concentration [11] and for biomolecule purification [12]. Covalent immobilization of lipase [13], and yeast alcohol dehydrogenase on magnetic nanoparticles increased the stability of the enzymes [14].

The immobilization of enzymes on magnetic nanoparticles could potentially result in unique properties of these bioactive magnetic particles. First, the increased surface area of nanoparticles compared with membranes, films, or micrometric particles, would enable immobilization of larger amounts of enzyme on the particles, which would lead to increased enzyme activity. Second, the ability to disperse the bioactive magnetic nanoparticles in solutions would enable rapid contact between the enzyme and its substrate, and reduce mass-transfer limitations. This would ultimately result in a lower limit of detection and faster analysis time. Finally, the magnetic properties would permit easy separation and reuse of the bioactive magnetic particles by cycles of magnetic separation, sample replacement and re-dispersion of the particles in the solution [9].

In this work the lignin peroxidase enzyme was covalently attached to modified magnetic nanoparticles. Magnetite (Fe_3O_4) nanoparticles averaging 16-20 nm in diameter were synthesized under alkaline conditions. The surface charges of Fe_3O_4 magnetic nanoparticles were modified using glutaraldehyde as cross-linker groups to support the covalent bioconjucation properties of enzyme immobilization. The modified Fe_3O_4 was used in immobilization and possible stabilization of LiP enzyme.

MATERIALS AND METHODS

Isolation and purification of lignin peroxidase (LiP) enzyme

One litter of bioremediations products from upflow fixed-film column (UFC) bioreactor and continuously stirred aerobic (CSA) bioreactor [5] was centrifuged at 3000 rpm for 10 min. The supernatant was pooled and solid ammonium sulphate was added to reach 40 % saturation and was left overnight at 4 °C. Then the mixture was centrifuged at 10000 rpm for 10 min, the supernatant was pooled and amended with solid ammonium sulphate to 80 % saturation. The solution was kept overnight at 4 °C and centrifuged at 10000 rpm for 10 min. The precipitate was dissolved in distilled water and dialyzed against 0.1 M phosphate buffer (pH 6) to remove ammonium sulphate. 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 using 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. The fractions exhibiting high peroxidase activity were pooled together and passed through diethyl amino ethyl (DEAE) cellulose columns(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 Bradford assay [15], and bovine serum albumin was used as standard.

Molecular weight determination of the isolated Lip enzyme

To determine the relative molecular weight of purified LiP enzyme, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a stacking and separating gel according to the method of Laemmli [16] 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 (Fe_3O_4) nanoparticles

The 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 [17].

Activation and modification of the magnetic nanoparticles

The synthesized magnetic nanoparticles (~2 g) were dispersed in ethanol and sonicated for about 10 min for getting the complete dispersion. Half ml of carbodiimide solution (25 mg/ml) and 1 ml of glutaraldehyde solution (10 %) were added to the above particles and incubated at room temperature for about 2 h. The particles were then washed with water to remove the excess glutaraldehyde [18].

Immobilization of lignin peroxidase on the magnetic nanoparticles

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

Examination of magnetic nanoparticles and immobilized enzyme

Examination using 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 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 [19].

Examination using scanning electron microscopy (SEM)

The morphology of the magnetic nanoparticles and immobilized enzyme were studied using scanning electron microscopy [20].

Examination using energy-dispersive X-ray spectroscopy

The structure and composition of magnetic nanoparticles and immobilized enzyme were studied using energy-dispersive X-ray spectroscopy (EDX) [21].

