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Formulation of Carboxyl-Functioned Magnetic Nanoparticles for Magnetically Guided *Aeromonos* sp. F3 Separation

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In this research, the magnetic nanoparticles were synthesized by a sequential process, involving the chemical coprecipitation of Fe(II) and Fe(III) salts with ammonium hydroxide, resuspension of the nanoparticles in water, and functioned on its surface by $KMnO_4$. The active group coated magnetic nanoparticles were loaded on Aeromonos sp.F3 to prepare the magetic Aeromonos sp.F3 which was easy to separate the enzyme from the broth for collagenase production. The magnetic nanoparticles were characterized in terms of their particle composition, size and size distribution by FT-IR spectrometer, X-ray and LPSA. The influences of the ratio of bacteria and magnetic nanoparticles, temperature, reaction time and pH on the formulation of magnetic *Aeromonos* sp.F3 were discussed.

Key words: Magnetic nanoparticles, Carboxyl-function, Modification, Size distribution.

Magnetic nanoparticles (MNPs) are increasingly being considered for a number of biomedical applications due to their inherent ultra fine size, biocompatibility and superparamagnetic properties (Lu AH *et al*. 2007; Ito A *et al*. 2005). The properties of the MNPs can be tailored for specific biological functions, such as drug delivery (Namdeo M *et al*. 2008), hyperthermia or magnetic targeting (Zhang L *et al*. 2009; Wilhelm C *et al*. 2007), magnetic resonance imaging (MRI) (Sun C *et al*. 2008), cell labeling and sorting (Wilhelm C *et al*. 2008), and bioseparations (Bruce IJ *et al*. 2005). Among the MNPs, iron oxide nanoparticles (magnetiteã-Fe_2O_3 or magnetite Fe_3O_4) are the most popular formulations (Namdeo M *et al*. 2008). The applicability of iron oxide nanoparticles depends upon nanoparticles size, functionality, stability, dispensability, and interfacial surfaces (Namdeo M *et al*. 2008; Osaka T *et al*. 2006; Dey T *et al*. 2006; Gupta AK *et al*. 2007; Lee SY *et al*. 2006). While a number of suitable methods have been developed for the synthesis of MNPs of various compositions, successful application of such MNPs in the areas listed above[1] is highly dependent on the size of the particles, magnetic moment, and its surface functionalization. The small particles tend to form agglomerates to reduce the energy associated with the high surface area to volume ratio of the nanosized particles. The individual nanoparticles with a large constant magnetic moment behave like a giant paramagnetic atom with a fast response to applied magnetic fields with negligible remanence (residual magnetism) and coercivity (the field required to bring the magnetization to zero). The nanoparticles surface can be further modified with functional groups. This treatment allows the linkage of functional ligands which may make the materials suitable for catalytic and optical applications.

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MATERIALS AND METHODS

Materials

Aeromonos sp. F3, laboratory keep; Spectrum One-B Infrared (FTIR & IR)

Spectroscopy, perkin elmer, USA; X-ray diffractometer (XRD), D/max-3B, rigaku corporation , Japan, Power, 100VA; Laser diffraction particle size analyzers (LPSA), zetasize 3000HSA , Malvern, UK, Measurement range, 2^ÿ3000nm, Light Source: He-Ne laser 633nm, Max 5Mw, Temperature: 25!.

Aeromonos sp. F3 culture

Bacteria Aeromonos sp. F3 was isolated from the marine deep beneath mud according to the method of Assis and Mariano(1999). The Aeromonos sp. F3 release the collagenase with the high activity outside the cell in the ferment, so the Aeromonos sp. F3 could be used to product the collagenase prodution. The Aeromonos sp. F3 was screened, gelatin as inducer, and identified with the classical method, 16S rDNA sequencing determination and BLST search. Throw the examination, the optimum ferment condition of the Aeromonos sp. F3 was determined, and the strain materiel was got under the culture condition. The optimum culture condition were 40°C, pH 8 and 80~100 mL/250 mL culture volume ratio for 24 h. The optimum culture medium contained 3 g/L glucose as carbon source, 9 g/L yeast extract powder as nitrogen source, 5g/L NaCl as inorganic salt and 10mg/L $MnSO_4$ as metal ion.

