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# Alteration in protein profile of *Pseudomonas aeruginosa* (PTSOX4) coated with magnetic Fe<sub>3</sub>O<sub>4</sub> nanoparticles

Fazeleh Bahrampour, Jamshid Raheb\* and Zohreh Rabiei

## Abstract

One of the remarkable features of bacterial species is their capacity for rapid growth when the appropriate environmental condition for growth is provided. Some bacteria, during their growth period, encounter stress factors in their natural environments, such as limitation in growth bioavailability, heat shock, heavy metal, etc. One stress factor not studied is the effect of magnetic Fe<sub>3</sub>O<sub>4</sub> nanoparticles on bacterial growth rate. The effect of magnetic Fe<sub>3</sub>O<sub>4</sub> nanoparticles on the protein profiles of genetically engineered bacterial strain *Pseudomonas aeruginosa* (PTSOX4), a strain with biological desulfurization characteristic, was investigated. The magnetic Fe<sub>3</sub>O<sub>4</sub> nanoparticles were synthesized using co-sedimentation method, and their morphology was observed by scanning electron microscopy (SEM). The topography of magnetic Fe<sub>3</sub>O<sub>4</sub> nanoparticles was detected by X-ray diffraction, and the average nanoparticle size measured was 40 to 50 nm. The bacterial cells were coated with magnetic nanoparticles, and the SEM electrographs of the bacterial cells indicated that the nanoparticles were uniformly coated on the cell surface. Proteins from both uncoated and coated bacterial cells were extracted by sonication and subjected to two-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis. Some novel protein bands appeared in the protein profiles of coated bacterial cells; however, some protein bands disappeared. The two-dimensional gel electrophoresis results highlighted the presence of two different polypeptide groups, with molecular weights of 30 to 56 kDa and 56 to 65 kDa.

**Keywords:** Desulfurization; Nanoparticle; Protein profile; Polypeptide; *Pseudomonas aeruginosa*

## Background

The synthesis of magnetic monodisperse nanoparticles has attracted great interest in the field of nanotechnology, and the investigation is developed with several types of iron oxides that have been carried out in the field of magnetic nanoparticles (NPs). The application of small iron oxide (NPs) *in vitro* diagnostics has been investigated for nearly half a century [1,2]. Nanotechnology has developed to such an extent that it has become possible to fabricate, characterize, and specially tailor the functional properties of NPs for biomedical and diagnostic applications [3,4]. At present, a number of different chemical and biological methods have been reported for magnetite Fe<sub>3</sub>O<sub>4</sub> NPs [5]. The study on chemical co-precipitation has been widely used to produce magnetite NPs due to its

facility and large-volume capability [6,7]. This method involves co-precipitation to synthesize iron oxides from aqueous Fe<sup>2+</sup> and Fe<sup>3+</sup> salt solutions by adding a base [8,9] as follows:  $\text{Fe}^{2+} + 2\text{Fe}^{3+} + 8\text{OH}^{-} \rightarrow \text{Fe}_3\text{O}_4 + 4\text{H}_2\text{O}$ .

Nano-sized particles have physical and chemical properties that are characteristic of neither the atom nor the bulk counterparts [10]. Among various types of NPs, magnetic iron oxide NPs have been explored increasingly by researchers for their applications in drug delivery, magnetic resonance imaging, tissue repair, hyperthermia, and cell separation [11,12]. In the field of nano-sized magnetic NPs (the single domain with diameter of about 5 to 20 nm), magnetite is a very promising candidate since its biocompatibility has already been proven [13,14]. In fact, two main types of iron oxides, magnetite (Fe<sub>3</sub>O<sub>4</sub>) and maghemite (γ-Fe<sub>2</sub>O<sub>3</sub>), are widespread in the environment and have been used in various biomedical applications [3,15]. Maghemite could

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be considered as a Fe(II)-deficient magnetite, and it is formed by the topotactic oxidation of magnetite [7]. The study on magnetic Fe<sub>3</sub>O<sub>4</sub> NPs is of increasing interest because of its special magnetic properties and low toxicity [8,16]. Moreover, it has been found to have various biomedical applications. On the other hand, bacterial genomes are ideal model systems for the development and application of proteomic technology because they are relatively small organisms [4]. A combination of proteomic technologies was used in this study for the global analysis of protein expression and regulation in the Gram-negative bacteria *Pseudomonas aeruginosa* (PTSOX4).