Decolourization of wastewater containing Reactive Blue azo dye

Free lignin Peroxidase (LiP) enzyme, immobilized LiP enzyme on magnetic nanoparticles, Fe_3O_4 magnetic nanoparticles, free *Pseudomonas aeruginosa* cells and *Pseudomonas aeruginosa* immobilized on magnetic nanoparticles were tested for decolourization of textile wastewater containing 300 ppm of Reactive Blue azo dye.For testing the free bacteria, 2 ml of *Pseudomonas aeruginosa* 2 days old culture were transferred to sterile 20 ml tubes. The tubes were filled to 18 ml working volume by sterile wastewater containing Reactive Blue dye (300 ppm) amended with yeast extract to give concentration of 0.5 g/l. The tubes

were sealed with screw caps to achieve anoxic conditions as described by Darwesh *et al.* [22+2]. For testing the immobilized bacteria, 1gm of immobilization product [2] was transferred to sterile 20 ml tubes. The tubes were filled to 18 ml working volume by sterile wastewater containing Reactive Blue dye (300 ppm) amended with yeast extract as above mentioned. The tubes were sealed with screw caps. For testing the free and immobilized LiP enzyme, 1 ml containing 10 units of the enzyme was transferred to sterile 20 ml tubes. The tubes were filled to 18 ml working volume by sterile wastewater containing Reactive Blue dye (300 ppm) amended with yeast extract. The tubes were sealed with screw caps. For testing the magnetic nanoparticles, 1 gm of Fe_3O_4 magnetic nanoparticles was transferred to sterile 20 ml tubes. The tubes were filled to 18 ml working volume by sterile wastewater containing Reactive Blue dye (300 ppm) amended with yeast extract. The tubes were sealed with screw caps. Tubes containing 18 ml of sterile wastewater containing Reactive Blue dye (300 ppm) and amended with yeast extract were used as a control. All tubes were incubated under static conditions at 28 $^{\circ}$C for 3 days. The decolourization of wastewater was measured at 24 hours intervals [2].

RESULTS AND DISCUSSION

Isolation and purification of lignin peroxidase (LiP)

Lignin peroxidase (LiP) enzyme is known to catalyse several oxidations in the side chains of lignin and related compounds resulting in mineralization of variety of recalcitrant aromatic compounds, polychlorinated biphenyls and dyes [1]. LiP enzyme was partially purified from the bioremediation products of textile wastewater containing Reactive Blue azo dye in a bioremediation UFC/CSA prototype bioreactor. The enzyme was isolated by precipitation using ammonium sulphate then dialyzed by dialysis bag against 0.1 M phosphate buffer (pH 6) to remove excess ammonium sulphate followed by passing the solution through Sephadex G-100 column. The fractions containing high activity of LiP enzyme were collected and purified by diethyl amino ethyl (DEAE) cellulose column. The molecular weight and purity of the LiP enzyme was assessed by SDS-PAGE electrophoreses. After Coomassie blue staining of the gel, a single band was detected (Figure 1). The purified LiP enzyme had molecular mass of approximately 38 kDa as compared with protein marker (Figure 1). This is likely to be near to complete purification of LiP enzyme. The molecular weight of the obtained 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 [23]. The LiP enzyme from *Hexagona tenuis* MTCC 1119 had molecular weight of 48 kDa [24], from *Loweporus lividus* MTCC-1178 had MW of 40 kDa [25] and from *Trametes versicolor* IBL-04 had MW of 30 kDa [26].

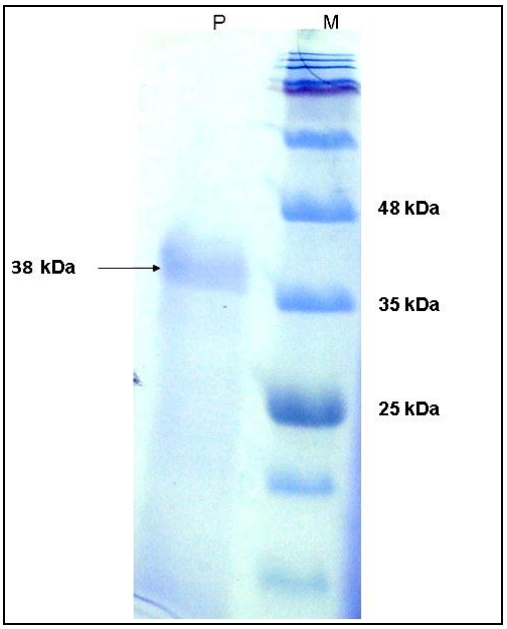

Figure 1: Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of purified LiP enzyme from *Pseudomonas aeruginosa* strain OS4.