Synthesis of MNPs

Magnetic particles Fe_3O_4 were prepared by co-precipitating and trivalent Fe ions by alkaline solution and treating under hydrothermal conditions (Ruoyu Hong *et al*. 2007). 30 mg $FeCl_3.6H_2O$ and 16.5 mg $FeSO_4.7H_2O$ in 140 ml doubly distilled water were thoroughly mixed and added to 18 ml solution of NH_4OH under continuous stirring (500 rpm) at 80°C for 30 min. The pH was maintained approximately at 10 during the reaction process. The magnetic particles on the mixture was sedimented and separated with magnetic field, the precipitate was washed with hot distilled water and alcohol respectively. The particles obtained were black in colour and exhibited a strong magnetic response. The 100 ml suspension of the particles in distilled water was slowly added to 4.66g oleic acid, and stirred at 500 rpm under nitrogen atmosphere for 1h. The impurity such as ions and redundant oleic acid were removed by washing with copious amounts of hot distilled water. Finally the MNPs were dispersed in slightly alkaline medium (pH 8.9, at 4°C) for further use.

Modification of MNPs

The pure MNPs were prepared by the previous method, and its surface was modified with $KMnO_4$. 160 ml of deionized water containing 200 mg the pure MNPs and 1.6 g $KMnO_4$ was mixed and stirred at 900 rpm for 1 h. The mixture was sedimented with magnetic field and the precipitate was washed with hot distilled water for three times. The ultrasonic irradiation was used to promote the dispersion of magnetic nanoparticles(Gao J *et al*. 2009; McCarthy JR *et al*. 2008).

Preparation and fermentation of Aeromonos sp. F3 loaded with modified MNPs

1.5g Aeromonos sp. *F3* were suspented in 50ml, pH 6 sterile buffer and 1ml (20mg"ml,w/v), the modified MNPs were mixed at room temperature (Michael Chorny *et al*. 2012), and stirred at 100 rpm for 30 min. Then washed three times with pH 6 sterile buffer. The Aeromonos sp. F3 loaded with modified MNPs were maintained on nutrient yeast dextrose agar (NYDA) slants containing 8 g nutrient broth, 5g yeast extract, 10g glucose, and 20 g agar. The cultures were stored at 4°C, until further study for collignase production.

The Aeromonos sp. F3 loaded with modified MNPs were incubated at 37°C, 160 rpm for 8-12h in fermentation medium. The fermentation medium contained 3g/L glucose as carbon source, 9g/L yeast extract powder as nitrogen source, 5g/L NaCl as inorganic salt and 10mg/L $MnSO_4$ as metal ion.

The Aeromonos sp. F3 Loaded with modified MNPs were collected by applying the external magnetic field, and washed two times with sterile distilled water and applied again in next process of the collagenase production.

Determination of collagenase activity

The magnetic Aeromonos sp. F3 was separated with external magnetic field from the fermentation broth. The supernatant was centrifuged at 20,000g for 30 min, and the resulting supernatant was added with ammonium sulfate (70% of saturation). Collagenase was collected by centrifugation at 12,000g for 20 min, resuspended in extraction buffer and desalted.

Collagenase activity was assayed by the modified method (Sung Kang S *et al*. 2005)using casein as substrate. 100 ìl of enzyme solution was added to 900ìl of substrate solution (2 mg/ml , w/v) casein in 10 mM Tris–HCl buffer, pH 8.0.The mixture

was incubated at 45°C for 30 min. Reaction was terminated by the addition of an equal volume of 10% (w/v) chilled trichloroacetic acid then the reaction mixture was allowed to stand in ice for 15 min. The supernatant was separated by centrifugation at 10,000 rpm for 10 min at 4°C; the acid soluble product in the supernatant was neutralized with 5 ml of 0.5 M Na_2CO_3 solution. The colour developed after adding 0.5 ml of 3-fold-diluted Folin–Ciocalteau reagent was measured at 560 nm. One protease unit is defined as the amount of enzyme that releases 1 µg of tyrosine per milliliter per minute under the optimum conditions.

RESULTS

Physical characterization of carboxyl functioned MNPs

Particle size distribution analysis of carboxyl-functioned MNPs

In this section the particle size distribution of carboxyl-functioned MNPs was investigated. The laser diffraction particle size analyzersÿLPSA ÿwas used to investigate the size and range of particles (Matthew *et al*. 2012) and the result was illustrated in figure 1. The range of particles size was from 80 nm to 290 nm and the averge diameter of particles was about 180nm.

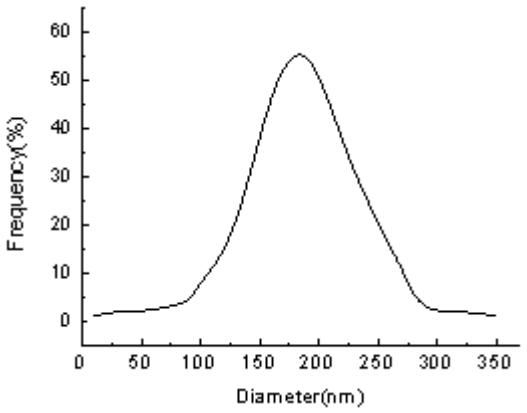

Fig. 1. Particle size distributionin of carboxyl-functioned MNPs

FT-IR spectra of carboxyl-functioned MNPs

Infrared spectra of microspheres were taken by using KBr pellet technique and were recorded on Bomem MB-II FT-IR spectrometer, Quebec, Canada.