The term proteomics was coined in 1995 by Marc Wilkins as a complement of proteins expressed by a genome. In the simplest term, it is the study on proteome, the collection of proteins in an organism [17]. Proteins are organic compounds that consist of 20 common amino acids joined by peptide bonds [18].

All amino acids have a central carbon atom to which a hydrogen atom, an amino group (NH<sub>2</sub>), and a carboxyl group (COOH) are attached in common [19]. In fact, sequences of amino acids fold to generate compact domains (three-dimensional structures: secondary, tertiary, and quaternary) from linear chains (primary structure) [19]. In protein molecules, electrostatic interactions, Van der Waals forces, hydrogen bonds, and hydrophobic interactions play an important role in defining and stabilizing the three-dimensional structure and adsorption of protein molecules [17,18]. In addition, many proteins have carbohydrate-binding sites on their surface [20]. They play an important role in bacterial adhesion and recognition of pathogens by specific surface carbohydrates of the immune system [20]. In fact, proteins are one group of recognizable organic matter from the environment [21]. Proteins play an important role in bacterial transport in natural environments. Therefore, it is important to understand the role of proteins in bacteria [21]. On the other hand, proteomics offers the most effective approach to identify protein markers specific to epidemic strains that could serve as targets for novel therapeutics [22,23]. Proteomics can have practical applications through the identification of proteins that may be potential targets for the biotechnology industry and through expansion of our understanding on the physiological action of these proteins [24]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) is an electrophoretic method for the separation of polypeptides according to their molecular weights. The technique is performed in polyacrylamide gels containing sodium dodecyl sulfate. SDS-PAGE is the most widely used in analytical methods to resolve separate components of a protein mixture. The technique is also a powerful tool for estimating the molecular weights of proteins [25]. In the present study, using proteomic strategies to achieve these aims includes

(a) analysis by SDS-PAGE and (b) two-dimensional (2-D) gel electrophoresis.

## Results and discussion

### Determination of nanoparticle size for bacterial cell coating

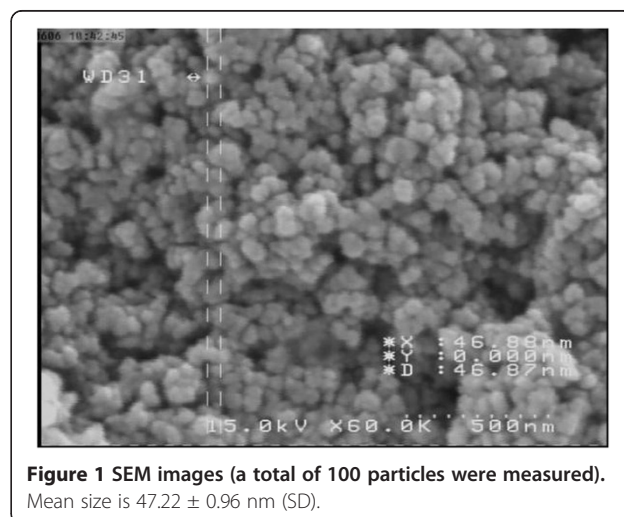
Scanning electron microscopy (SEM) was used to characterize the nanoparticle size suitable for bacterial cell coating. The nanoparticle diameter in the range of 45 to 55 nm was determined directly from the SEM images, as shown in (Figure 1). An average diameter of 40 nm was selected for nanoparticles since it is small enough to have super paramagnetic characteristics. The SEM electrophotograph of the cells coated with Fe<sub>3</sub>O<sub>4</sub> nanoparticles is shown in (Figure 2), in which it is clearly demonstrated that the Fe<sub>3</sub>O<sub>4</sub> nanoparticles were adsorbed on the surface of bacterial cells.