Where; P, purified LiP enzyme and M, protein marker.

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Properties of prepared magnetic nanoparticles

Magnetic (Fe_3O_4) nanoparticles were prepared by co-precipitation technique. The obtained nanoparticles changed in colour, size, shape, magnetic properties and surface area charge as compared with Fe^{+3} molecule. The colour of prepared magnetic nanoparticles was black (Figure 2A). The Fe_3O_4 nanoparticles were separated from suspension by permanent magnet as shown in Figure (2B).

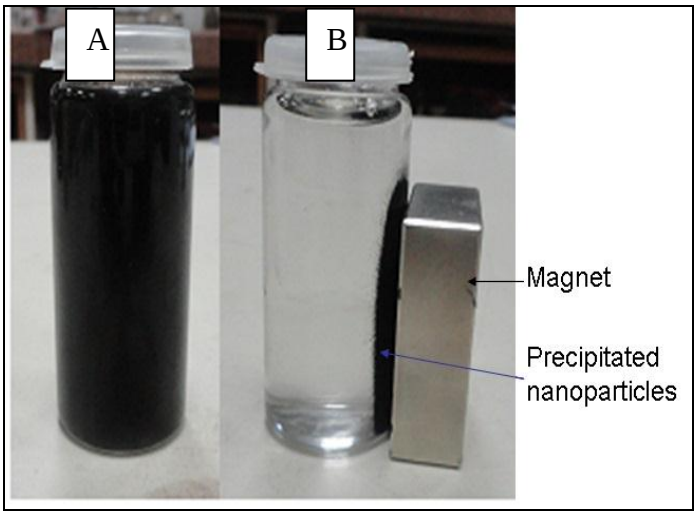

Figure 2. Fe_3O_4 Magnetic nanoparticles prepared by co-precipitation technique, A; dispersed nanoparticles, B; precipitated nanoparticles.

The size and shape of Fe_3O_4 magnetic nanoparticles were estimated by transmission electron microscopy (TEM). Figure (3) illustrates the transmission electron micrograph of Fe_3O_4 nanoparticles. The average particle size is 16- 20 nm and the particles had spherical shapes. The magnetic properties of Fe_3O_4 were reported as superparamagnetic material [27]. The advantages of these nanoparticles for industrial enzyme immobilization were reported by Wahajuddin and Arora [28].