IR spectrum a, b and c shown in figure 2. represented the characteristic of the pure MNPs, the carboxyl-functioned MNPs and Aeromonos sp.F3 loaded with the modified MNPs respectively. The three IR spectrums showed the characteristic peaks at 584 cm^{H1} for Fe_3O_4 and the peaks at 3424 cm^{H1} also support the presence of -OH group respectively. The strong peaks in the region of 1600–1700 cm^{H1} on the IR spectrum a and b represents the stretching vibration mode of lattice –COOH. It is main characteristic peaks of the modified microspheres. There are more obvious characteristic peaks at 1063 cm^{H1} for nucleic acid on IR spectrum b which revealed the bacterium loaded by the modified MNPs on its surface (CI *et al*. 2002).

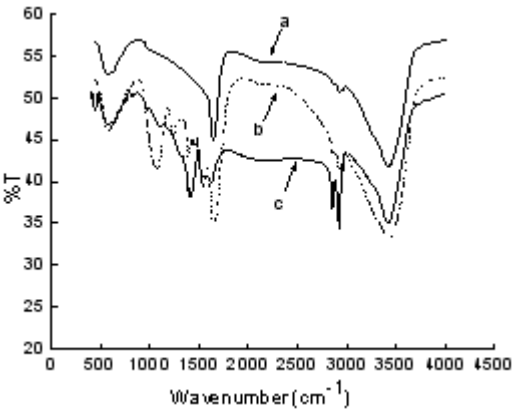

Fig. 2. FT- IR spectra of MNPs a, carboxyl-functioned MNPs; b, Aeromonos sp. F3 loaded with MNPs; c, pure MNPs

X-ray diffraction (XRD) analyse of carboxyl-functioned MNPs

X-ray diffraction (XRD) patterns of the modified MNPs were recorded employing a D/Max–3B Rikagu diffractometer using Cu radiation at λ = 0.1546 nm and operating at 40 kV and 20 mA.

The figure 3. shows that the samples were mounted on six main diffraction peaks and measurements were performed at 2è from 20 to 70°, which is same with the standard Fe_3O_4 crystal to indicate the high purity and crystallinity of the Fe_3O_4 obtained. In addition, the peaks were widened, implying the small size of the modified MNPs.

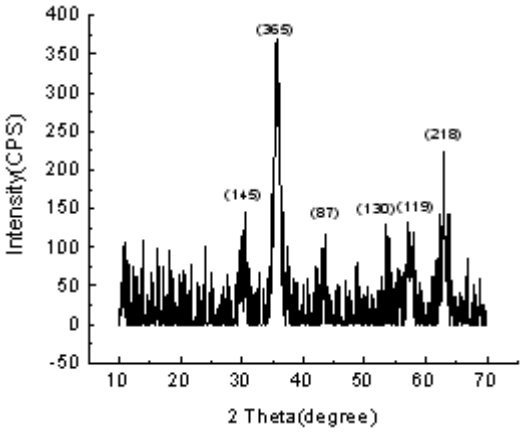

Fig. 3. XRD patterns for samples

Preparation of Aeromonos sp. F3 loaded with carboxyl-functioned MNPs

Effect of ratio of bacteria and particle on preparation

The bacteria and the modified MNPs were suspended on 0.05mol/L Tric-HCl buffer respectively, mixed in the ratio 1.9, 3.1, 4.0, 4.9, 5.4, 6.2 (W/W) and shaked at room temperature for 1h. The bacteria loaded with the modified MNPs on the mixture was precipitated with magnetic field, remove the supernatant containing unfixed bacteria and the precipitate was washed with deionized water. The bacteria loaded with the modified MNPs was cultivated on the basal culture medium for 8h. The enzyme activity in the brothand the influence of the ratio of bacteria and the modified MNPs on preparation were determined. The results shown in the figure 4.