### Structural characterization of the magnetic Fe<sub>3</sub>O<sub>4</sub> nanoparticles

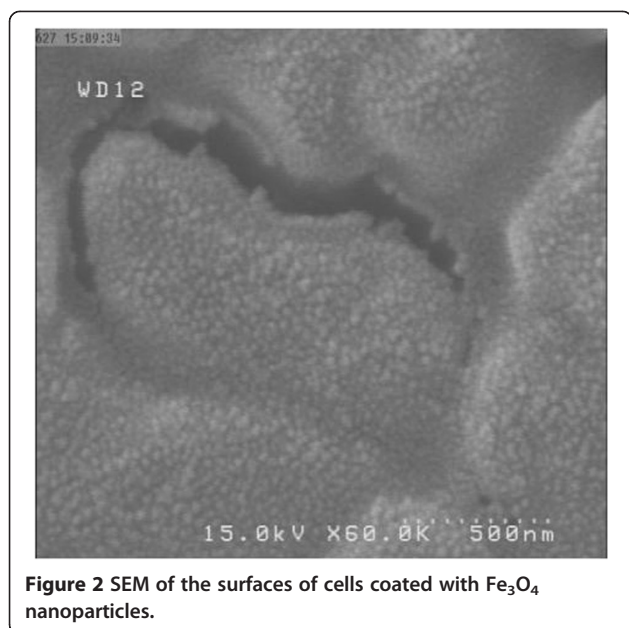
The X-ray diffraction (XRD) pattern (Figure 3) of the Fe<sub>3</sub>O<sub>4</sub> nanoparticles prepared under standard conditions revealed diffraction peaks at (220), (311), (400), (422), (511), (440), etc., which were characteristic peaks of Fe<sub>3</sub>O<sub>4</sub> in a crystal form with a cubic spinel structure. It is clear that only the phase of Fe<sub>3</sub>O<sub>4</sub> is detectable and there was no other phase such as Fe(OH)<sub>3</sub> or Fe<sub>2</sub>O<sub>3</sub>, as usual products in a chemical co-precipitation procedure (Figure 4). It was also found that particle size was quite small from the relatively wide half-peak breadth. According to the XRD pattern, the average diameter could be evaluated by the Scherrer equation [9].

### SDS-PAGE analysis of total bacterial cell protein

SDS-PAGE was applied for total cell protein analysis. The total proteins include the control and those treated with Fe<sub>3</sub>O<sub>4</sub> nanoparticle, and the marker is demonstrated in



**Figure 1** SEM images (a total of 100 particles were measured). Mean size is 47.22 ± 0.96 nm (SD).



**Figure 2** SEM of the surfaces of cells coated with Fe<sub>3</sub>O<sub>4</sub> nanoparticles.

Figure 5. The results indicated the presence of two different polypeptide groups, with molecular weights of 30 to 45 kDa and 17 to 30 kDa. The polypeptide with the molecular weight of 78 kDa was more upregulated than the others. Moreover, the effects of Fe<sub>3</sub>O<sub>4</sub> nanoparticles on the cell surface of the bacteria could be considered as the main reason for the elimination of polypeptide available per bacterial cells to yield a suitable level of cell coating.

#### 2-D gel electrophoretic analysis of total bacterial cell protein

In order to define protein expression and regulation in *P. aeruginosa* grown under Fe<sub>3</sub>O<sub>4</sub> nanoparticle stress,