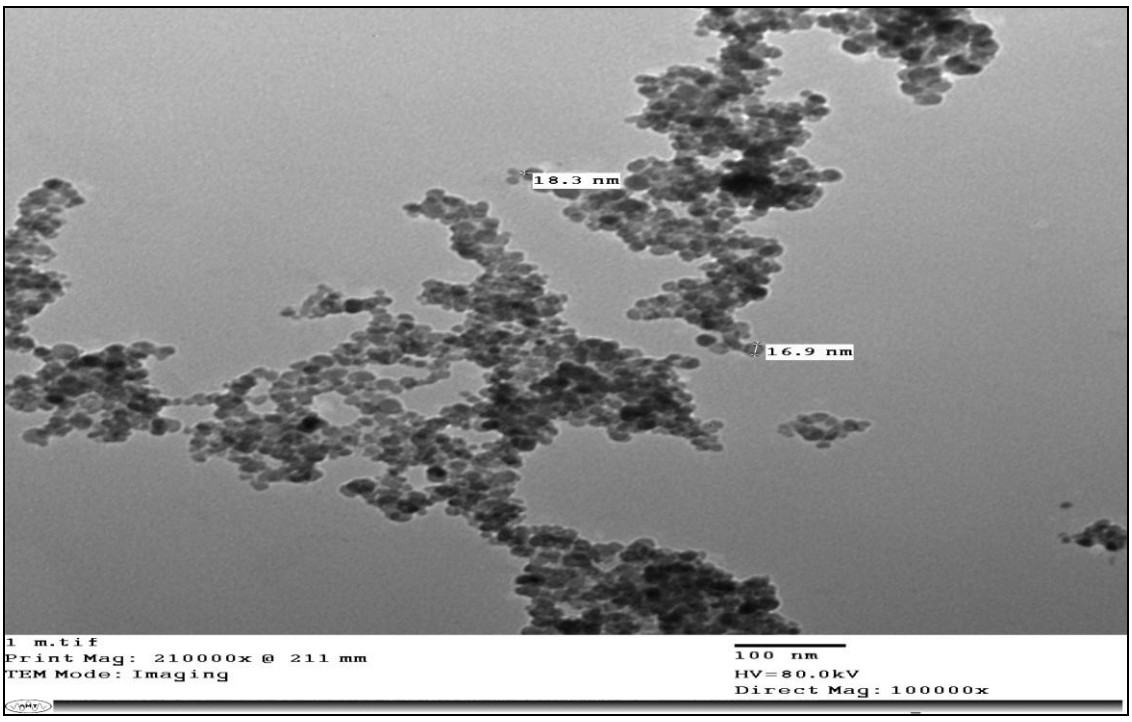

Figure 3: Transmission electron micrographs of Fe_3O_4 nanoparticles (210000 x magnifications).

Enzyme immobilization and charactarization of immobilized LiP enzyme

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One of the main objectives of this study is to enhance the stability and efficiency of LiP azo dye degrading enzyme. 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. The crude surface of magnetic nanoparticles modified by using glutaraldehyde as cross-linker to synthesise aldehyde as an active group with a positive charge. These active groups support the covalent bioconjucation of Fe_3O_4 nanoparticles and enzyme as well as whole bacterial cells. The modified Fe_3O_4 was used in immobilization and stabilization of both LiP enzyme and whole bacterial cells. Same approach was used by Mahdizadeh *et al.* [29] for preparation of Fe_3O_4 magnetic nanoparticles for immobilization of Glucose Oxidase and application of immobilized enzyme for Water Deoxygenation. Bahrami and Hejazi [30] prepared modified magnetic Fe_3O_4 nanoparticles and used it for immobilization of Pectinase enzyme. This technique facilitated the economic use of several enzymes.

To check the immobilization success, two techniques were applied: TEM microscopy and scanning electron microscopy with energy dispersive X-Ray analysis (SEM/EDX). The size and shape of immobilized LiP enzyme were measured by TEM. Figure (4) illustrates the TEM micrographs of immobilized LiP on Fe_3O_4 nanoparticles. The average particles size was 16.5- 24 nm. The TEM micrographs show the formation of film surrounding the magnetic nanoparticles (Figure 4) 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.* [31] who reported enhanced lipase stability and catalytic activity by covalently immobilization on surface of silica-coated modified magnetic nanoparticles.

