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The Effect of Magnetic Fe_3O_4 Nanoparticles on the Growth of Genetically Manipulated Bacterium, *Pseudomonas aeruginosa* (PTSOX4)

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ABSTRACT

Background: Magnetite (Fe_3O_4) nanoparticles are currently one of the important and acceptable magnetic nanoparticles for biomedical applications. To use magnetite nanoparticles for bacteria cell separation, the surface of nanoparticles would be modified for immobilizing of nanoparticles on the surface of bacteria. Functionalization of magnetite nanoparticles is performed by different surfactants such as glycine or oleic acid to attach on the bacteria cell surface simultaneously. The magnetic nanoparticles have very low toxicity on the living cells. There are some studies on evaluating the toxicity of magnetite nanoparticles on eukaryote cells, which their results showed negligible toxicity in eukaryote cells of the modified magnetite nanoparticles with different surfactants. But the toxicity of magnetite nanoparticles on bacteria cells is not reported.
Objectives: in this study, the effect of the magnetic nanoparticles iron oxide (Fe_3O_4) on the growth rate of the genetically engineered *Pseudomonas aeruginosa* (PTSOX4) cells in different media with different magnetic nanoparticles concentration have been investigated.
Materials and Methods: In this study, the genetically manipulated bacterial cells, *Pseudomonas aeruginosa* (PTSOX4), were coated with magnetic Fe_3O_4 nanoparticles to evaluate the toxicity effect of these nanoparticles on the growth rate of this strain in Laurial Bertany (LB) and Basal Salt media (BSM) separately. In addition the minimal inhibitory concentration (MIC) and the minimal bactericidal concentration (MBC) tests of these nanoparticles were examined.
Results: A low concentration of nanoparticles has little toxicity effect on the cell growth in this bacterium. Maximal level of the growth obtained in the late stationary phase, using a concentration of 500 ppm or more of Fe_3O_4 nanoparticles, but a high concentration of these nanoparticles, more than 1000 PPM, resulted in reducing the cell growth rate. However, there was not a considerable lethal effect on the cell viability. Moreover, using a high nanoparticle concentration leads to a high level of bacterial cell coating due to more contact of the nanoparticles to bacterial cell surface.
Conclusions: It is concluded that magnetite nanoparticles have negligible toxicity on the living bacteria cells and they are so applicable in different parts of biotechnology fields.

Implication for health policy/practice/research/medical education:
Implication for research and industrial applications.

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1. Background

Magnetic nanoparticles with a super paramagnetic behavior are excellent for a variety of interdisciplinary technology and biomedical application (1). According to the unique chemical and physical properties, super paramagnetic nanoparticles have a high quality for several biomedical applications, such as: 1) cell and biomacromolecule separation 2) gene and drug delivery 3) magnetic resonance imaging (MRI) 4) hyperthermia and some others.(2). Magnetite (Fe_3O_4) nanoparticles are currently one of the important and acceptable magnetic nanoparticles for biomedical applications (3). The surface of magnetite nanoparticles is modified by surfactants, biomacromolecules and some others for using in biomedical areas. Using of each surfactant to functionalize and stabilize nanoparticles is related to their application, because each surfactant is able to give the magnetite nanoparticles special properties (4). To use magnetite nanoparticles for bacteria cell separation, the surface of nanoparticles would be modified for immobilizing of nanoparticles on the surface of bacteria. For that, the surface of magnetite nanoparticles should be modified with a surfactant that immobilizes nanoparticles on the surface of cells spontaneously. Compared to other conventional techniques of bacteria separation, magnetic sorting enables higher throughput and could use less specialized tools while keep the cell viability (5). Recently, magnetic bacteria cell separation has been interested to industrial application such as microbial desulfurization of oil (6). For that, bacteria cells are firstly coated with magnetite nanoparticles; after performing of desulfurization reaction, they are isolated from reaction solution by application of external magnetic field (7). Functionalization of magnetite nanoparticles is performed by different surfactants such as glycine or oleic acid to attach on the bacteria cell surface simultaneously. The toxicity of nanoparticles in contact to living cells could be due to several reasons such as: 1) the toxicity of ions of heavy metal atoms which can impress on the macromolecules, organelles and other parts of cells 2) due to small size of nanoparticles, they can penetrate to living cells and impress them (8). The magnetic nanoparticles with a super paramagnetic behavior have very low toxicity on the living cells (9). There are some studies on evaluating the toxicity of magnetite nanoparticles on eukaryote cells, which their results showed negligible toxicity in eukaryote cells of the modified magnetite nanoparticles with different surfactants (10). But the toxicity of magnetite nanoparticles on bacteria cells is not reported. Nonetheless, magnetite nanoparticles are naturally produced by some of organisms such as several microorganisms and in the some part of developed organisms (11). Because of using Fe atom in several pathways of metabolism, low iron toxicity is expected.