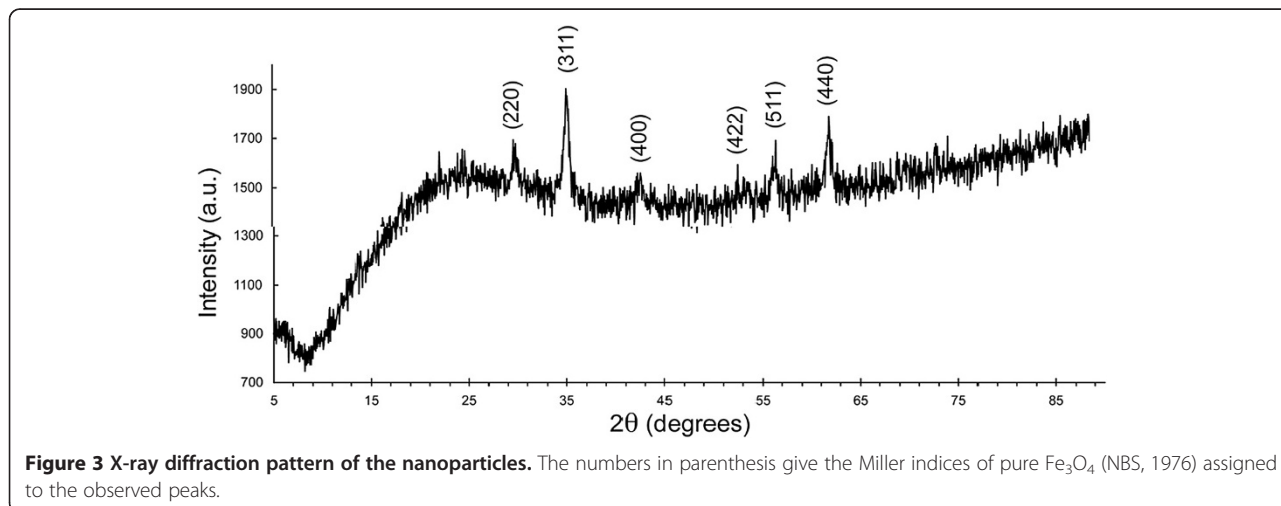
proteomic technology was utilized. The total cell protein of *P. aeruginosa* was extracted by a 7-cm Immobiline DryStrip (GE Life Sciences, Chalfont St. Giles, England), pH 3 to 10. The Ettan IPGphor isoelectric focusing system (Figure 6) and treatment sample (Figure 7) showed complete differentiation of protein quantity in the control and treatment samples, which was distinguished by the white area. The standard ladder (Bio-Rad, Hercules, CA, USA) used in the 2-D technique was determined between 97 and 21.5 kDa.

Ishikawa and coworkers have purified an iron-binding protein from *Campylobacter jejuni*, cloned and sequenced the dps gene encoding this protein, and investigated the role of the dps gene in hydrogen peroxide stress resistance [26,27]. In this work, the protein was purified from *P. aeruginosa* to investigate the role of iron oxide nanoparticles in stress resistance.

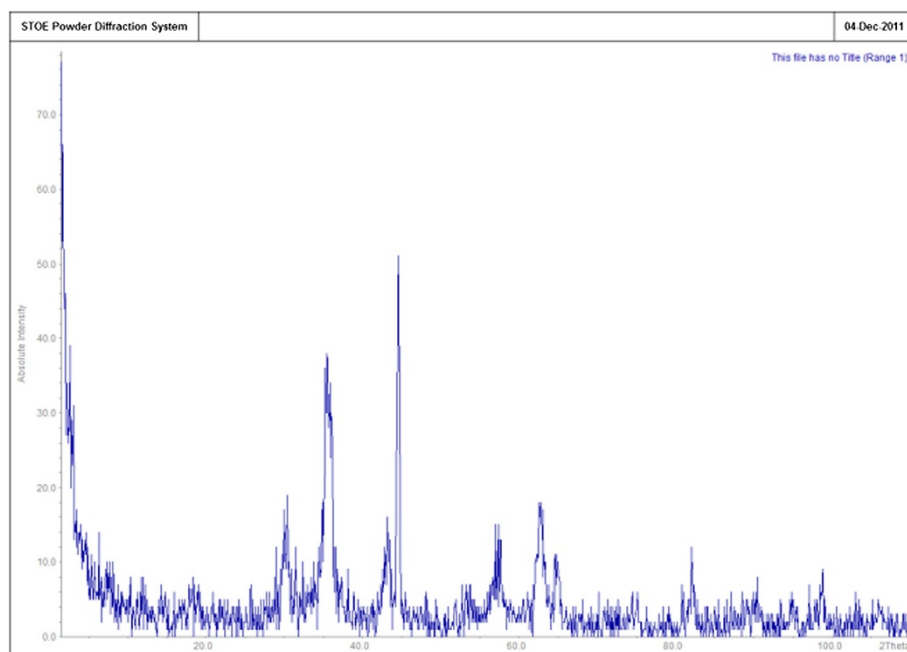
Proteins can be quantitatively precipitated from a variety of sources without interference from detergents, chaotropes, and other common reagents used to solubilize protein (Figure 6). Recovery is generally greater than 50%. The procedure does result in spot gain or loss or changes in spot position relative to untreated samples (Figure 7). The precipitated proteins are easily re-suspended in a 2-D sample solution. On the other hand, it showed that in the SDS-PAGE, some bands disappeared, but majority of the protein banding patterns in both were stable, which introduce the effect of Fe<sub>3</sub>O<sub>4</sub> nanoparticles on the bacterial protein profile. Many proteins were differentially expressed between each mutant strain situation.