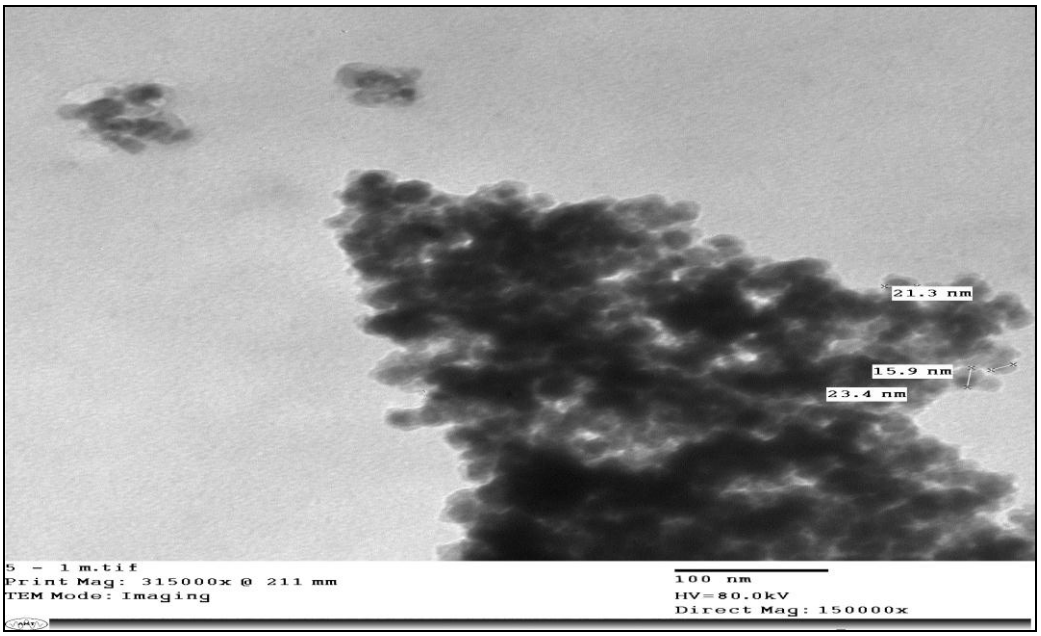

Figure 4: Transmission electron micrograph of Fe_3O_4 nanoparticles at magnification of 315000 x.

The scanning electron microscopy is probably the most widespread analytical instrument available in analytical laboratories to characterize physical properties such as morphology, shape, size or size distribution of materials at the nanoscale. Figure (5 A&B) shows clear defferences between magnetic nanoparticles before and after the enzyme immobilization. These changes clearly indicate the immobilization of the LiP enzyme to the magnetic nanoparticles. Direct evidence for this linking could be seen in Table (1) that shows the elemental analysis of the nanoparticles before and after immobilization. While, no carbon, nitrogen and phosphorous are present in the prepared magnetic nanoparticles, the immobilized enzyme on nanoparticle surfaces resulted in the presence of these three elements.The only reason for elemental analysis changes could be due to the immobilization of the enzyme on the nanoparicles. This indicates the successful immobilization of the enzyme protien particles on Fe_3O_4 magnetic nanoparticeles used.

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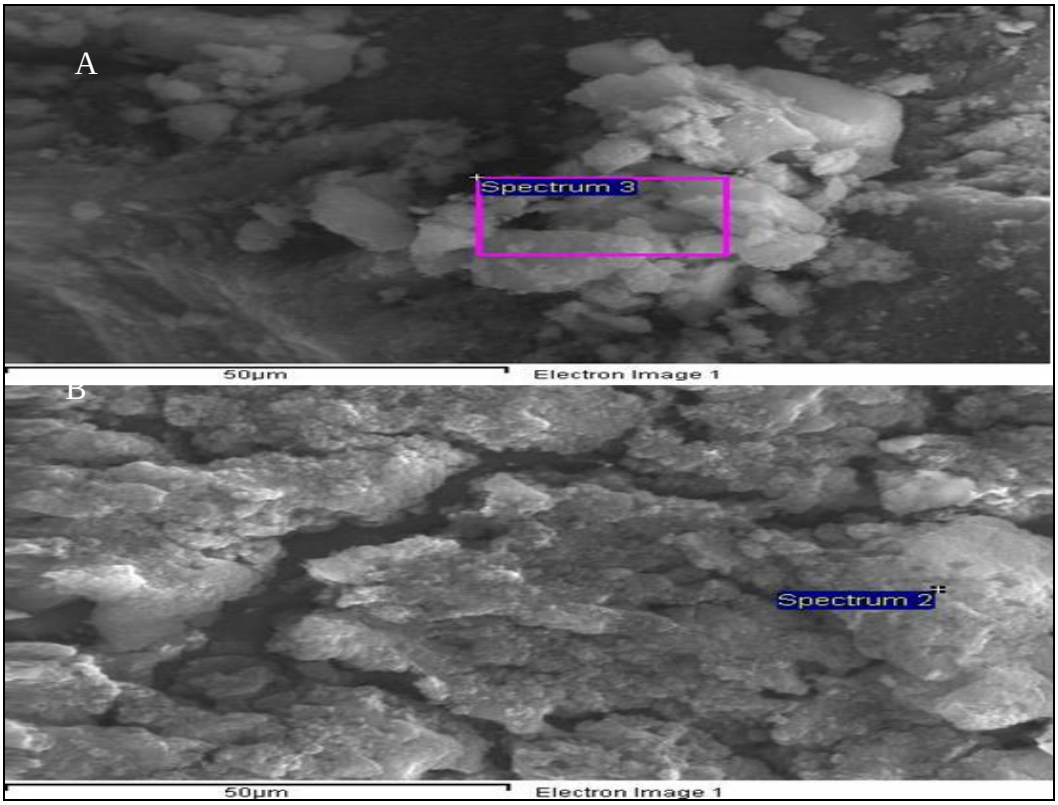