2. Objectives

the effect of the magnetic nanoparticles iron oxide (Fe_3O_4) on the growth rate of the genetically engineered *Pseudomonas aeruginosa* (PTSOX4) cells (12) in different media with different magnetic nanoparticles concentration have been investigated.

3. Materials and Methods

3.1. Chemicals

$FeCl_2$, $FeCl_3$, Glycine, NaOH and other materials were purchased from Merk (Germany).

3.2. Bacterial Strains and Medium

P. aeruginosa (PTSOX4) (13) was provided from National Institute of Genetic Engineering and Biotechnology (NIGEB) and have the ability to convert Dibenzothiophene (DBT) to 2-Hydroxy-biphenyl (2-HBP) and sulfate. This organism was grown on a sulfur free culture medium comprising 2.44 g KH_2PO_4, 5.47 g Na_2HPO_4, 0.2 g $MgCl_2.6H_2O$, 0.001 g $CaCl_2.2H_2O$, 0.001 g $FeCl_3.6H_2O$, 0.004 g $MnCl_2.4H_2O$ and 2 mL Glycerol in 1 liter deionized water, in addition, DBT solution was added to form the final solution of 100 ppm/liter. Pseudomonas strain was grown at 30°C.

3.3. Synthesis of Magnetite Nanoparticles

Magnetic Nanoparticles were synthetized by the following method. 0.045 g $FeCl_2.4H_2O$ and $FeCl_3.6H_2O$ were dissolved in 150mL deionized water with mechanical stirring at 1100 rpm and 65°C which was previously acidified with1mL of HCl (37%), then, NH_4OH (1 M) was quickly added until the pH reached to 11. After 0.09 g Glycine was added over a period of 10 minutes. After 20 minutes, the magnetic precipitate was separated by a centrifuge process (4000 rpm). The sample was washed two times and dried at 80°C with a vacuum drying. Pseudomonas cells were coated with magnetic nanoparticles. The magnetic suspension (20 mg) was mixed with 100 mL of a cell suspension (100mg [dry weight] of cells per liter of Basal salt Medium). The samples were incubated for 30 min at 37°C with 180 rpm.

3.4. Analytical Method

Magnetite nanoparticles size and morphology were evaluated with Transmission Electron Microscopy (TEM) (Philips CM 200, 200 kV TEM, ATM 2k * 2k CCD Camera). The samples were prepared by evaporating of dilute nanoparticles suspension on a carbon copper grid. Then cells were coated with magnetite nanoparticles which were fixed with 3% glutaralde in 0.1 M phosphate buffer, pH 7.0, for 2 h, dehydrated in an alcohol series for 2 h, embedded in an acrylic resin, and allowed to polymerize for two days at 60°C. Ultrathin cell sections were viewed and