#### Conclusions

High resistance to environmental stress factors is one of the excellent characteristics of bacterial responses. Stimulation of these factors in different bacteria is triggered for some production, leading to the formation of a series of proteins



**Figure 3** X-ray diffraction pattern of the nanoparticles. The numbers in parenthesis give the Miller indices of pure Fe<sub>3</sub>O<sub>4</sub> (NBS, 1976) assigned to the observed peaks.



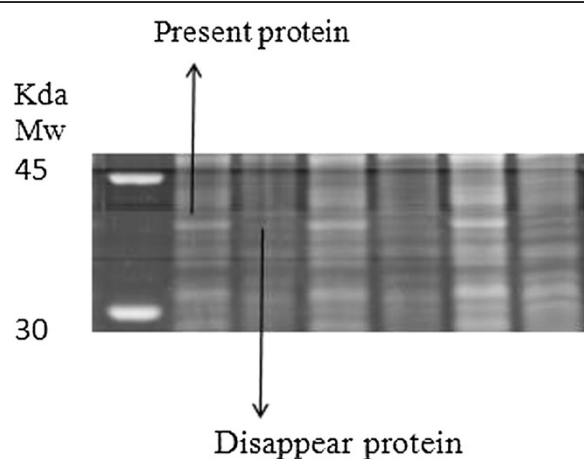
**Figure 4** XRD pattern indicating the sole existence of inverse cubic spinel phase of  $\text{Fe}_3\text{O}_4$ . The average size was about 40 nm.

in response to the environmental stress factors such as nanoparticles. Magnetic nanoparticles with a super paramagnetic behavior have very low toxicity on living cells [28]. However, these nanoparticles as an environmental stress factor cause the production of a number of proteins in response to resist the toxicity of these nanoparticles. This mechanism occurs due to the development of a more resistant state in most bacterial cells. Exposure to nonlethal levels of magnetic nanoparticles also increases

the resistance of actively growing cells to subsequent lethal level of the same and, occasionally, other stressors. This enhanced resistance which is developed during exposure to the magnetic nanoparticle requires newly synthesized proteins.

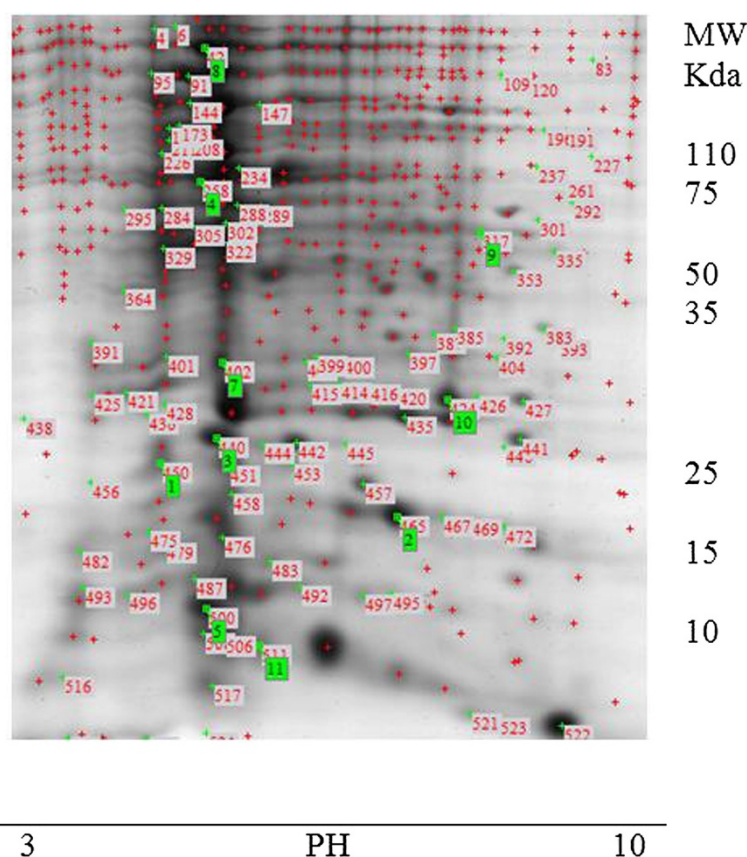
In *Vibrio furnissii*, in response to some environmental factors, cell mass and rRNA decreased rapidly while the total cell counts remained stable. This reduction in cell mass is seen as miniaturization of cell, similar to the phenomena observed in other *Vibrio* species [29]. The overall rate of proteins synthesized in *Vibrio* upon exposure to one of the stressors after 24 h was about 10% of that of cells growing exponentially in a complex medium, and the global rate of protein synthesis was reduced by 95% in *Escherichia coli* [30]. In both cases, the proteins being synthesized were those associated with one environmental factor and the development of resistance to other factors in these bacteria. The rate of total protein synthesis decreased immediately at the onset of stress, and after 48 h, it was less than 1% of the synthesis rate during growth [31,32].