Figure 5: Micrographs of scanning electron microscopic analysis (SEM) of Fe_3O_4 magnetic nanoparticles (A) and immobilized LiP enzyme on the nanoparticles (B).

Table 1: Energy-dispersive X-ray spectrum (EDX) analysis of magnetic nanoparticles before and after immobilization with LiP enzyme.

Element	Weight of elements %	
	Magnetic nanoparticles	Immobilized enzyme
C	-	14.83
N	-	10.92
O	63.12	39.86
P	-	3.79
Fe	36.88	30.61
Totals	100.00	100.00

Energy dispersive X-ray diffraction (EDXD) analysis was very effective tool used for investigation of the elemental composition of the nanoparticles [32]. Figure (6 A&B) shows the energy-dispersive X-ray spectrum (EDX) analysis of magnetic nanoparticles (Fe_3O_4) before and after enzyme immobilization. The results revealed the presence of N, C and P elements in the samples of immobilized LiP on nanoparticles. This once more indicates the presence of enzyme protein the surface of magnetic nanoparticles. These elements were not found on the nanoparticles without enzyme immobilization. Siemieniec *et al.* [33] reported that the EDX spectrum analysis is a helpful tool to confirm the magnetic nanoparticles attached to organic molecules.

The successful LiP enzyme immobilization was further assessed by testing the enzyme activity using pyrogallol as specific substrate. The immobilized enzyme turns pyrogallol to dark yellow colour indicating the successful enzymatic reaction. It is likely that the conjugation between enzyme and immobilization matrix was out of the enzyme active site [34].

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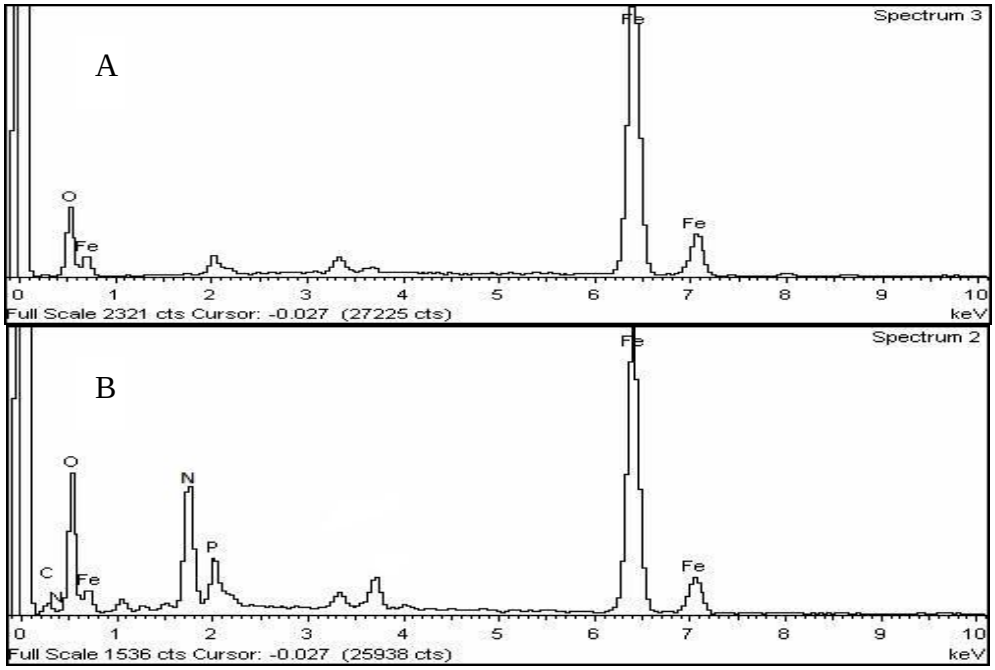