photographed with a TEM at 200 kV. The morphology of coated cells was determined using a Scanning Electron Microscopy (SEM). After several times washing with deionized water and drying the samples were ready for SEM photomicroscopy. The phase structure of the synthesized iron oxide nanoparticles was analyzed with X-Ray diffractometer. The study of the growth rate of free cells in genetically engineered *P. aeruginosa* (PTSOX4). In this experiment bacterial cells were cultured in BHI medium for an overnight incubation at 35°C. The next day an identical colony was transferred to 20 mL of LB medium and incubated for 18 h at 35°C. Cells were washed two times by Basal Salt Medium (BSM) solution and then a suspension of 100mg dry weight cells per liter was provided in both LB and BSM media. After that samples were incubated for 30 h at 33°C with shaking in180 rpm and the OD was measured spectrophotometrically at 600 nm. The experiment was repeated three times. The effects of the magnetic nanoparticles Fe_3O_4 was evaluated on the (Minimal Inhibitory Concentration) MIC and (Minimal Bactericidal Concentration) MBC in genetically engineered *P. aeruginosa* (PTSOX4) cells. In this experiment bacterial cells were cultured in BHI medium for 24 h and incubation at 35°C. The next day an identical colony was transferred to 20 mL of LB medium and incubated for 18 h at 35°C. Cells were washed two times with BSM solution and then a suspension of 100 mg dry weight cells per liter provided in LB medium. Then a serial dilution of 0, 100, 500, 1000, 7500, 9000 and 10000 ppm of magnetic nanoparticles iron oxide Fe_3O_4 with the above LB medium was provided. In each case a cell free suspension was prepared as the control. The samples with the control were incubated for 20 h at 35°C with shaking in180 rpm and the OD was measured spectrophotometrically at 620 nm. Study of the growth rate of genetically engineered *P. aeruginosa* (PTSOX4) cells coated with magnetic nanoparticles. In this experiment, bacterial cells were cultured in BHI medium, incubated overnight at 35°C. The next day, the identical colonies were transferred to 20 mL of LB medium and incubated for 18 h at 35°C. Cells were washed two times by BSM solution and then a suspension of 100 mg dry weight cells per liter was provided in both LB and BSM media. Then a serial dilution of 0, 100, 200 and 500 ppm of magnetic nanoparticles iron oxide with the above LB medium was provided. At the same time another serial dilution of 0.100 and 200 ppm of magnetic nanoparticles iron oxide with the above BSM medium was provided. In each case a cell free suspension was prepared as the control. The samples with the control were incubated at 35°C with shaking in180 rpm and the OD was measured spectrophotometrically at 620 nm. The growth curve of the each case has been provided separately (Figure 5 and Figure 6).

4. Results

4.1. Characteristics of the Synthesized Magnetic Nanoparticles

The magnetic nanoparticles were synthesized using co-precipitation method and the size and morphology were analyzed with TEM. As it is demonstrated in Figure 1, particle sizes ranged from 10 to 50 nm using TEM. The nanoparticles solution was stable for several months. Figure 2 demonstrated the XRD of the magnetic nanoparticles iron oxide.

4.2. Analysis of the Bacterial Cells Coated With Magnetic Nanoparticles

The bacterial cells coated with magnetic nanoparticles were analyzed by SEM analysis. Figure 3 demonstrates the coated genetically engineered *P. aeruginosa* (PTSOX4) by magnetic Fe_3O_4 nanoparticles.

4.3. The Study of the Growth Rate of Free and Coated Bacterial Cells

The comparison of the growth conditions of genetically engineered *P. aeruginosa* (PTSOX4) cells in LB and BSM media are shown in Figure 4. Table 1 demonstrates

Table 1. Identity Percentage of the Immunodominant Membrane Protein Gene With Closely Related Sequences in the NCBI Database

Logarithmic Decrement of Bacterium *P. aeruginosa* (PTSOX4)	Means of Optical Absorption of *P. aeruginosa* (PTSOX4) (λ=620 nm)	Standard error Means of Optical Absorption of *P. aeruginosa* (PTSOX4) (λ=620 nm)	Sample Dilution (Treatment), ppm
0	2.5267	0.00882	0
0	2.6767	0.00667	100
0	2.8900	0.00000	200
0	0.4900	0.00577	500
1	0.0100	0.00000	1000
1	0.0033	0.00333	5000
2	0.0000	0.00000	7500
3	0.0000	0.00000	9000
3	0.0000	0.00000	10000