In the experimental reported here, the total number of proteins seen in the profiles of *P. aeruginosa* (PTSOX4) cells run on SDS-PAGE showed several changes over an exposure period to magnetic nanoparticles. The changes observed depend on the time period of exposure, and after a longer time, the changes were more considerable. No measurements of the total amount of proteins in the cells or the overall rate of the protein synthesis were made in this experiment. However, it is evident from an



**Figure 5** Data obtained from SDS-PAGE showing the separation of bacterial protein. Column 1, ladder; columns 2, 4 and 6 represent the uncoated bacteria as control, and columns 3, 5 and 7 represent coated bacteria.





**Figure 6** 2-D protein of *P. aeruginosa*. Extracted by a 7-cm Immobiline DryStrip, pH 3 to 10, Ettan IPGphor isoelectric focusing system. Protein profiles of uncoated bacterial cells analyzed by Melani software.

examination of the gels that the total amount of cellular protein decreased and that there were reproducible changes observed in the protein profile of cells when exposed to the nanoparticles. As SDS-PAGE only analyze the proteins based on molecular weight, it is impossible to identify many proteins which may have a different isoelectric point but the same molecular weight. Many of the bands seen on this gel could be due to the multiple proteins. Consequently, two-dimensional gel electrophoresis was used to demonstrate in more detail the changes in the total protein profiles of cells under different stresses. The study on cells coated with nanoparticles showed that a number of proteins were eliminated. These proteins are responsible for overall integrity, protection against oxidative stress, and change in osmotic pressure. Previously, we showed that these nanoparticles are less toxic to PTSOX4 bacteria. We suggest that the coating of  $\text{Fe}_3\text{O}_4$  NPs has no effects on the osmotic equilibrium of the membrane. Some report that the synthesis of some proteins is most critical when the stress effect response in *E. coli* occurred in the first period of incubation and that these proteins play a major role in the response to long-term effects of environmental factors [33-35].