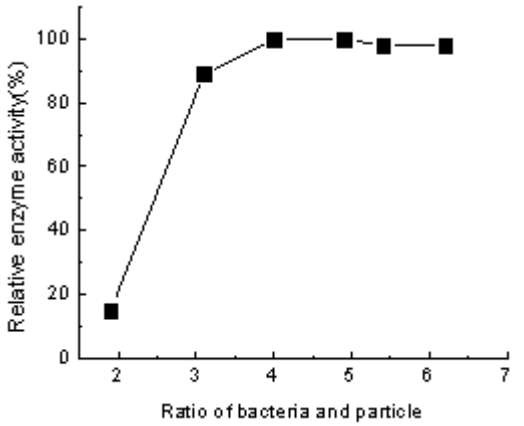

Fig. 4. Effect of ratio of bacteria and particle on preparation

The figure illustrated that the effects of the ratio of bacteria and the modified MNPs on preparation of the bacteria loaded with the modified MNPs were more obvious. The enzyme activity had reached to the highest at 100%. When the mass ratio is 4 compared with the ratio of 1.9, enzyme activity increased by 85%. The number of bacteria on the modified MNPs surface is increased with the cell/particle mass ratio increasing, gradually the enzymatic activity increased. After the ratio of bacteria/particle reach at 4 there is no influence to enzymatic activity by increasing the quantity of bacteria.

Effect of reaction temperature on preparation

The effect of temperature on preparation of batieria loaded with the modified MNPs by assaying collagenase activity was studied in the range 5 to 60!, and illustrated in figure 4. These results suggest that the preparation of bacteria onto the modified MNPs is less dependent on the reaction temperature. The enzyme activity remained 98% of its initial activity at 5°C and 60°C. So we suggested that the room temperature was optimum temperature.

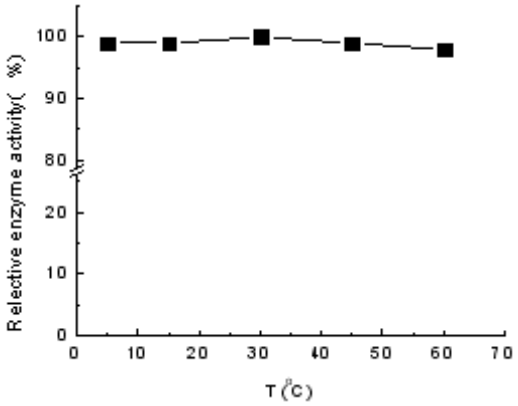

Fig. 5. Effect of reaction temperature on preparation

Effect of reaction time on preparation

The effect of the reaction time on the preparation of bacteria loaded with the modified MNPs was examined from 15 to 90 min by assaying collagenase activity under room temperature as shown in figure 6

Figure 6. suggested that the influence of the reaction time was minor for enzyme activity in general. Take the cell viability into account. We contemplated that the shorter time for preparation the better to the follow-on operational test and evaluation of the preparation cell.

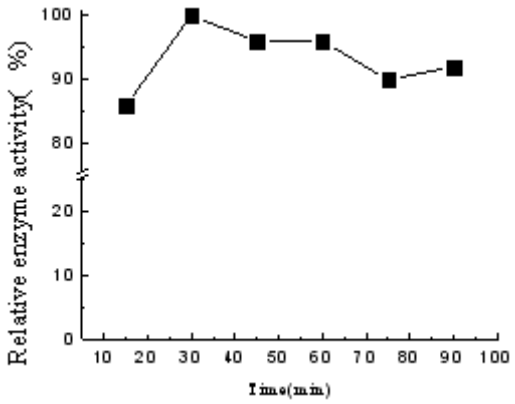

Fig. 6. Effect of reaction time on preparation

Effect of pH on preparation

The effect of pH on preparation of bacteria loaded with the modified MNPs was determined in the pH range 6– 10 by assaying collagenase activity and the results are presented in figure 7.

The result indicted that the pH affect the preparation of bacteria loaded with the modified MNPs was obviously and the maximum activity reached at pH 8. So the pH 8 of the reaction system was the more favour pH value of the preparation.

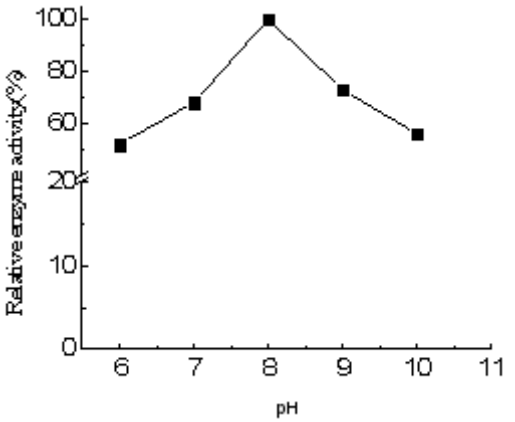

Fig. 7. Effect of pH on preparation

DISCUSSION

The modified MNPs formed under the above mentioned method have more narrow size distribution, the range of particles diameter from 80 nm to 290 nm and the averge size was about 180 nm. After modified with KMnO4, the surface of the MNPs was linked with the active group –COOH efficiently.

The best conditions of preparation of bacteria loaded with the modified MNPs is as follows that the ratio of bacteria and the modified MNPs was 4, the reaction temperature was room temperature, the reaction time was 30 min and the optimum pH was 8.

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