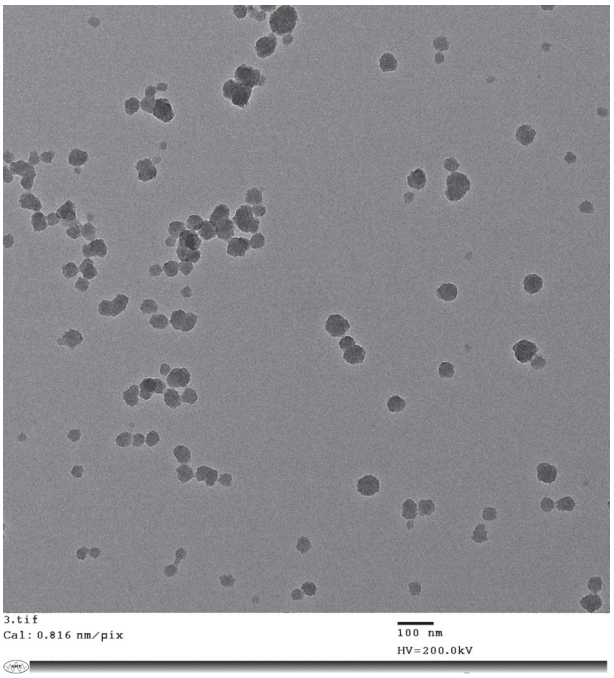

Figure 1. TEM Images of Synthesized Magnetite Nanoparticles

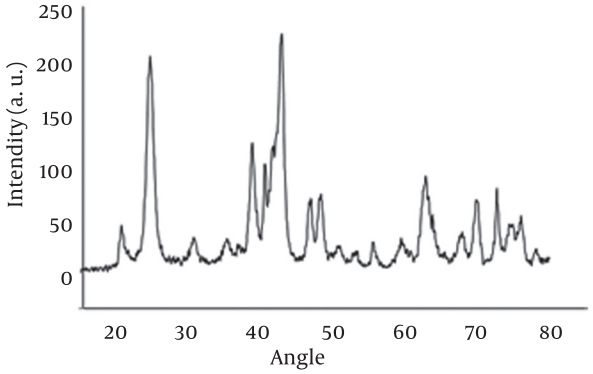

Figure 2. XRD Pattern of Synthesized Magnetite Nanoparticles

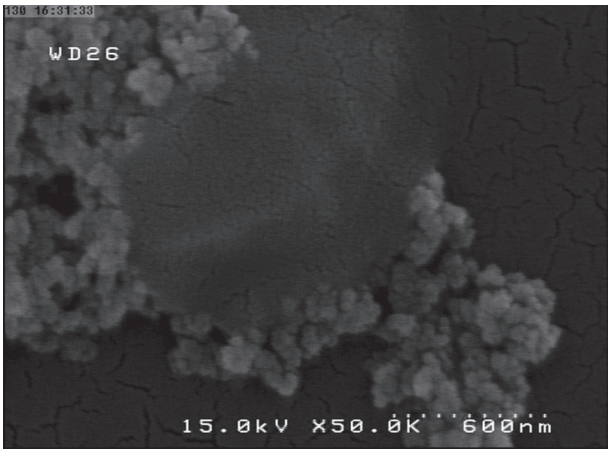

Figure 3. SEM Images of Coated Bacteria With Fe_3O_4 Nanoparticles

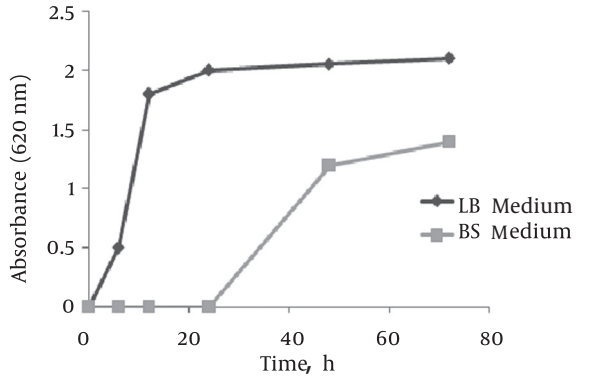

Figure 4. Growth Curve of Free Bacteria Cells in Two Different BHI and BSM Media

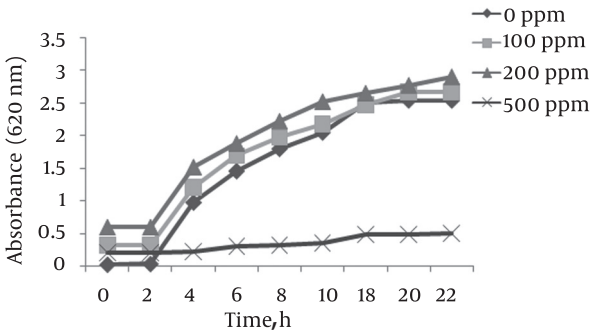

Figure 5. Growth Curve of Bacteria Cells on BHI Medium With Different Concentrations of Nanoparticles