## Methods

### Materials

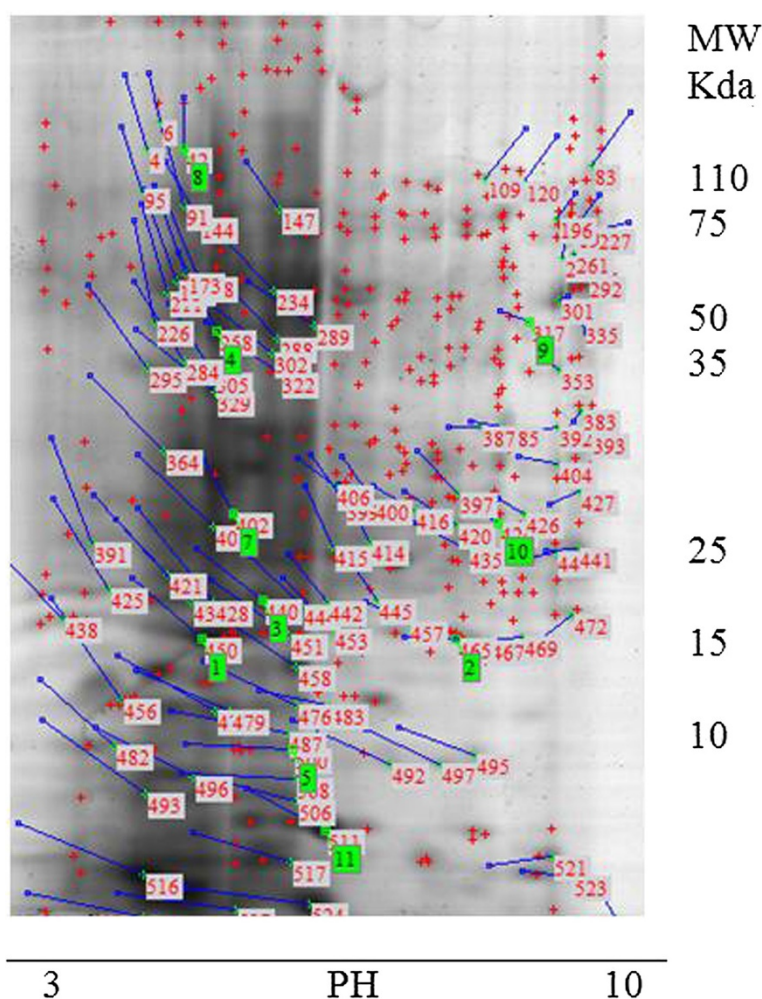
#### *Bacterial strain and medium*

*P. aeruginosa* (PTSOX4) and *E. coli* DH5 $\alpha$  were provided by the National Institute of Genetic Engineering and Biotechnology and have the ability to convert dibenzothiophene (DBT) to 2-hydroxy-biphenyl and sulfate. The bacteria were grown on a sulfur-free culture medium (bifidus selective medium (BSM) composed of 2.44 g  $\text{KH}_2\text{PO}_4$ , 5.47 g  $\text{Na}_2\text{HPO}_4$ , 0.2 g  $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ , 0.001 g  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.001 g  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ , 0.004 g  $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ , and 2 mL glycerol in 1 L deionized water. In addition, DBT solution was added to obtain the final solution of 100 ppm  $\text{L}^{-1}$ . *Pseudomonas* strain was grown in BSM at 30°C. *E. coli* strains were grown in Luria-Bertani (LB) medium (Difco, Becton, Dickinson and Company, Franklin Lakes, NJ, USA) at 37°C.

### Methods

#### *Nanoparticle synthesis*

The synthesis of magnetic nanoparticles was done by the following method. We dissolved 0.045 g  $\text{FeCl}_2 \cdot 4\text{H}_2\text{O}$  and  $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$  in 150 mL deionized water, previously



**Figure 7** 2-D protein of *P. aeruginosa* coated with iron oxide nanoparticles. Extracted by a 7-cm Immobiline DryStrip, pH 3 to 10, Ettan IPGphor isoelectric focusing system. Protein profiles of coated bacterial cells analyzed by Melani software.

acidified by 1 mL HCl (37%), with mechanical stirring at 1,100 rpm and 65°C, then quickly added NH<sub>4</sub>OH (1 M) until the pH reached 11. To the resulting suspension, 0.09 g glycine was added over a period of 10 min. After 20 min, the magnetic precipitate was separated by a centrifuge (4,000 rpm). The sample was washed two times and dried at 80°C by vacuum drying.

#### Methods of NP characterization

In contrast, the surface coated with nanoparticles is more freely aligned with the external field than the uncoated surface. The repulsive force between hydrophobic surfactant molecules coated on individual particles can prevent them from agglomeration.

#### Structure of the crystal

A crystallographic study on magnetic Fe<sub>3</sub>O<sub>4</sub> NPs was performed with an X-ray powder diffractometer using a CuK $\alpha$  ( $\lambda = 0.15406$  nm) target, graphite monochromator.

The working voltage was 40 kV, and the working electrical current was 30 mA with a scan speed of 8°C min<sup>-1</sup>.