Figure 6: Energy-dispersive X-ray spectrum (EDX) analysis of magnetic nanoparticles (A) and immobilized enzyme (B).

Efficiency of enzyme immobilization

The immobilization of enzymes onto nanoparticles usually depends on various reaction factors such as immobilization time, quantity of nanoparticles, reaction temperature and buffer solution [18]. In this study, the efficiency of LiP enzyme immobilization was studied with respect to immobilization time. In Figure (7), immobilized lignin peroxidase showed maximum activity after 24 h of the start of the immobilization. The activity of immobilized enzyme showed gradual increase as the reaction time proceeds. On contrary, the activity of the free enzyme decreased gradually with time. The maximum activity of the immobilized LiP was set to 100 % as a reference value. The immobilization efficiency of 100 % means that, all LiP free enzymes added was immobilized onto the nanoparticles (**Figure 7**).

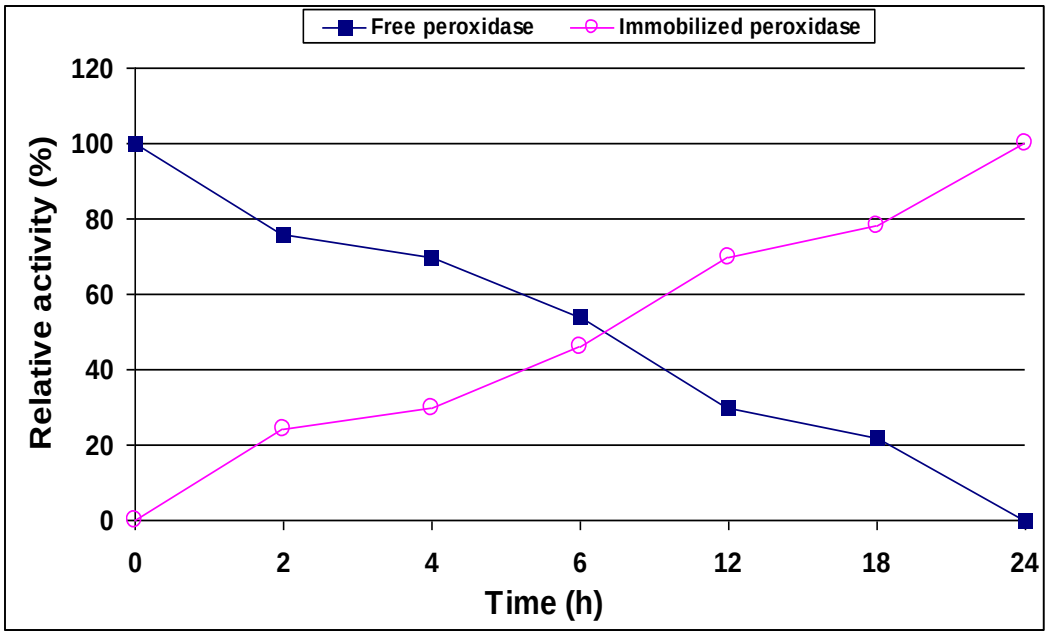

Figure 7: The LiP activity performance as a factor of time and the enzyme immobilization efficiency with respect to the immobilization time.