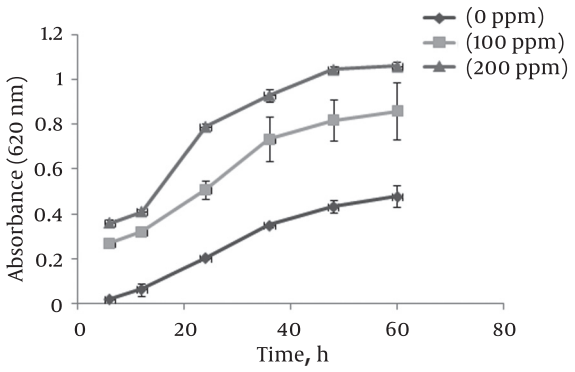

Figure 6. Growth Curve of Bacteria Cells on BSM Medium With Different Concentrations of Nanoparticles

the results for the experiments of the MIC and the MBC in the strain genetically engineered *P. aeruginosa* (PTSOX4) cells. Obtained results from these tests showed that there is no cell growth in the samples media with 5000, 7500, 9000, 10000 ppm of magnetic nanoparticles. Therefore these samples were selected for the following method to determine the MBC experiment. 1 mL from each samples and controls was mixed with Brain Heart Infusion (BHI)

agar medium at 48°C and immediately was poured in the petri dish. After that the culture plates were incubated for an overnight at 35°C. Next day, the plates were collected for colony counting. Study of the growth rate of genetically engineered *P. aeruginosa* (PTSOX4) cells coated with Magnetic nanoparticles. The bacterial cells growth was evaluated in the presence of different concentrations of bacteria and during 22 h. Obtained results from this analysis show that magnetite nanoparticles in low concentration have not toxicity or inhibitory on bacteria growth.

5. Discussion

Synthesis of the magnetic nanoparticles by co-precipitation method is simple, economic and reusable under stable conditions in comparison to other methods. Shan et al., 2005, applied this method to synthesize magnetite nanoparticles and coat bacterial cells, they reported that replacement of air by N_2 has the advantage to prevent the oxidation of ferrous iron during preparation of nanoparticles in the aqueous solution and also has the ability of the size control (6). The surface of nanoparticles has to be modified with a suitable surfactant to use magnetite nanoparticle to coat bacteria. Shan et al. used oleic acid as a surfactant to functionalize and immobilize magnetite nanoparticles on the surface of bacteria; however, Ansari et al. used glycine to modify the surface of nanoparticles (7). Fe atom of magnetite nanoparticles has a strong tendency to COOH groups, so that the Fe atom of nanoparticle reacts with COOH of oleic acid or glycine, therefore oleic acid form a bilayer shell on the surface of nanoparticles (14), and glycine produce an amine layer on the surface of magnetite nanoparticles (7) which leads to the dispersion of magnetic nanoparticles iron oxide in water phase with hydrophilic characteristics. On the other hand, it is reported that this functionalized magnetite nanoparticles are absorbed on the surface of bacteria simultaneously (6, 7). The absorbance of glycine-modified magnetite nanoparticles on the negative-surface of bacteria cells is due to the positive charge of nanoparticles. Previous reports have been showed that functionalized magnetite nanoparticles with different surfactant shave low toxicity on living eukaryote cells in comparison to free nanoparticles (15). Here we have evaluated the effect of glycine-modified nanoparticles on bacterial cells with MBC and MIC tests. This organism lives in soil, water, plant and animal tissues and even can survive on nonliving materials (16) and also is able to survive in diverse environments, therefore can adapt to a free living or biofilm lifestyle (17-19). Although this strain is coated with magnetic Fe_3O_4 nanoparticles for cell separation, it is also a suitable strain to study the enhancement of biodesulfurization activity (6, 7), as the immobilization of this biocatalyst for its localization in a support medium in a commercial bioreactor system (6). The obtained results from growth of this bacterium in different media of BSM and LB showed that the growth rate on the beginning of culture in the LB is more than BSM. LB is a rich medium while BSM is a poor one. Ordinary, lag phase of bacteria in the poor medium is more than rich ones, because it would need more time for synthesis of new enzymes to use presence materials. The obtained results from MIC and MBC analysis showed that nanoparticles have low toxicity on the pseudomonas bacteria cells. According to this analysis, pseudomonas bacteria cells do not grow in the presence of magnetite nanoparticles of more than 5000 ppm concentration. It can be due to surface saturating of bacteria cells with magnetite nanoparticles and increasing the contact of nanoparticle to cell membrane. Thus, cell membrane is injured by them. Accordance to evaluation of viability of bacteria cells in the presence of different concentrations of magnetite nanoparticles; it is appeared to the growth of bacteria cells enhanced by increasing of concentration of magnetite nanoparticles. This can be due to stimulation effect of nanoparticles on the growth of bacteria cells. On the other hand, the magnetite nanoparticles might have absorbance in the applied wave length. It is concluded that magnetite nanoparticles have negligible toxicity on the living bacteria cells and regarding super paramagnetic behavior of these nanoparticles, they are so applicable in different parts of biotechnology fields.

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