#### Cell growth and coating

The bacterial cells were grown in 100 mL LB until the mid-exponential growth phase, harvested by centrifugation at 1,400 $\times$ g for 10 min, and concentrated to obtain 5.6 mg of dry cells per liter. The cells were coated with magnetic nanoparticles through the following method: 10 mL of a suspension containing 100  $\mu$ g mL<sup>-1</sup> Fe<sub>3</sub>O<sub>4</sub> NPs in water solution was mixed with 100 mL of the cell suspension in LB at a final concentration of 0.5 mM. The ratio of nanoparticle mass to biomass was measured, which was 1.78 w/w and considered appropriate to make sufficient particles.

#### Protein extraction and purification

Bacterial cells were grown in a suitable liquid medium for 24 h at 30°C. The cells were harvested by centrifugation

and sonicated in 1 mM Tris buffer (pH 8.0). The sonicated cells were centrifuged at 1,500×g for 20 min at 4°C. The pellet was discarded, and the supernatant was centrifuged at 48,400×g in a JA20 rotor (Beckman Coulter, Brea, CA, USA) for 60 min at 4°C to harvest the membrane proteins and leave the soluble proteins in the supernatant. The membrane proteins were re-suspended in 3 mL Tris buffer (pH 8.0) and stored at -20°C.

The supernatant was concentrated to about 5 mL using an Amicon (Millipore Co., Billerica, MA, USA) ultrafiltration apparatus. The filters were able to retain proteins with molecular weight greater than 10,000 Da. An equal amount of saturated ammonium sulfate was added to the concentrated supernatant fraction to give a final ammonium sulfate concentration of 50%, which was left overnight to allow the proteins to precipitate. The precipitate was pelleted by centrifugation at 35,000×g for 30 min, re-suspended in 5 mL of sterile distilled water, and dialyzed overnight against at least 4 L of distilled water to remove any ammonium sulfate from the samples.

#### **Two-dimensional gel electrophoresis**

This technique separated proteins according to their isoelectric point in the first dimension and according to their molecular weight in the second dimension by SDS-PAGE. These two unrelated parameters give the maximum resolution of the proteins present in a complex mixture.

Approximately 75 µg of bacterial protein was placed in a rehydration buffer that was immobilized by a dry strip (pH 3 to 10 for 16 h) and then placed in an Ettan IPGphor isoelectric focusing system (92.5 kV h for 21 h; GE Life Sciences). After that, the equilibrated IPG strips for the second-dimension gel between the two glass plates were applied. The IPG plastic backing was placed against one of the glass plates in room temperature on one side of the strip with the filter paper and protein marker.

#### **Rehydration buffer**

The buffer was prepared for one IPG strip of 7 cm, 50 µL of buffer including 12 mg (8 M) urea, 0.075 mg thiourea, 2 mg 4% CHAPS, 2.5 µL 100% (1 M) DTT, 0.2 µL 1% IPG buffer (ampholines), 20 µL Bromophenol blue, and deionized water up to 125 µL.

#### **Equilibration buffer**

The following buffer was applied as an equilibration buffer in this work: Tris (1 M), pH 8.8 5 mL, 36.03 g urea, 30 mL 100% glycerol, 20 mL 10% SDS, 200 µL 1% Bromophenol blue, and deionized water up to 100 mL.

#### **SDS polyacrylamide gel**

Gel electrophoresis was carried out in a vertical SDS polyacrylamide gel containing 10% SDS, with stacking gel of 6% acrylamide and a resolving gel of 12% acrylamide. Electrophoresis was performed for 2 to 3 h at 100 mA. The standard protein markers (Bio-Rad) were included. Gels were fixed and stained with Coomassie blue as previously described. Photographs of each gel were taken by a gel documentation system (LabScan TM 6.0, Swiss Institute of Bioinformatics, Geneva, Switzerland).

#### **Competing interests**

The authors declare that they have no competing interests.

#### **Authors' contributions**

JR and FB carried out the nanobiotechnology and proteomics studies. JR, FB and ZR participated in writing the manuscript.

#### **Authors' information**

FB is a PhD student at the National Institute of Genetic Engineering and Biotechnology. JR and ZR are academic staff and associate professor at the National Institute of Genetic Engineering and Biotechnology.

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