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Bioremediation of textile industry wastewater containing Reactive Blue azo dye

The Reactive Blue azo dye is one of the widely used textile dyes. Unfortunately the dying basins wastewaters were found to contain large amounts of dye residues amounting up to 50 % of the used dye [22]. The save disposal of the dye wastewater requires special technology to remove the dye residues to reach the acceptable levels by environmental authorities. The cost of physical and/or chemical techniques used for this purpose was found to be uneconomic. In this study we test new approaches for removal of textile dye residues using nanobiotechnological bioremediation approaches. These approaches are based on the selection of potent microbial agents capable to remove the dye in rather short time and use these microbes either as it is or LiP enzyme isolated from their cells and known to contribute significantly in azo dye biodegradation/bioremoval [2]. The partially purified enzyme obtained from microbial cells is cross-linked to magnetic nanoparticles prepared specially for this bioremediation purpose. The bacterium used in this study is *Pseudomonas aeruginosa* strain OS4. This strain was proven in our previous studies [2,5] to be highly efficient in dye removal in rather short time. The Fe_3O_4 magnetic nanoparticles were prepared according to the method described in details in materials and methods section. In order to test the efficiency of the suggested nanobiotechnological approach several controls were included for comparison. The results in Table (2) show that in controls where the dye alone and or in the presence of nanoparticles alone no change in solution colour was observed till the end of the experiment (72 hours). The treatment of dye containing wastewater with LiP enzyme either in free state or immobilized on Fe_3O_4 magnetic nanoparticles induced colour removal at various degrees. The free LiP enzyme started to remove dye colour after 24 hours where it could remove only 6.7% of the colour .The colour removal in this treatment increased to 15.4 after 72 hours. The immobilization of the enzyme on magnetic nanoparticles induced early decolourization and enhanced the removal markedly as compared with the free Lip enzyme. The application of whole bacterial cells either in free state or immobilized on Fe_3O_4 nanoparticles resulted in extremely higher colour removal starting at 24 hours. The colour removal at 48 hours sampling showed that the immobilized whole bacterial cells were superior in colour removal as compared with the free bacterial cells being 93.4% in the first compared to 58.4% in the second. After 72 hours, the last two treatments almost removed most of the dye colour resulting in 95.2% of colour removal in the first and 97.5% in the second (Table 2). This clearly shows that the bioremediation by either *Pseudomonas aeruginosa* whole cells or the LiP enzyme extracted from it play important role in dye bioremoval. The immobilization of Free *Pseudomonas aeruginosa* strain OS4 whole cells on magnetic nanoparticles has dramatically improved the performance of this bacterium in bioremediation of a commonly used textile dye residues. The results are close to those obtained by using partially purified LiP enzyme. However the advantage of the enzyme conjugated to nanoparticles is mainly linked to the repeated use of nanoparticles –enzyme complex to effectively perform the bioremediation. The limited cycles of immobilized bacterial cells as compared to the immobilized enzymes were previously reported [2,35,36]. In addition, the tolerance of enzymes to the storage and the adaptation of the industries to the use of chemicals including enzymes in different forms render the use of enzyme immobilized nanoparticles more easy and practical than using living microbial cells.

Table 2: Decolourization % of textile wastewater containing Reactive Blue azo dye.

Treatments	Incubation time (hours)		
	24	48	72
Control	0.0	0.0	0.0
Fe_3O_4 magnetic nanoparticles	0.0	0.0	0.0
Free LiP enzyme	0.0	6.7	15.4
Immobilized LiP on Fe_3O_4	10.2	30.6	54.3
Free *Pseudomonas aeruginosa* strain OS4	23.7	58.4	95.2
Immobilized *Pseudomonas aeruginosa* on Fe_3O_4	56.8	93.4	97.5

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CONCLUSION

The enzymatic bioremediation technology using magnetic nanoparticles represents promising nanobiotechnological approach that has wide range of application in industry in generated and bioremediation of recalcitrant chemical residues in particular.

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